

# Illuminating the Role of *Leishmania* Extracellular Vesicles in Parasite Infection and Survival

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## Abstract

Leishmaniasis is a widespread neglected tropical disease that affects 0.9–1.6 million people annually, causing up to 40 000 deaths. The disease is caused by the protozoan parasite *Leishmania*, which is transmitted by sandflies. *Leishmania* is an ancient human pathogen that has evolved alongside humanity, able to evade mammalian immune responses using virulence factors like the metalloprotease GP63 to infect host macrophages. Our lab has previously demonstrated that extracellular vesicles play a crucial role in *Leishmania* infection. The study of extracellular vesicles has emerged as a critical area of research, yet their inherent heterogeneity continues to pose challenges. *Leishmania* parasites, with their genetic diversity and established protocols for growth and vesicle isolation, offer a unique model for investigating the variability among vesicle populations secreted by different clonal lines. *Leishmania mexicana* was cultured on solid SDM agar plates, and extracellular vesicles from eight clonal colonies were analyzed using nanoparticle tracking analysis, transmission electron microscopy, and proteomics. Despite similar growth rates and indistinguishable physical characteristics among the eight clones, proteomic analysis revealed distinct protein profiles for vesicles isolated from each clonal culture. These profiles differed significantly from those of vesicles derived from a heterogeneous *L. mexicana* liquid culture. Infection experiments in C57BL/6 mice demonstrated that vesicles from clone CL7 caused less footpad swelling compared to those from CL1, CL8, and heterogeneous *L. mexicana*. However, this trend was not observed in mice infected with parasites alone. These results suggest that extracellular vesicles from clonal *L. mexicana* colonies exhibit unique protein compositions that can influence infection outcomes. To further investigate extracellular vesicle biogenesis in *Leishmania*, we disrupted the *Vps36* gene, a key component of the ESCRTIII complex involved in extracellular vesicle production, in *L. major* using CRISPR/Cas9. Comparative analyses of vesicle production between *Vps36*<sup>(-/-)</sup> and WT *L. major* using nanoparticle tracking analysis, transmission electron microscopy, and proteomics showed that *Vps36*<sup>(-/-)</sup> mutants secreted significantly fewer small extracellular vesicles with altered protein cargo. Furthermore, *Vps36*<sup>(-/-)</sup> parasites exhibited

reduced infectivity in Balb/c mice, even when co-injected with WT vesicles. Restoring *Vps36* rescued vesicle production and parasite infectivity, underscoring its role in vesicle biogenesis. However, the reduced infectivity in *Vps36*<sup>(-/-)</sup> parasites may be attributed to defects in amastigote and metacyclic transformation, as well as disruptions in metabolic shifts upon macrophage phagocytosis, rather than impaired vesicle production alone. These findings together highlight the critical role of extracellular vesicles in *Leishmania* infection and leishmaniasis disease progression and suggest that defective ESCRTII can cause disruptions in parasite transformation and metabolic adaptation, leading to reduced infectivity and intramacrophage survival.

## Résumé

La leishmaniose est une maladie tropicale négligée et répandue qui touche entre 0,9 et 1,6 million de personnes chaque année, causant jusqu'à 40 000 décès. Cette maladie est provoquée par le parasite protozoaire *Leishmania*, transmis par les phlébotomes. *Leishmania* est un pathogène humain ancien qui a évolué parallèlement à l'humanité, capable d'échapper aux réponses immunitaires des mammifères grâce à des facteurs de virulence tels que la métalloprotéase GP63, lui permettant d'infecter les macrophages hôtes. Notre laboratoire a précédemment démontré que les vésicules extracellulaires jouent un rôle crucial dans l'infection par *Leishmania*. L'étude des vésicules extracellulaires s'est imposée comme un domaine de recherche essentiel, bien que leur hétérogénéité inhérente continue de poser des défis. Les parasites *Leishmania*, grâce à leur diversité génétique et à des protocoles bien établis pour leur culture et l'isolement de vésicules, offrent un modèle unique pour étudier la variabilité des populations de vésicules sécrétées par différentes lignées clonales. *Leishmania mexicana* a été cultivée sur des plaques d'agar SDM solide, et les vésicules extracellulaires de huit colonies clonales ont été analysées à l'aide de l'analyse de suivi des nanoparticules, de la microscopie électronique à transmission et de la protéomique. Bien que les huit clones présentent des taux de croissance similaires et des caractéristiques physiques indiscernables, l'analyse protéomique a révélé des profils protéiques distincts pour les vésicules isolées de chaque culture clonale. Ces profils différaient significativement de ceux des vésicules issues d'une culture liquide hétérogène de *L. mexicana*. Des expériences d'infection chez les souris C57BL/6 ont montré que les vésicules provenant du clone CL7 provoquaient moins de gonflement de la patte que celles des clones CL1, CL8 et de *L. mexicana* hétérogène. Cependant, cette tendance n'a pas été observée chez les souris infectées uniquement par les parasites. Ces résultats suggèrent que les vésicules extracellulaires des colonies clonales de *L. mexicana* présentent des compositions protéiques uniques pouvant influencer les résultats de l'infection. Pour approfondir l'étude de la biogenèse des vésicules extracellulaires chez *Leishmania*, nous avons perturbé le gène *Vps36*, un composant clé du

complexe ESCRTII impliqué dans la production des vésicules extracellulaire, chez *L. major* à l'aide de CRISPR/Cas9. Des analyses comparatives de la production des vésicules entre *Vps36*<sup>(-/-)</sup> et *L. major* sauvage utilisant l'analyse de suivi des nanoparticules, la microscopie électronique à transmission et la protéomique ont montré que les mutants *Vps36*<sup>(-/-)</sup> sécrétaient significativement moins des petites vésicules extracellulaires avec un contenu protéique altéré. De plus, les parasites *Vps36*<sup>(-/-)</sup> présentaient une infectiosité réduite chez les souris Balb/c, même lorsqu'ils étaient co-injectés avec des vésicules sauvages. La restauration du gène *Vps36* a permis de rétablir la production des petites vésicules extracellulaires et l'infectiosité du parasite, confirmant son rôle dans la biogenèse des vésicules. Cependant, la réduction de l'infectiosité chez les parasites *Vps36*<sup>(-/-)</sup> pourrait être attribuée à des défauts de transformation en amastigotes et métacycliques, ainsi qu'à des perturbations des changements métaboliques suite à la phagocytose par les macrophages, plutôt qu'à une simple altération de la production des vésicules. Ces résultats mettent en évidence le rôle essentiel des vésicules extracellulaires dans l'infection par *Leishmania* et la progression de la leishmaniose, tout en suggérant que des défauts dans le complexe ESCRTII peuvent entraîner des perturbations dans la transformation et l'adaptation métabolique des parasites, conduisant à une infectiosité réduite et à une survie intramacrophagique limitée.

## Acknowledgements

It is with great joy and relief that I have come to the end of my journey as a PhD student. I will forever be grateful for the guidance and support I have received over these years from my supervisor, Dr. Martin Olivier, as well as my co-supervisor, Dr. Christopher Fernandez-Prada. Dr. Olivier has served as a constant support and guide throughout this degree, always available to discuss my ideas and worries, be they related to my degree or to life at large. He has been there to celebrate both my breakthroughs and failures, always making sure I am progressing in the right direction. Dr. Fernandez-Prada has graciously provided me with a second home, as much as a lab can healthily be considered a home. His expertise, ambition, and friendship have been indispensable to both my research and life. It goes without saying that this thesis would not have been possible without these two fine gentlemen and their mentorship has also shaped my understanding of what it means to be a scientist, a scholar, and a colleague. Both my supervisors have welcomed me into their labs and their homes, and it is with great pride that I may dedicate this thesis in part to them.

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## Preface

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation". The candidate has chosen to present the thesis in a "Manuscript-based thesis" format following these recommendations:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis"

All the work in this thesis was done under supervision of Dr. Martin Olivier and co-supervision of Dr. Christopher Fernandez-Prada, who also helped with final revisions of this thesis. The candidate wrote and edited the body of this thesis alone and is the first author of the two manuscripts presented in this thesis. Author contributions for each manuscript are detailed below and are also included in their respective chapters.

The manuscript presented in chapter 2 of this thesis is finalizing revisions in *Frontiers of Immunology*, a peer-reviewed open-access online journal. The project was conceptualized by MO and GD. All experiments and analysis, including the EV purifications, NTA analysis, microBCA, TEM imaging, proteomic analysis, and *in vivo* mice infections, were performed by GD. Mice footpad measurements were performed with support from MO. All figures were generated by GD except figure 7, which was generated by ND, who also processed the GO data necessary for the figure. Manuscript was written by GD and reviewed/edited by GD and MO.

The manuscript presented in chapter 3 of the thesis is ready for submission for publication. The project was conceptualized by MO, GD, and CFP. All experiments and analysis, including the EV purifications, NTA analysis, microBCA, TEM imaging, proteomic analysis, *in vitro* macrophage infections, and *in vivo* mice infections, were performed by GD. Mice footpad measurements were performed with support from MO. Vps36 addback and PCR

confirmation was performed by AC. RNA sequencing and analysis was performed by DL. Sandfly experiments and dissections were performed by GG in the lab of NP. All figures were generated by GD except figure 4, which was generated by LM, and figure 13, which was generated by GG.

Below are publications the candidate significantly contributed to but are not presented in the thesis:

Chan, A., Ayala, J. M., Alvarez, F., Piccirillo, C., Dong, G., Langlais, D., & Olivier, M. (2021). The role of *Leishmania* GP63 in the modulation of innate inflammatory response to *Leishmania major* infection. *PLoS One*, 16(12), e0262158. <https://doi.org/10.1371/journal.pone.0262158>

Dong, G., Filho, A. L., & Olivier, M. (2019). Modulation of Host-Pathogen Communication by Extracellular Vesicles (EVs) of the Protozoan Parasite *Leishmania*. *Front Cell Infect Microbiol*, 9, 100. <https://doi.org/10.3389/fcimb.2019.00100>

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Douanne, N., Dong, G., Amin, A., Bernardo, L., Blanchette, M., Langlais, D., Olivier, M., & Fernandez-Prada, C. (2022). *Leishmania* parasites exchange drug-resistance genes through extracellular vesicles. *Cell Rep*, 40(3), 111121. <https://doi.org/10.1016/j.celrep.2022.111121>

Douanne, N., Dong, G., Douanne, M., Olivier, M., & Fernandez-Prada, C. (2020). Unravelling the proteomic signature of extracellular vesicles released by drug-resistant *Leishmania infantum* parasites. *PLoS Negl Trop Dis*, 14(7), e0008439. <https://doi.org/10.1371/journal.pntd.0008439>

Ibarra-Meneses, A. V., Amin, A., Dong, G., Olivier, M., Langlais, D., & Fernandez-Prada, C. (2023). Identification and analysis of the DNA content of small extracellular vesicles isolated

from *Leishmania* parasites. *STAR Protoc*, 4(2), 102248.  
<https://doi.org/10.1016/j.xpro.2023.102248>

Siddiq, A., Dong, G., Balan, B., Harrison, L. G., Jex, A., Olivier, M., Allain, T., & Buret, A. G. (2023). A thermo-resistant and RNase-sensitive cargo from *Giardia duodenalis* extracellular vesicles modifies the behaviour of enterobacteria. *J Extracell Biol*, 2(9), e109.  
<https://doi.org/10.1002/jex2.109>

Vucetic, A., Filho, A., Dong, G., & Olivier, M. (2020). Isolation of Extracellular Vesicles from *Leishmania* spp. *Methods Mol Biol*, 2116, 555-574. [https://doi.org/10.1007/978-1-0716-0294-2\\_33](https://doi.org/10.1007/978-1-0716-0294-2_33)

## List of abbreviations

### A

|       |                                    |
|-------|------------------------------------|
| ADP   | adenosine diphosphate              |
| Alix  | ALG-2-interacting protein X        |
| AmB   | amphotericin B                     |
| AP-1  | activator protein 1                |
| ARRDC | arrestin domain-containing protein |
| ARF   | ADP-ribosylation factor            |
| ATP   | adenosine triphosphate             |

### B

|        |                                |
|--------|--------------------------------|
| B-cell | bursa-derived-cell             |
| BMDM   | bone-marrow-derived macrophage |
| Bro    | brother                        |

### C

|            |                                                                 |
|------------|-----------------------------------------------------------------|
| C-terminal | carbon-terminal                                                 |
| Cas9       | CRISPR-associated protein 9                                     |
| CCD        | charge-coupled device                                           |
| CCL        | CC chemokine ligand                                             |
| CCR        | CC chemokine receptor                                           |
| CD         | cluster of differentiation                                      |
| CHMP       | chromatin-modifying protein/charged multivesicular body protein |
| CL         | cutaneous leishmaniasis                                         |
| CNV        | copy number variation                                           |
| CP         | cysteine protease                                               |
| CpG        | cytidine monophosphate guanosine                                |
| CR         | complement receptor                                             |
| CRISPR     | clustered regularly interspaced short palindromic repeats       |

### D

|          |                                                              |
|----------|--------------------------------------------------------------|
| DALY     | disability-adjusted life years                               |
| DC       | dendritic cells                                              |
| DCL      | diffuse cutaneous leishmaniasis                              |
| DNA      | deoxyribonucleic acid                                        |
| DsCL     | disseminated cutaneous leishmaniasis                         |
| <b>E</b> |                                                              |
| Eap      | ESCRT-associated protein                                     |
| ECM      | extracellular matrix                                         |
| EGF      | epidermal growth factor                                      |
| EGFR     | epidermal growth factor receptor                             |
| EM       | electron microscopy                                          |
| ER       | endoplasmic reticulum                                        |
| ESCRT    | endosomal sorting complex required for transport             |
| <b>G</b> |                                                              |
| GLUE     | GRAM-like ubiquitin-binding in EAP45                         |
| GP63     | glycoprotein 63                                              |
| GPI      | glycophosphatidyl-inositol                                   |
| GTP      | guanosine triphosphate                                       |
| <b>H</b> |                                                              |
| HEK293   | human embryonic kidney 293                                   |
| HIV      | human immunodeficiency virus                                 |
| HRS      | hepatocyte growth factor-regulated tyrosine kinase substrate |
| HSC      | heat shock cognate                                           |
| HSP      | heat shock protein                                           |
| Ht       | heterogeneous                                                |
| <b>I</b> |                                                              |
| IFN      | interferon                                                   |
| IL       | interleukin                                                  |
| ILV      | intraluminal vesicles                                        |

|          |                                                           |
|----------|-----------------------------------------------------------|
| Indels   | insertions or deletions                                   |
| iNOS     | inducible nitric oxide synthase                           |
| IRAK-1   | interleukin-1 receptor associated kinase 1                |
| ITIM     | immunoreceptor tyrosine-based inhibitory motifs           |
| <b>J</b> |                                                           |
| JAK      | Janus kinase                                              |
| <b>K</b> |                                                           |
| KTIM     | tyrosine-based inhibitor motif                            |
| <b>L</b> |                                                           |
| LAmB     | liposomal amphotericin B                                  |
| LCMSMS   | liquid chromatography with tandem mass spectrometry       |
| LPG      | lipophosphoglycan                                         |
| LPS      | lipopolysaccharide                                        |
| LR       | leishmaniasis recidivans                                  |
| <b>M</b> |                                                           |
| MA       | meglumine antimoniate                                     |
| MBP      | mannan-binding protein                                    |
| Mbps     | megabase pairs                                            |
| MCF-7    | Michigan Cancer Foundation-7                              |
| MCL      | mucocutaneous leishmaniasis                               |
| MHC      | major histocompatibility complex                          |
| MIP      | macrophage inflammatory protein                           |
| MISEV    | minimal information for studies of extracellular vesicles |
| MLC      | myosin light chain                                        |
| mRNA     | messenger ribonucleic acid                                |
| MSP      | major surface protein                                     |
| mTOR     | mammalian target of rapamycin                             |
| mTORC1   | mammalian target of rapamycin complex 1                   |
| MV       | microvesicle                                              |

|                |                                                                |
|----------------|----------------------------------------------------------------|
| MVB            | multivesicular body                                            |
| MyD88          | myeloid differentiation primary response gene (88)             |
| <b>N</b>       |                                                                |
| N-terminal     | nitrogen-terminal                                              |
| NET            | neutrophil extracellular traps                                 |
| NF- $\kappa$ B | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK             | natural killer                                                 |
| NKG            | natural killer group                                           |
| NLR            | nod-like receptor                                              |
| NLRP3          | NOD-, LRR- and pyrin domain-containing protein 3               |
| NO             | nitric oxide                                                   |
| NOD            | nucleotide-binding oligomerization domain                      |
| nSMase2        | neutral sphingomyelinase 2                                     |
| NTA            | nanoparticle tracking analysis                                 |
| NTD            | neglected tropical disease                                     |
| NW             | new world                                                      |
| <b>O</b>       |                                                                |
| ORP1L          | oxysterol-binding protein-related protein 1L                   |
| OW             | old world                                                      |
| <b>P</b>       |                                                                |
| PAMP           | pathogen-associated molecular patterns                         |
| PBS            | phosphate buffered saline                                      |
| PC             | prostate cancer                                                |
| PH             | pleskin homology                                               |
| PKC            | protein kinase C                                               |
| PKDL           | post-kala-azar dermal leishmaniasis                            |
| PMEL           | premelanosome protein                                          |
| PMN            | polymorphonuclear neutrophil granulocyte                       |
| PPG            | proteophosphoglycan                                            |

|          |                                                              |
|----------|--------------------------------------------------------------|
| PRR      | pattern recognition receptors                                |
| PSG      | promastigote secretory gel                                   |
| PTM      | post-translational modification                              |
| PTP      | protein tyrosine phosphatase                                 |
| <b>R</b> |                                                              |
| rab      | ras-associated binding                                       |
| Rel      | v-rel avian reticuloendotheliosis viral oncogene homolog A   |
| RNA      | ribonucleic acid                                             |
| RNAi     | ribonucleic acid interference                                |
| ROCK     | rho-associated coiled-coil-containing protein kinase         |
| ROS      | reactive oxygen species                                      |
| <b>S</b> |                                                              |
| SDM      | Schneider's drosophila medium                                |
| SEC      | size exclusion chromatography                                |
| SEM      | scanning electron microscopy                                 |
| Ser      | serine                                                       |
| sEV      | small extracellular vesicle                                  |
| SHP-1    | Src homology region 2 domain-containing phosphatase-1        |
| SNAP     | Soluble N-ethylmaleimide-sensitive factor attachment protein |
| SNARE    | SNAP receptor                                                |
| Snf      | Sucrose non-fermentable                                      |
| SNP      | single-nucleotide polymorphism                               |
| SSG      | sodium stibogluconate                                        |
| STARD3   | StAR related lipid transfer domain containing 3              |
| STAT     | signal transducer and activator of transcription             |
| <b>T</b> |                                                              |
| T-cell   | Thymus-cell                                                  |
| TCPTP    | T-cell protein tyrosine phosphatase                          |
| TEM      | transmission electron microscopy                             |

|          |                                             |
|----------|---------------------------------------------|
| TGN      | trans-Golgi network                         |
| TF       | transcription factor                        |
| Th       | T helper                                    |
| Thr      | threonine                                   |
| TLR      | toll-like receptor                          |
| TS       | temperature shock                           |
| TSG      | tumor susceptibility gene                   |
| TSPN     | tetraspanin                                 |
| Tyr      | tyrosine                                    |
| <b>U</b> |                                             |
| UC       | ultracentrifugation                         |
| <b>V</b> |                                             |
| Vps      | vacuolar protein sorting-associated protein |
| VL       | visceral leishmaniasis                      |
| <b>W</b> |                                             |
| WHO      | World Health Organization                   |
| WP       | whole parasite                              |
| WT       | wild type                                   |

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# Chapter 1. Introduction and scientific objectives

## 1.1 Leishmaniasis and the life of the *Leishmania* parasite

Leishmaniasis comprises a group of diseases caused by blood-borne protozoan parasites of the *Leishmania* genus and are transmitted by *Phlebotomus* and *Lutzomyia* sandflies. There are about 20 *Leishmania* species identified that cause leishmaniasis in humans and other mammals (Cecilio et al., 2022). These diseases are prevalent in impoverished tropical and subtropical regions and remain classified as neglected tropical diseases (NTDs) by the World Health Organization (WHO), despite ongoing efforts to reduce their incidence (WHO, 2023). In mammalian hosts, infections with various *Leishmania* species result in diverse clinical manifestations, ranging in severity. Current prevention and treatment options are limited and often entail significant side effects. Moreover, the expanding impact of climate change, leading to increased temperatures, may facilitate the geographical spread of leishmaniasis to previously unaffected areas, including parts of Europe and North America (Gonzalez et al., 2010; Ready, 2010). Thus, there is a pressing need for deeper insights into the pathological mechanisms underlying these diseases. This section offers a comprehensive overview of current knowledge concerning the immunology, vectors, and causative agents of leishmaniasis.

### 1.1.1 Disease manifestations

One of the most intriguing facets of leishmaniasis is that, despite there being few unique genes separating different species of *Leishmania*, clinical manifestations of leishmaniasis can greatly vary depending on the causative *Leishmania* species (Figure 1) (Burza et al., 2018). Thusly, leishmaniasis can be broadly divided into 3 distinct forms defined by where *Leishmania* parasites typically accumulate and cause damage: visceral (VL), cutaneous (CL), and mucocutaneous (MCL) (Table 1).



Figure 1. Clinical manifestations of leishmaniasis. Post Kala-Azar Dermal Leishmaniasis (PKDL) and MCL images are reproduced from (Burza et al., 2018), Diffuse Cutaneous Leishmaniasis (DCL) and CL images are reproduced from (Mann et al., 2021), VL image is reproduced from (PAHO, 2018), and LR image is reproduced from (Gitari et al., 2018).

VL, also known as Kal-Azar, is caused by *Leishmania donovani* and *Leishmania infantum*, formerly known as *Leishmania chagasi* (Ready, 2014). Skin lesions will appear at the site of the sandfly bite like with CL and visceral inflammation will follow. The incubation period of this disease can range from 2 weeks to 18 months and once symptoms appear, untreated VL is mostly fatal with up to 95% mortality rate (Barrett & Croft, 2012). VL is characterized by fever, weight loss, and the notable enlargement of the spleen and liver. This leads to extensive immunosuppression and increased susceptibility to secondary bacterial infections. In specific regions, notably Sudan and India, individuals who recover from VL may subsequently develop PKDL, where skin lesions caused by *L. donovani* appear on the skin (Zijlstra et al., 2003).

CL is the most common form of leishmaniasis and is caused by *L. donovani*, *L. infantum*, *Leishmania major*, *Leishmania tropica* and *Leishmania aethiopica* in the old world (OW) (eastern hemisphere) and *Leishmania braziliensis*, *Leishmania panamensis*, *Leishmania*

*guyanensis*, *Leishmania amazonensis*, *Leishmania mexicana* and *Leishmania peruviana* in the new world (NW) (western hemisphere) (Reithinger et al., 2007). In a typical infection, the area where the sandfly bites develops erythema (redness of skin) and eventually forms a lesion within 2 to 6 weeks. This lesion is typically self-healing and will clear in 2 to 9 months and often confers immunity against future infections. Healing time depends on a variety of factors, especially host immune response and the specific infecting *Leishmania* species. However, the healing process results in the formation of a cutaneous scar, which can be a cause of lifelong social stigma. Furthermore, infection by *L. tropica* can cause a rare form of CL known as leishmaniasis recidivans (LR), defined by a recurrence of lesions at the original infection site (Gitari et al., 2018). More problematically, infection by *L. amazonensis*, *L. aethiopica*, and *L. mexicana* can lead to diffuse cutaneous leishmaniasis (DCL), a severe form of CL with uncontrolled parasite growth in diffuse legions across the skin, resembling lepromatous leprosy (Christensen et al., 2019; Fikre et al., 2017; Velasco et al., 1989). In comparison, disseminated cutaneous leishmaniasis (DsCL) is more manageable due to its sensitivity to drug treatment (Carvalho et al., 1994).

MCL is a form of CL that occurs exclusively in the NW and can be observed in infections caused by *Leishmania* parasites of the *Viannia* subgenus, including *L. V. panamensis*, *L. V. braziliensis*, and *L. V. guyanensis*. The progression from CL to MCL is characterized by disfiguring and potentially fatal damage to the upper respiratory mucosal tissues, resulting in the destruction of oronasopharyngeal mucosa and cartilage (Abadias-Granado et al., 2021). In rare cases, airway obstruction and long-term sequela can occur due to the development of laryngeal lesions. The exact mechanisms of CL progression to MCL are unclear, but it has been found that *Leishmania* RNA virus (LRV) is a major contributor to MCL severity (Ives et al., 2011).

| <b><i>Leishmania</i> species</b>                                | <b>Clinical form</b>           | <b>Main clinical features</b>                                                                                                 | <b>Natural progression</b>                                                                                         | <b>Risk groups</b>                                                                                                          | <b>OW vs. NW</b> |
|-----------------------------------------------------------------|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|------------------|
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>donovani</i>   | VL and PKDL                    | Persistent fever, splenomegaly, weight loss, and anemia in VL; multiple painless macular, papular, or nodular lesions in PKDL | VL is fatal within 2 years if untreated; PKDL lesions self-heal in up to 85% of cases in Africa but rarely in Asia | Predominantly adolescents and young adults for VL; young children in Sudan and no clearly established risk factors for PKDL | OW               |
| <i>Leishmania</i><br>( <i>Leishmania</i> ) <i>tropica</i>       | CL, LR, and rarely VL          | Ulcerating dry lesions, painless, and frequently multiple                                                                     | CL lesions often self-heal within 1 year                                                                           | No well-defined risk groups                                                                                                 | OW               |
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>aethiopica</i> | CL, DCL, DsCL, and oronasal CL | Localized cutaneous nodular lesions; occasionally oronasal; rarely ulcerates                                                  | Self-healing, except for DCL, within 2–5 years                                                                     | Limited evidence; adolescents                                                                                               | OW               |
| <i>Leishmania</i><br>( <i>Leishmania</i> ) <i>major</i>         | CL                             | Rapid necrosis, multiple wet sores, and severe inflammation                                                                   | Self-healing in >50% of cases within 2–8 months; multiple lesions slow to heal, and severe scarring                | No well-defined risk groups                                                                                                 | OW               |
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>infantum</i>   | VL and CL                      | Persistent fever and splenomegaly in VL; typically single nodules and minimal                                                 | VL is fatal within 2 years if untreated; CL lesions self-heal within 1 year                                        | Children under 5 years and immunocompromised adults for VL; older children and young adults for CL                          | OW and NW        |

|                                              |                      |                                                                                                                                       |                                                                |                             |    |
|----------------------------------------------|----------------------|---------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|-----------------------------|----|
|                                              |                      | inflammation in CL                                                                                                                    | conferring individual immunity                                 |                             |    |
| <i>Leishmania (Leishmania) mexicana</i>      | CL, DCL, and DsCL    | Ulcerating lesions, single or multiple                                                                                                | Often self-healing within 3–4 months                           | No well-defined risk groups | NW |
| <i>Leishmania (Leishmania) venezuelensis</i> | CL                   | Ulcerating lesions                                                                                                                    | Not well described                                             | No well-defined risk groups | NW |
| <i>Leishmania (Leishmania) amazonensis</i>   | CL, DCL, and DsCL    | Ulcerating lesions, single or multiple                                                                                                | Not well described                                             | No well-defined risk groups | NW |
| <i>Leishmania (Viannia) braziliensis</i>     | CL, MCL, DCL, and LR | Ulcerating lesions can progress to mucocutaneous form; local lymph nodes are palpable before and early on in the onset of the lesions | Might self-heal within 6 months; 2.5% of cases progress to MCL | No well-defined risk groups | NW |
| <i>Leishmania (Viannia) guyanensis</i>       | CL, DsCL, and MCL    | Ulcerating lesions, single or multiple that can progress to mucocutaneous form; palpable lymph nodes                                  | Might self-heal within 6 months                                | No well-defined risk groups | NW |
| <i>Leishmania (Viannia) peruviana</i>        | CL                   | Ulcerating lesions, single or multiple                                                                                                | Not well described                                             | No well-defined risk groups | NW |
| <i>Leishmania (Viannia) panamensis</i>       | CL, MCL and DCL      | Ulcerating lesions, single or multiple that can progress to                                                                           | Not well described                                             | No well-defined risk groups | NW |

|                                             |           |                                        |                    |                             |           |
|---------------------------------------------|-----------|----------------------------------------|--------------------|-----------------------------|-----------|
|                                             |           | mucocutaneous form                     |                    |                             |           |
| <i>Leishmania (Viannia) lainsoni</i>        | CL        | Ulcerating lesions, single or multiple | Not well described | No well-defined risk groups | NW        |
| <i>Leishmania (Viannia) lindenbergi</i>     | CL        | Ulcerating lesions                     | Not well described | No well-defined risk groups | NW        |
| <i>Leishmania (Viannia) naiffi</i>          | CL        | Ulcerating lesions, single and small   | Not well described | No well-defined risk groups | NW        |
| <i>Leishmania (Viannia) shawi</i>           | CL        | Ulcerating lesions                     | Not well described | No well-defined risk groups | NW        |
| <i>Leishmania colombiensi</i> #             | CL and VL | Not well described                     | Not well described | No well-defined risk groups | NW        |
| <i>Leishmania (Mundinia) martiniquensis</i> | CL and VL | Not well described                     | Not well described | No well-defined risk groups | OW and NW |
| <i>Leishmania (Mundinia) orientalis</i>     | CL and VL | Not well described                     | Not well described | No well-defined risk groups | OW and NW |

Table 1. Human pathogenic *Leishmania* species and their known reservoirs, transmission, and epidemiology. Adapted from (Cecilio et al., 2022).

### 1.1.2 Disease epidemiology and prevention

Leishmaniasis is a NTD endemic to 98 countries across Africa, Asia, and South and Central America (Georgiadou et al., 2015; Mann et al., 2021). This disease is closely bound to poverty and the lack of hygiene that results from poor infrastructure and development, largely affecting remote and rural regions of developing countries. Globally, there are approximately 12 million cases of leishmaniasis, with an additional 1.5-2 million new cases and 70,000 deaths reported annually (Burza et al., 2018; WHO, 2023). As mentioned previously, the bulk of deaths associated with leishmaniasis result from VL. The lack of mandatory reporting in endemic countries along with the high prevalence of subclinical/asymptomatic infections continues to inhibit accurate reporting of the real number of cases globally (Ibarra-Meneses

et al., 2022). For example, the seroprevalence of VL-causing *L. donovani* ranges from 7-63% in endemic regions, indicating high rates of asymptomatic infection. Along with mortality, leishmaniasis incurs significant morbidity, with some estimates as high as 2.4 million disability-adjusted life years (DALYs), and important health expenditure for both individuals and healthcare systems (Okwor & Uzonna, 2016).

CL, accounting for up to 80% of leishmaniasis cases, is endemic in 89 countries, with approximately 70% of global cases concentrated in Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Sudan, and the Syrian Arab Republic (Torres-Guerrero et al., 2017). Recent instability in these regions, especially Syria, has caused mass migration of at-risk populations of refugees and represents a pressing challenge on rapid health response (Du et al., 2016; Ozkeklikci et al., 2017). Furthermore, as noted previously, cases have started to climb in southern Europe and United States due to the spread of sandflies northward (Curtin & Aronson, 2021; Ready, 2010). While OW CL is caused only by *Leishmania* parasites of the *Leishmania* subgenus, the prevalence of *Viannia* subgenus *Leishmania* parasites in the NW presents a unique challenge to treating CL; in 1-10% of CL cases in South America, MCL can develop in patients even up to 5 years after initial lesion healing, with 90% of these cases occurring in Bolivia, Brazil, and Peru (Torres-Guerrero et al., 2017). While VL is the much less common and less widespread form of the disease, it accounts for the majority of the deaths. 90% of VL cases occurred in Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan. The millions of people living at the border of Nepal and India are especially at-risk of VL with an incidence of 2.67 per 10 000 people in 2006-2007 (Pandey et al., 2011).

In endemic areas, the incidence of CL rises until the age of 15, after which immunity develops and the risk of infection decreases (Burza et al., 2018). This trend can also be observed to some extent with VL, though VL susceptibility seems much more dependent on socioeconomic factors like malnutrition (Abdullahi et al., 2024; Harhay et al., 2011). Both CL and VL tended to affect males at a higher ratio compared to females, and although it is unclear the exact factors at play, it has been suggested that male-biased occupations tend

to involve greater exposure to *Leishmania* vectors (Cloots et al., 2020; Soares et al., 2014; Strazzulla et al., 2013). Other risk factors include poor housing conditions, which can be conducive to sandfly resting and mating, and the presence of peridomestic animals, which can act as reservoirs (Singh et al., 2014). In fact, most *Leishmania* species classified as human pathogens are associated with zoonotic transmission, with only *L. donovani* parasites lacking a definitive alternate reservoir other than humans (Table 2) (Cecilio et al., 2022). Even then, there is evidence that dogs and mongooses (*Herpestes ichneumon*) may serve as *L. donovani* animal reservoirs (Dereure et al., 2003; Elnaiem et al., 2001; Jambulingam et al., 2017). Common animal reservoirs for *Leishmania* are mostly sylvatic and include canines, rodents, bats, marsupials, and even some reptile species (Belova, 1971; Roque & Jansen, 2014).

| <b><i>Leishmania</i> species</b>          | <b>Clinical form</b>           | <b>Main reservoir</b> | <b>Transmission</b>    | <b>OW vs. NW</b> | <b>High-burden countries or regions</b>                                      | <b>Estimated annual worldwide incidence</b>          |
|-------------------------------------------|--------------------------------|-----------------------|------------------------|------------------|------------------------------------------------------------------------------|------------------------------------------------------|
| <i>Leishmania (Leishmania) donovani</i>   | VL and PKDL                    | Humans                | Epidemic anthroponotic | OW               | India, Bangladesh, Ethiopia, Sudan, and South Sudan                          | 50,000–90,000 VL cases; unknown number of PKDL cases |
| <i>Leishmania (Leishmania) tropica</i>    | CL, LR, and rarely VL          | Humans, hyraxes       | Urban anthroponotic    | OW               | Eastern Mediterranean, the Middle East, and northeastern and southern Africa | 200,000–400 000 CL                                   |
| <i>Leishmania (Leishmania) aethiopica</i> | CL, DCL, DsCL, and oronasal CL | Hyraxes               | Rural zoonotic         | OW               | Ethiopia and Kenya                                                           | 20,000–40,000 CL                                     |
| <i>Leishmania (Leishmania) major</i>      | CL                             | Rodents               | Rural zoonotic         | OW               | Iran, Saudi Arabia, north Africa, the Middle East,                           | 230,000–430,000 CL                                   |

|                                                                    |                               |                                                                                    |                          |                 |                                                                                                           |                                                                                                                           |
|--------------------------------------------------------------------|-------------------------------|------------------------------------------------------------------------------------|--------------------------|-----------------|-----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|
|                                                                    |                               |                                                                                    |                          |                 | central Asia,<br>and west Africa                                                                          |                                                                                                                           |
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>infantum</i>      | VL and<br>CL                  | Dogs,<br>rodents,<br>rabbits and<br>hares,<br>foxes,<br>opossums,<br>and<br>humans | Peridomestic<br>zoonotic | OW<br>and<br>NW | China,<br>southern<br>Europe, Brazil,<br>and South<br>America for VL<br>and CL; Central<br>America for CL | 6200–12,000<br>cases of Old<br>World VL and<br>4500–6800<br>cases of New<br>World VL;<br>unknown<br>number of CL<br>cases |
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>mexicana</i>      | CL, DCL,<br>and<br>DsCL       | Rodents<br>and<br>marsupials                                                       | Sylvatic<br>zoonotic     | NW              | South America                                                                                             | Limited number<br>of cases,<br>included in the<br>187,200–<br>300,000 total<br>cases of New<br>World CL                   |
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>venezuelensis</i> | CL                            | Unknown                                                                            | Zoonotic                 | NW              | Venezuela                                                                                                 | Limited number<br>of cases,<br>included in the<br>187,200–<br>300,000 total<br>cases of New<br>World CL                   |
| <i>Leishmania</i><br>( <i>Leishmania</i> )<br><i>amazonensis</i>   | CL, DCL,<br>and<br>DsCL       | Opossums<br>and<br>rodents                                                         | Sylvatic<br>zoonotic     | NW              | South America                                                                                             | Limited number<br>of cases,<br>included in the<br>187,200–<br>300,000 total<br>cases of New<br>World CL                   |
| <i>Leishmania</i><br>( <i>Viannia</i> )<br><i>braziliensis</i>     | CL,<br>MCL,<br>DCL,<br>and LR | Dogs,<br>humans,<br>rodents,<br>and horses                                         | Sylvatic<br>zoonotic     | NW              | South America                                                                                             | Majority of the<br>187 200–<br>300 000 total<br>cases of New<br>World CL                                                  |
| <i>Leishmania</i><br>( <i>Viannia</i> )<br><i>guyanensis</i>       | CL,<br>DsCL,<br>and MCL       | Opossums,<br>sloths, and<br>anteaters                                              | Sylvatic<br>zoonotic     | NW              | South America                                                                                             | Limited number<br>of cases,<br>included in the<br>187,200–<br>300,000 total                                               |

|                                         |                 |                     |                   |    |                                |                                                                                      |
|-----------------------------------------|-----------------|---------------------|-------------------|----|--------------------------------|--------------------------------------------------------------------------------------|
|                                         |                 |                     |                   |    |                                | cases of New World CL                                                                |
| <i>Leishmania (Viannia) peruviana</i>   | CL              | Unknown, dogs?      | Zoonotic          | NW | Peru, Bolivia                  | Limited number of cases, included in the 187,200–300,000 total cases of New World CL |
| <i>Leishmania (Viannia) panamensis</i>  | CL, MCL and DCL | Rodents, dogs?      | Sylvatic zoonotic | NW | Central and South America      | Limited number of cases, included in the 187,200–300,000 total cases of New World CL |
| <i>Leishmania (Viannia) lainsoni</i>    | CL              | Rodents, porcupines | Sylvatic zoonotic | NW | Brazil, Bolivia, Peru, Ecuador | Limited number of cases, included in the 187,200–300,000 total cases of New World CL |
| <i>Leishmania (Viannia) lindenbergi</i> | CL              | Unknown             | Zoonotic          | NW | Brazil                         | Limited number of cases, included in the 187,200–300,000 total cases of New World CL |
| <i>Leishmania (Viannia) naiffi</i>      | CL              | Rodents, Anteaters  | Sylvatic zoonotic | NW | Brazil, French Guyana          | Limited number of cases, included in the 187,200–300,000 total cases of New World CL |
| <i>Leishmania (Viannia) shawi</i>       | CL              | Rodents, sloths     | Sylvatic zoonotic | NW | Brazil                         | Limited number of cases, included in the 187,200–300,000 total                       |

|                                             |           |         |                          |           |                                           |                         |
|---------------------------------------------|-----------|---------|--------------------------|-----------|-------------------------------------------|-------------------------|
|                                             |           |         |                          |           |                                           | cases of New World CL   |
| <i>Leishmania colombiensi</i> s             | CL and VL | Sloths  | Sylvatic zoonotic        | NW        | Colombia                                  | Limited number of cases |
| <i>Leishmania (Mundinia) martiniquensis</i> | CL and VL | Unknown | Likely sylvatic zoonotic | OW and NW | Martinique, Thailand, Central Europe, USA | Limited number of cases |
| <i>Leishmania (Mundinia) orientalis</i>     | CL and VL | Unknown | Likely sylvatic zoonotic | OW and NW | Thailand                                  | Limited number of cases |

*Table 2. Human pathogenic Leishmania species and their known reservoirs, transmission, and epidemiology. Adapted from (Cecilio et al., 2022)*

Primary prevention against leishmaniasis bears much similarity with strategies used for malaria, namely providing at-risk populations with interventions against sandfly bites (Montenegro Quinonez et al., 2021; Stockdale & Newton, 2013). This includes the use of bug nets for beds and effective insecticides and insect repellants, though emerging insecticide resistance amongst sandflies is a cause for concern (Arzamani et al., 2017; Chowdhury et al., 2018). Unfortunately, due to the sheer amount of *Leishmania* animal reservoirs, especially sylvatic reservoirs, interventions targeting reservoir-human transmission are seemingly impossible (Gonzalez et al., 2015). However, there have been several studies attempting to control leishmaniasis in canine populations using vaccination, culling, and insecticide-collars (Morales-Yuste et al., 2022; Werneck et al., 2014). In human communities, educational programs may be as important as primary prevention; by strengthening positive beliefs and removing stigmas related to leishmaniasis, reporting rates and early recognition can be greatly improved in these communities and also encourage healthcare-seeking behavior (Alidosti et al., 2021).

### 1.1.3 Treatment

The effective management of leishmaniasis heavily relies on early diagnosis and prompt treatment for both CL and VL, though it becomes a matter of life or death in the case of VL (Modabber et al., 2007; Moore & Lockwood, 2010). The development of leishmaniasis in immunocompromised individuals, especially those with human immunodeficiency virus (HIV), presents a unique challenge due to the high likelihood and rapid onset of worsening disease due to uncontrolled parasite expansion in both CL and VL, often requiring combined long-term therapies (Lindoso et al., 2016). Regarding CL specifically, successful early treatment not only accelerates healing and minimizes scarring but also reduces the risk of complications and further disease development (MCL and DCL/DsCL). However, treatment outcomes can vary depending on factors such as the parasite species responsible for infection, its virulence, host-related factors, and geographic location (Pinart et al., 2020). Various treatments for leishmaniasis have been introduced, including chemotherapy (Berman, 1997), thermotherapy (Mosleh et al., 2008; Silva et al., 2021), and less commonly used alternative methods (Orabi et al., 2023). Still, leishmanicidal drug therapy remains the intervention of choice.

Amongst the many antileishmanial drugs that have been or are currently in use, antimonials remain the first-line CL treatment in most countries, especially when the causative agent and clinical presentation are not yet known. Sodium stibogluconate (SSG) and meglumine antimoniate (MA) are the two major antimonials still in use against CL (Madusanka et al., 2022). The exact mechanism of action is unknown, but it is suggested that antimonials can activate infected macrophages to kill intracellular *Leishmania* (Mookerjee Basu et al., 2006) and are also inherently antileishmanial themselves, whether it be as a prodrug or through its action on trypanothione reductase (Haldar et al., 2011; Krauth-Siegel & Comini, 2008). Other common drugs include amphotericin B, miltefosine, paromomycin, as well as topical and systemic azoles (Madusanka et al., 2022). Problematically, the efficacy of many of these drugs is species specific, especially pentavalent antimony. Despite this, these chemotherapeutic agents have significantly contributed to reducing CL transmission and

alleviating disease burden over many years. However, their effectiveness is limited by challenges such as the emergence of drug resistance leading to reduced efficacy, prolonged treatment durations, and frequent relapses, in addition to the serious side effects of these drugs (Ponte-Sucre et al., 2017).

MCL treatment is mostly the same as CL treatment, though treatment regimens often require higher dosage. Antimonials remain the gold standard of treatment, with SSG being used for intramuscular and intravenous injections whereas MA is used for intramuscular injections only (Palumbo, 2010). Pentamidine, an aromatic diamidine, is also used to treat MCL as an alternative to antimonials and is in fact the first-line treatment against *L. guyanensis* (Nacher et al., 2001). While it has noted toxicity against protozoa and fungi, its mechanism of action is unknown. In Colombia, pentamidine isethionate was demonstrated to have a similar cure rate to MA in CL treatment (96% vs 91%) using a short-course low-dose regimen, using only 4 2mg/kg doses over the course of 8 days (Soto et al., 1994). However, the risk of diabetes mellitus development during MCL treatment is elevated due to the higher dose requirement (Amato et al., 1998). One major challenge in MCL treatment is that many topical treatments available for CL, such as cryotherapy, are ineffective in treating the extensive mucosal damage that can result from MCL lesions (Gurei et al., 2000; Palumbo, 2010). However, the topical application of antileishmanial ointment, specifically containing paromomycin and methylbenzethonium chloride, has been demonstrated to be effective (Arana et al., 2001). Additionally, the direct injection of antimonials into lesions maximizes local efficacy with little systemic side effects, though this method does not reach metastatic infections (Faghihi & Tavakoli-kia, 2003; Palumbo, 2010).

The treatment of VL relies almost entirely on antileishmanial drugs, unlike CL, where several alternative treatment methods have been tested and validated. In addition to the use of antimony, amphotericin B (AmB) is a common treatment for VL as well as for CL, commonly used as a second-line treatment in leishmaniasis cases that are unresponsive to SSG treatment (Berman, 1997). It is a polyene antifungal agent that also possesses antileishmanial properties, binding to ergosterol in the cell membrane leading to ion leakage

and cell death (Stone et al., 2016). Problematically, AmB exhibits high toxicity, and while this is not a great issue with CL because it can be given as an intralesional injection, it must be injected intravenously to treat VL (Moore & Lockwood, 2010). With the new formulation of liposomal amphotericin B (LAmB), however, not only was toxicity reduced but tissue penetration was also enhanced, making LAmB an effective treatment option for VL (Adler-Moore & Proffitt, 2003). The only downside of LAmB is its high cost due to it being a private formulation under the trade name AmBisome®, so it is often kept as a second-line drug for VL as with CL. However, the significantly higher cure rates associated with LAmB compared to SSG (100% compared to 70%) makes research into lowering LAmB dosage while maintaining high clearance rate highly important (Mishra et al., 1994). Indeed, several studies in India have shown that a single dose regimen of 7.5-10mg/kg LAmB is sufficient to achieve clearance rates comparable with traditional multidose regimens (Sundar et al., 2010; Sundar et al., 2003). Unfortunately, this success may not translate well to other settings like Africa, where *Leishmania* parasite load tends to be higher in comparison (Moore & Lockwood, 2010). It has already been shown that LAmB efficacy against *L. braziliensis* is dose dependent (Motta & Sampaio, 2012). Comparatively, while the treatment of PKDL is much the same as VL treatment, the main challenge is the detection of PKDL as it is commonly asymptomatic, leading to patients rarely seeking treatment (Datta et al., 2021).

Considering the lack of a universally effective treatment for leishmaniasis as well as the high toxicity and severe side effects of currently available antileishmanial drugs, new treatment and prevention strategies are desperately needed, with many studies assessing the viability of *Leishmania* vaccines and alternate antileishmanial mechanisms of action (Dinc, 2022; Olivier & Fernandez-Prada, 2019).

#### 1.1.4 *Leishmania* life cycle

*Leishmania* parasites exhibit a digenic lifecycle and reproduce primarily through binary fission (Figure 2). They alternate between a sandfly insect vector and mammalian host, transforming from a motile flagellated promastigote form to an immotile elliptical amastigote form (Sunter & Gull, 2017). *Leishmania* promastigotes, approximately 8-10µm in

length, reside within the gut of female sandflies of the genera *Phlebotomus* in the old world (OW) and *Lutzomyia* in the new world (NW). Additionally, it is well known that specific species of *Leishmania* are adapted to the midguts of specific species of sandfly (Sacks, 2001; Volf & Myskova, 2007).

Over a 7-day period after *Leishmania* parasites enter its sandfly vector via bloodmeal, procyclic promastigotes will travel through the midgut and undergo transformation into metacyclic promastigotes. This process is developmentally regulated by changes in surface molecules such as lipophosphoglycan (LPG), which mediates attachment and detachment from galectin receptors on the surface of midgut cells and allows the developing promastigotes to move from the posterior to anterior midgut (Kamhawi et al., 2004; Sacks et al., 2000). Additionally, expression of viral factors like the major surface protease GP63 is augmented, preparing the parasites for host-infection by enhancing resistance to complement-mediated lysis and overall infectivity of the parasite (Bates, 2007). Autophagy has also been implicated as a key process necessary for metacyclogenesis (Besteiro et al., 2006).

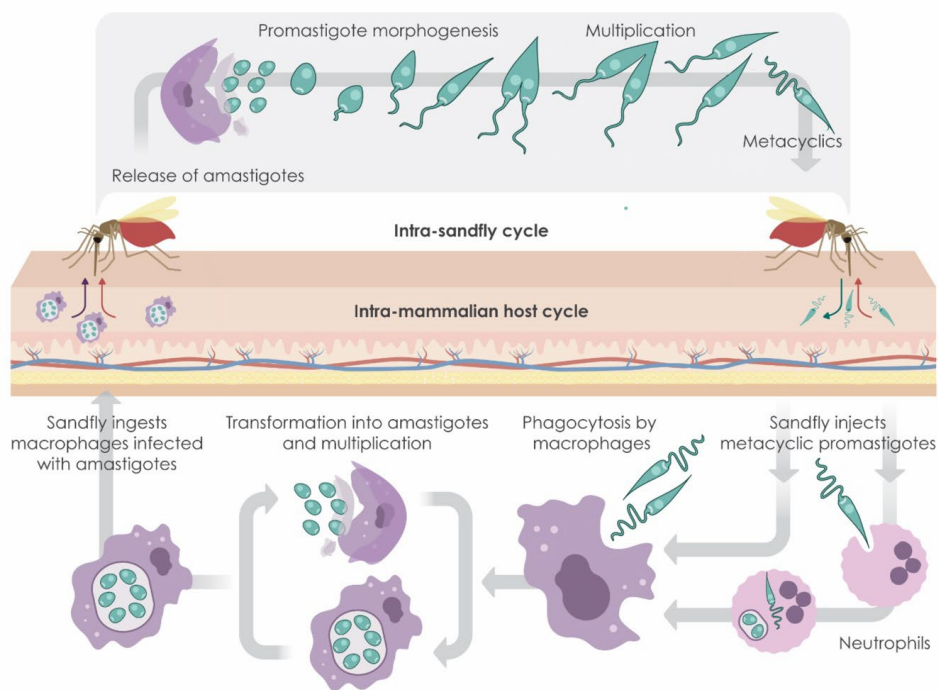


Figure 2. Digenic life cycle of *Leishmania*. Adapted from (Olivier et al., 2019)

When an infected sandfly takes a bloodmeal from a mammalian host, it regurgitates anywhere from 10-10000 highly infective metacyclic promastigotes (Kimblin et al., 2008). This is mediated by the secretion of promastigote secretory gel (PSG) by leptomonad promastigotes, leading to the formation of a PSG plug in the anterior midgut, promoting sandfly regurgitation during bloodmeal (Bates, 2007; M. E. Rogers et al., 2002). Along with the *Leishmania* parasites, the sandfly saliva itself contains proteins that are anti-hemostatic and highly immunogenic, influencing the host immune response to *Leishmania* infection (Gomes & Oliveira, 2012). Immediately after injection, innate immune cells such as macrophages, neutrophils, and dendritic cells phagocytose the parasites. Although macrophages are the primary host cells for *Leishmania* parasites during its mammalian life cycle, neutrophil phagocytosis has been suggested to play an important role in establishing a successful initial infection by acting as a trojan horse of sorts for the parasites (Peters et al., 2008).

Inside the macrophage's phagolysosome, *Leishmania* parasites transform into elliptical, aflagellated, nonmotile amastigotes, approximately 2-3 $\mu$ m in diameter. This transformation is triggered by a variety of factors, the most important of which being an increase in temperature to 37°C and a decrease in pH to 5.5 within the phagolysosome (Barak et al., 2005). In its amastigote form, *Leishmania* undergoes a shift in metabolism and enters a quiescent state (McConville et al., 2015). This shift is accomplished by a rewiring of the parasite carbon metabolism resulting in decreased glucose and amino acid intake, leading to a so called “stringent response” (Saunders et al., 2011; Saunders et al., 2014). This stringent response protects the amastigote from cellular stress resulting from host-immune response, drug pressure, and nutrient excess (McConville et al., 2015). The following replication of amastigotes inside the phagolysosome eventually ruptures the host macrophage, releasing the parasites to infect other macrophages and spread the infection. Amastigotes within infected cells can also be ingested during another sandfly bloodmeal and transform back into procyclic promastigotes, thereby completing the lifecycle. Even with robust host immune response, *Leishmania* parasites are almost never fully cleared, with tissue-resident non-phagocytic fibroblasts serving as the likely long term hosts. This

likely contributes to the acquired long-term immunity to *Leishmania* re-infection (Kaye & Scott, 2011). Contrastingly, mannose receptor high M2-like dermal macrophages have been suggested to serve as a safe haven for *Leishmania* in the skin of infected hosts (Lee et al., 2018).

### 1.1.5 The sandfly: vectors and deliverers of *Leishmania*

Most *Leishmania* spp. that are pathogenic towards humans are associated with zoonotic transmission, with only *L. donovani* being an exception as previously discussed. The zoonotic nature of *Leishmania* spp., especially since many species also have at least one sylvatic reservoir (Table 2), contributes to the epidemiological complexity of leishmaniasis, making control efforts challenging. The risk of leishmaniasis spreading to non-endemic regions is heightened by the movement of parasites and vectors into new areas, driven by changing weather patterns, globalization, and human migration (Berry & Berrang-Ford, 2016; Curtin & Aronson, 2021; Georgiadou et al., 2015; Ready, 2010). As a result, a comprehensive understanding of leishmaniasis requires not only the study of clinical aspects, particularly parasite-host interactions, but also an understanding of the sandfly vectors and their interactions with both *Leishmania* parasites and animal/human hosts.

Sandflies are arthropods belonging to the order Diptera (two-winged flies), within the suborder Nematocera, family *Psychodidae*, and subfamily *Phlebotominae* (Akhoundi et al., 2016). The sandfly life cycle consists of four main stages: eggs, larvae, pupae, and adults, and unlike mosquitoes and other Diptera, there is no aquatic stage (Maroli et al., 2013). Both male and female adult sandflies feed on sugary secretions, though females require at least one bloodmeal in order to complete egg development. Oviposition occurs 5-8 days after bloodmeal, although some species are known to feed multiple times before successfully developing viable eggs while others are known to not require any bloodmeal at all (Cecilio et al., 2022; Volf & Volfova, 2011). Typically, a female sandfly lays between 30 to 70 eggs in sheltered locations selected for the availability of organic matter and sufficient humidity (Killick-Kendrick, 1999). Both humidity and temperature play crucial roles in sandfly development, with adult sandflies preferring humid resting places and breeding grounds

(Benkova & Volf, 2007; Erguler et al., 2019). This dependency on specific environmental conditions also explains why sandflies are typically found in regions where temperatures exceed 15.6°C for at least three months of the year. This temperature range covers most of the world, spanning from a latitude of 50° N to 40° S, although sandflies are notably absent from New Zealand and the Pacific islands (Figure 3).

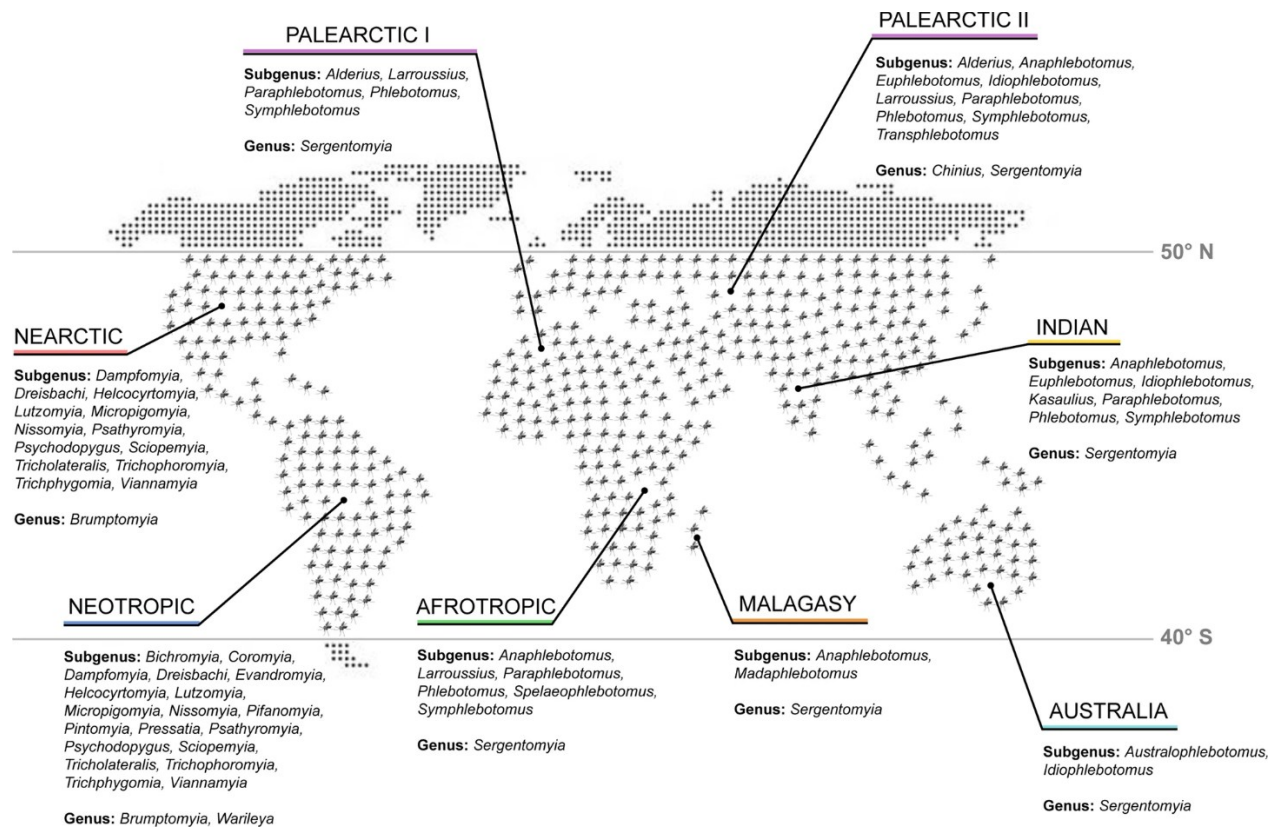


Figure 3. Global distribution of sandfly species. Sandflies have a global distribution between latitude 50° N and 40° S (demarked by the gray horizontal lines), excluding New Zealand and the Pacific islands. Reproduced from (Cecilio et al., 2022).

As mentioned above, *Leishmania* spp. infecting sandflies are known to form a PSG plug in the anterior midgut (Bates, 2007). This process starts with the secretion of PSG, composed of *Leishmania* proteophosphoglycans (PPGs), by leptomonad promastigotes and blocks efficient blood feeding by the infected sandfly. One important effect of this is that infected sandflies are observed to increase bloodmeal frequency, taking longer to feed and yet often taking incomplete meals compared to uninfected sandflies (Killick-Kendrick et al., 1977; M.

E. Rogers et al., 2002). These effects together increase exposure of potential hosts and reservoirs to *Leishmania* infection and are augmented by parasite burden, as more parasites can generate a larger PSG plug (Serafim et al., 2018). Next, during the bloodmeal itself, the PSG plug causes regurgitation in the sandfly, increasing *Leishmania* reflux and metacyclic promastigote deposition in the wound following the “blocked fly hypothesis” (Rogers, 2012). Along with the metacyclic promastigotes, an infectious inoculum is also regurgitated at the bloodmeal site. This inoculum contains both parasite- and vector-derived factors that can augment *Leishmania* infection and survival. The most immediately obvious parasite-derived factor is PSG itself, known to exacerbate both CL and VL disease progression (Rogers, 2012). Other parasite-derived factors are secreted, typically as extracellular vesicles (EVs) into the sandfly midgut and egested along with *Leishmania* parasites themselves (will be covered in depth at a later point) (Atayde et al., 2015). The most apparent vector-derived factor is the sandfly saliva, which is known to enhance infection and promote anti-inflammatory local immune responses (Belkaid et al., 1998; Gomes & Oliveira, 2012; Titus & Ribeiro, 1988). With recent intense interest in gut microbiota, it has been demonstrated that the sandfly microbiome is also an important vector-derived factor (Telleria et al., 2018). The microbiome of the infected sandfly not only influences *Leishmania* development but also is co-egested with the parasites and enhance infection during transmission (Dey et al., 2018; Kelly et al., 2017). This can also lead to the increased recruitment of neutrophils, which are known to potentially serve as “trojan horses” for the infecting *Leishmania* (Laskay et al., 2003; Peters et al., 2008).

Approximately 1000 species and subspecies of sandflies have been identified and described to date (Shimabukuro et al., 2017). Of these, only 10% are proven/suspected vectors of *Leishmania* according to the vector incrimination criteria proposed by Killick-Kendrick and the WHO (Bates, 2008; Killick-Kendrick, 1990; Maroli et al., 2013; World Health, 2010). The most significant vectors of human-pathogenic *Leishmania* spp. are recognized as genus *Phlebotomus* sandflies in the OW and genus *Lutzomyia* sandflies in the NW (Lewis, 1971). In laboratory studies, *Leishmania* sandfly vectors have been categorized into two main groups: restrictive and permissive vectors. Restrictive vectors, such as *Phlebotomus papatasi* and

*Phlebotomus sergenti*, exhibit a high specificity for the *Leishmania* species they transmit in nature (*L. major* and *L. tropica*, respectively) (Volf & Myskova, 2007). In contrast, permissive vectors, such as *Phlebotomus arabicus* and *Lutzomyia longipalpis*, can support the development of multiple *Leishmania* species. For example, recent research demonstrated that *L. longipalpis*, the natural vector of *L. infantum*, can also act as a competent vector for *L. major* under laboratory conditions (Cecilio et al., 2020). This dichotomy between restrictive and permissive vectors is thought to be linked to parasite attachment to the sand fly midgut, a crucial step for the infection to progress within the vector (Dostalova & Volf, 2012). As discussed above, attachment is mediated by the binding of LPG, one of the most abundant *Leishmania* surface proteins, to galectin, surface receptors present on sandfly midgut and mammalian immune cells (Kamhawi et al., 2004; Pelletier et al., 2003; Pelletier & Sato, 2002). Through the study of LPG, its polymorphic nature quickly emerged as a potential mechanism for vector restrictiveness and parasite development within the sandfly midgut, as observed with *P. papatasi* and *L. major* (Forestier et al., 2014). Conversely, permissive vector interactions are less understood, though they likely involve broader or non-specific binding processes mediated by “sticky proteins” in the sandfly midgut, such as mucin-like O-linked glycoproteins (Myskova et al., 2016).

#### 1.1.6 Genetic characteristics of *Leishmania*

The *Leishmania* genome is approximately 33 megabase pairs (Mbps), encoding over 8300 coding genes across 36 chromosomes (Ivens et al., 2005; Wincker et al., 1996). *Leishmania* spp. appear to be genetically similar due to the lack of identifying unique genes between species; for example, gene synteny is conserved for over 99% of genes between *L. major*, *L. braziliensis*, and *L. infantum* (Rogers et al., 2011). Despite this, each *Leishmania* spp. is quite distinct from one another when considering the diverse vectors and reservoirs specific to each *Leishmania* spp. as well as the unique clinical presentations associated with each pathogenic *Leishmania* spp. This can be largely attributed to the genetic organization of *Leishmania* genes as well as their gene expression. Like other trypanosomatids, the *Leishmania* genome contains no introns and little non-coding regions compared to most

eukaryotes (Smith et al., 2007). Like other trypanosomatids, *Leishmania* protein-coding genes are organized in large polycistronic units and transcribed bidirectionally from transcriptional start sites located at strand switch regions (Martinez-Calvillo et al., 2004; Martinez-Calvillo et al., 2003). Messenger RNA (mRNA) transcription is then achieved by trans-splicing a spliced leader RNA (39 nucleotides (nt)) to the 5' end of each mRNA along with 3' polyadenylation (Matthews et al., 1994). Without transcriptional control, *Leishmania spp.* instead regulate gene expression through a combination of translational control, mRNA instability, DNA/chromosomal copy number variation (CNV), and single-nucleotide polymorphisms (SNPs). This is necessary for the parasites to transform throughout their life cycle as well as adapt to changing environments, host immune response, and drug pressure.

In *Leishmania* parasites, mRNA instability plays a crucial role in regulating gene expression throughout their life cycle. This mechanism involves the rapid degradation of mRNA molecules, which helps in tightly controlling the levels of specific proteins needed at different stages of development and is often regulated by 3' untranslated region (UTR) elements (Aly et al., 1994; Charest et al., 1996; Quijada et al., 2000; Wu et al., 2000). The Papadopolou group were able to elucidate one such 3'UTR element through the study of amastin mRNA throughout *Leishmania* development, determining that such stage-specific regulation occurred independent of deadenylation (Boucher et al., 2002; Haile et al., 2008). Eventually, they identified these short U-rich elements (UREs) as short interspersed degenerated retroposons (SIDERs), specifically demonstrating that SIDER2 retroposons promote rapid mRNA degradation through the recruitment of site-specific endonucleases while SIDER1 directly regulates translation (Muller et al., 2010; Smith et al., 2009). Amastin is a transmembrane glycoprotein that is a key marker of amastigote-stage trypanosomatid parasites, making it an exceptional target to study parasite development (Jackson, 2010). This in addition to the many trans-acting RNA-binding proteins identified in *Leishmania* contribute to the post-transcriptional regulation of gene expression (Kramer & Carrington, 2011).

Switching over to DNA, the genome and genetic organization of *Leishmania* are much better understood. Although *Leishmania* parasites were originally considered to be diploid, extensive evidence shows that aneuploidy is the norm (Downing et al., 2011; Leprohon et al., 2009; Rogers et al., 2011; Sterkers et al., 2011; Ubeda et al., 2008). In populations of *Leishmania* parasites, there is evidence of distinct aneuploidy patterns occurring at the single cell level. This phenomenon is known as mosaic aneuploidy, where the cumulative ploidy derived from next-generation DNA sequencing data suggests an average diploid population. However, this masks the reality that only a few parasites share identical ploidy states for individual chromosomes, resulting in a surprisingly high degree of heterogeneity within a single culture or population of *Leishmania* (Lachaud et al., 2014). Without transcription control, this aneuploidy serves to regulate gene expression by increasing or decreasing gene copy number based on environmental and developmental cues. This can happen on the scale of entire chromosomes and is well supported by the strong correlation between DNA and RNA expression levels (Leprohon et al., 2009). CNV also occurs at a smaller scale, mediated by gene rearrangement. In fact, SIDERs are again implicated in this process by providing a backbone to facilitate gene rearrangement (Laffitte et al., 2016).

On top of CNVs, SNPs and small nucleotide insertions or deletions (indels) are another source of *Leishmania* genetic heterogeneity and contribute to drug resistance. This has been observed with experimental drugs as well as with antimony-resistant field isolates (Downing et al., 2011; Imamura et al., 2016; Perez-Victoria et al., 2003; Ritt et al., 2013; Vasudevan et al., 2001). Heightened understanding and accurate sequencing of *Leishmania* CNVs, SNPs, and small nucleotide indels can serve as an important diagnostic tool for predicting drug resistance and clinical outcomes. As mentioned previously, *Leishmania* are thought to replicate by binary fission. However, the extensive level of intra and inter-species genetic heterogeneity makes it tempting to hypothesize a sexual form of reproduction. Indeed, previous studies have suggested that *Leishmania* do have a meiotic stage of their life cycle (Rougeron et al., 2010). More recently, HOP1 and HAP2 have been identified as key components of meiosis-related machinery in *Leishmania* (Catta-Preta et al., 2023).

## 1.2 Host immune response to *Leishmania* infection

One of the most problematic aspects of leishmaniasis is the diverse range of potential clinical presentations of the disease. Disease progression depends on a large number of factors involving the environment, vector, parasite, and host. One of the most important determinants of leishmaniasis progression is the reaction of the host immune system to *Leishmania* infection, with parasite clearance and disease healing depending on a delicate balance of inflammatory response and immune regulation. It is imperative to understand the host immune response to determine potential clinical outcomes as well as the development of new treatments and the improvement of existing treatments for leishmaniasis. At the same time, it is through the study of *Leishmania* infection itself that many aspects of the host immune response have been illuminated.

### 1.2.1 Host immune response to CL and disease progression

The immunopathogenesis of CL is shaped by intricate interactions between the *Leishmania* parasite and the innate and adaptive immune systems of its host; “proper” immune response leads to parasite clearance whereas “improper” immune response can lead to disease progression, resulting in acute and/or chronic disease. Following deposition by sandflies into their mammalian hosts, *Leishmania* parasites engage with various immune cells. Both traditional needle injection studies and newer approaches utilizing infected sandflies indicate the rapid recruitment of a mixed population of neutrophils and monocytes to the site of infection within hours (Matte & Olivier, 2002; Peters et al., 2008). Upon parasite internalization, inactivated macrophages release an initial burst of ROS to control the infection (Volpedo et al., 2021). Macrophages are innate immune cells that are required for parasitic clearance but are also the primary host cells for *Leishmania*, with the parasites surviving, differentiating, and replicating inside the macrophage phagolysosome

(McConville et al., 2007). Macrophages can be classically activated (M1) upon exposure to microbial products and pro-inflammatory cytokines, or alternatively activated (M2) by anti-inflammatory cytokines like interleukin-4 (IL-4), IL-10, and IL-13 (Tomiotto-Pellissier et al., 2018; N. Wang et al., 2014). The initial ROS burst in inactivated macrophages is often inadequate to kill parasites, but M1 macrophages are T helper type 1-skewed (Th1) and strongly active against intracellular threats including *Leishmania*, activating additional microbicidal mechanisms, including increased ROS and nitric oxide (NO) production, in order to achieve parasite clearance (Scott & Novais, 2016). M2 macrophages on the other hand are considered to be in a “resting” state, inhibiting further inflammatory response and providing a permissive environment for *Leishmania* parasites (Almeida et al., 2023).

Other immune cells at the site of infection release chemokines such as MIP-1 $\alpha$ , MIP-1 $\beta$ , and MIP-2, which recruit more inflammatory cells, especially monocytes (Atayde et al., 2016). This process is greatly influenced by the genetic background of the host and the infecting *Leishmania* species (Matte & Olivier, 2002). Neutrophils represent one of the highest populations of phagocytes present in the tissue and have long been overlooked despite their potential to phagocytose *Leishmania*. The group of Laskay et al. confirmed that neutrophils can phagocytose *Leishmania* promastigotes, which then remain intact within apoptotic neutrophils. These apoptotic neutrophils, containing viable parasites, are subsequently phagocytosed by macrophages, facilitating the silent internalization of *Leishmania* (Laskay et al., 2003). However, these experiments were performed with polymorphonuclear neutrophil granulocytes (PMNs), whereas Peters et al. observed that intact parasites can escape apoptotic neutrophils and directly infect macrophages, suggesting that parasites exiting apoptotic neutrophils may be better adapted for macrophage parasitism (Peters et al., 2008). The role of neutrophils is further complicated by studies demonstrating their ability to target and kill *Leishmania* promastigotes using neutrophil extracellular traps (NETs), as well as their important role in recruiting dendritic cells (DCs) through CCL3 (Charmoy et al., 2010; Guimaraes-Costa et al., 2009).

After this initial infection phase, antigen presentation follows, with DCs that have phagocytosed *Leishmania* parasites migrating to primary lymphoid tissues to present *Leishmania* antigens to naïve thymus (T) cells (Th0), initiating an adaptive immune response. Naïve T cells differentiate into T helper type 1 or type 2 (Th1/Th2) effector cluster of differentiation 4+ (CD4+) T cells, determining the course of the *Leishmania* infection (Volpedo et al., 2021). Research into the immune response during CL in mice generally identifies a dichotomy between Th1 and Th2 responses, where Th1 responses typically lead to infection control and healing, while Th2 responses exacerbate the infection (Tripathi et al., 2007). The Th1-mediated control of CL relies on IFN- $\gamma$ , produced by CD4+ T cells, CD9+ T cells, and natural killer (NK) cells, which activates macrophages to eliminate parasites through the production of nitric oxide (NO). IL-12 plays a critical role in promoting the Th1 response and IFN- $\gamma$  production, and its inhibition skews the immune response towards Th2, contributing to disease exacerbation and can lead to DCL (Burza et al., 2018; Tripathi et al., 2007). In contrast, a Th2 immune response characterized by IL-4 production results in an antibody response that does not effectively resolve the infection. IL-10 has been identified by several studies as a pivotal cytokine that dampens immune activation and promotes infection persistence (Kaye & Scott, 2011; K. A. Rogers et al., 2002). Resulting CL disease outcomes from a predominantly Th2 immune response resemble those stemming from immunosuppression, whether due to disease states like HIV/AIDS or through medical treatments. HIV-infected individuals are particularly susceptible to developing severe and atypical forms of CL due to their compromised immune systems (Burza et al., 2018; Lindoso et al., 2016). The diminished ability to mount effective Th1 responses and control parasite replication increases the risk of chronic and disseminated leishmaniasis, posing challenges for treatment and management.

While the Th1 response is crucial for parasite clearance, it has a limited window of protective immunity (Hartley et al., 2013). Excessive skewing of the Th1/Th2 balance towards an inflammatory phenotype can be detrimental, as seen in MCL, which is marked by excessive production of pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  (Scott & Novais, 2016). The etiology of parasitic dissemination in MCL is not fully understood, but the condition is

characterized by uncontrolled inflammatory cascades and cytotoxicity. During lesion ulceration, the ratio of CD4+:CD8+ T cells shifts in favor of CD8+ T cells, which express high levels of granzyme and natural killer group 2D (NKG2D), leading to cell death within the lesions (Crosby et al., 2014). MCL biopsies often show large populations of neutrophils and Th17 cells, high levels of IL-17-inducing cytokines, and increased antileishmanial antibody titers, all contributing to apoptosis and tissue damage (Boaventura et al., 2010; Nylen & Eidsmo, 2012). IL-17 is produced by a subset of T cells that establish a Th17 response, which has both protective and pathogenic roles depending on the context of infection (Goncalves-de-Albuquerque et al., 2017). In some cases, excessive Th17 activity can exacerbate tissue inflammation and contribute to disease severity (Gonzalez et al., 2020). This is commonly noted to occur together with high levels of neutrophil infiltration and activity, suggesting a synergistic effect on disease progression from CL to MCL (Boaventura et al., 2010; Goncalves-de-Albuquerque et al., 2017).

### 1.2.2 Host immune modulation by *Leishmania*

As discussed above, *Leishmania* parasites have uniquely evolved to infect macrophages, key components of mammalian immune response, and the success of the parasite depends entirely upon whether its mammalian host mounts the “proper” immune response for parasite clearance. Thus, it is no surprise that *Leishmania* spp. have evolved to modulate the host immune response. This begins during the early stages of infection, when *Leishmania* promastigotes come into contact with phagocytic immune cell surface receptors. Toll-like receptors (TLRs) are among the most important and earliest discovered of these (Lemaitre et al., 1996). They are considered pattern-recognition receptors (PRRs), a broad category of innate immune cell receptors that identify pathogen-associated molecular patterns (PAMPs) (Li & Wu, 2021).

TLRs, along with other PRRs like nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs), are specialized for detecting common PAMPs like bacterial lipopolysaccharide (LPS), viral double-stranded RNA, and non-methylated cytidine monophosphate guanosine (CpG) DNA motifs. These receptors trigger

different downstream signaling pathways depending on the specific TLR triggered, usually leading to immune activation, cytokine and interferon production, and the enhancement and polarization of adaptive immunity suitable for the detected pathogen (Schenten and Medzhitov, 2011). TLRs are expressed by almost all innate immune cells, as well as T- and B-cells, and each TLR and cell type is specialized in recognizing specific PAMPs. Studies have highlighted the involvement of TLRs 2, 3, 4, 7, and 9 in *Leishmania* infections, observed both *in vitro* and *in vivo* with macrophages, dendritic cells (DCs), neutrophils, and natural killer (NK) cells (de Carvalho et al., 2019; Halliday et al., 2016; Murray et al., 2013; Paun et al., 2011; Pratti et al., 2019; Regli et al., 2020; Sacramento et al., 2015; Tolouei et al., 2013). It remains unclear whether TLRs play a protective role against the parasite or if they are exploited by the parasite for its benefit, seemingly depending on the infecting *Leishmania* species, model used, and host genetic background (Faria et al., 2012). Additionally, it is well reported that signaling molecules downstream of TLRs are directly modulated during *Leishmania* infection, complicating TLRs' role in host-parasite interactions.

One such downstream signalling molecule is nuclear factor kappa-light-chain-enhancer of activated B (bursa-derived) cells (NF- $\kappa$ B) is a fundamental transcription factor (TF) found in animal cells and plays a crucial role in immune activation. It responds to a range of extracellular signals, including pathogens, cytokines, and mitogens, driving the production of various cytokines, chemokines, and immune receptors in macrophages and other immune cells (Liu et al., 2017). Given its extensive role, NF- $\kappa$ B members are often referred to as “master regulators” of immune cell function. The NF- $\kappa$ B family includes NF- $\kappa$ B1 (P50), NF- $\kappa$ B2 (P52), v-rel avian reticuloendotheliosis viral oncogene homolog A (RelA) (P65), cRel, and RelB. The activity of NF- $\kappa$ B is regulated by the dimerization of these proteins, either as homodimers or heterodimers (Lawrence, 2009). In their inactive state, NF- $\kappa$ B proteins are sequestered in the cytoplasm by Inhibitors of NF- $\kappa$ B (I $\kappa$ B). Phosphorylation of I $\kappa$ B by I $\kappa$ B Kinases (IKK) leads to its dissociation from NF- $\kappa$ B, allowing the TFs to dimerize and move into the nucleus (Israel, 2010). IKKs are activated through various upstream signaling pathways, including those mediated by MAPKs and TLRs. *Leishmania* spp. can directly modify NF- $\kappa$ B signalling through the cleavage of p65 RelA by GP63, resulting in a truncated p35 RelA. This

modified RelA subsequently translocates into the nucleus and can still bind p50, forming a unique and active heterodimer that induces a unique set of chemokines, inhibiting standard NF- $\kappa$ B signalling and inflammatory response (Gregory et al., 2008). Additionally, Cameron et al. found that NF- $\kappa$ B translocation is directly inhibited following infection with *L. mexicana* amastigotes, along with the degradation of IKKs and p65 and resulting in IL-12 inhibition (Cameron et al., 2004).

In macrophages, mitogen-activated protein kinases (MAPKs) are also downstream signalling molecules of TLR and thus are crucial for activating macrophages and driving inflammatory responses upon PAMP recognition. MAPKs are highly conserved enzymes that play a critical role in translating a range of external signals into essential cellular functions such as growth, proliferation, differentiation, migration, and apoptosis (Liu et al., 2007; Zhang & Liu, 2002). MAPK activation involves a cascade of phosphorylation events: a MAPK kinase (MAPKK) is phosphorylated by a MAPK kinase (MAPKK), which activates MAPK itself. Once activated, MAPKs can phosphorylate various targets and significantly alter cellular activation states. Additionally, MAPKs can move into the nucleus to directly modify and phosphorylate TFs (Plotnikov et al., 2011). The MAPK family includes three main types: extracellular signal-regulated kinases (ERK) 1/2, Jun N-terminal kinases (JNK), and p38, though new members continue to be identified (Cargnello & Roux, 2011). *Leishmania* have been demonstrated to inhibit MAPK-mediated signalling by reducing MAPK phosphorylation, specifically implicating LPG (Prive & Descoteaux, 2000). This helps explain how *Leishmania* infection impairs macrophage response to stimuli like LPS (Nandan et al., 1999). This MAPK dephosphorylation has been hypothesized to be a result of protein tyrosine phosphatase (PTP) activation by *Leishmania* infection.

Phosphorylation is a crucial and widespread post-translational modification (PTM) that plays a key role in regulating protein function and signal propagation through signalling pathways, as demonstrated above. This modification typically occurs on residues with polar -OH groups, such as serine (Ser), threonine (Thr), and tyrosine (Tyr). The addition or removal of phosphate groups can lead to significant conformational changes in proteins, which can

either activate or inhibit their function as well as affect their localization (Ardito et al., 2017). The processes of phosphorylation and dephosphorylation are tightly regulated by kinases and phosphatases, with these two enzyme-types serving as major building blocks of signalling pathways (Christian et al., 2016). *Leishmania* infection has been shown to directly affect immune cell phosphorylation in order to modulate host immune response, as briefly described above. *Leishmania*-induced inhibition of ERK phosphorylation leads to the suppression of NF- $\kappa$ B as well as activator protein 1 (AP-1), another important activator of immune cell response and propagation (Eferl & Wagner, 2003; Ghosh et al., 2002). It was also shown that *L. donovani*-induced inhibition of NO production is dependent on host Src homology region 2 domain-containing phosphatase 1 (SHP-1), an important host PTP (Forget et al., 2006). SHP-1 is expressed in a variety of immune cells, including those of both myeloid and lymphoid origin, and it is found at lower levels in other cell types. It works in conjunction with SHP-2 to regulate immune signalling and response through the dephosphorylation of key signalling molecules and receptors (Lorenz, 2009). Abu-Dayyeh et al. found interleukin-1 receptor-associated kinase 1 (IRAK-1), a signaling protein downstream of MyD88 and many TLRs, to be dephosphorylated by SHP-1 following infection, rendering the macrophage unresponsive to many TLR ligands such as LPS and CpG (Abu-Dayyeh et al., 2008). The authors postulate that *Leishmania*-induced SHP-1 dephosphorylation acts on a conserved kinase tyrosine-based inhibitor motif (KTIM) and can therefore inactivate other KTIM-bearing kinases such as Janus kinases (JAKs) and MAPKs (Abu-Dayyeh et al., 2010; Blanchette et al., 1999; Forget et al., 2006; Nandan et al., 1999).

JAKs together with signal transducer and activator of transcription (STAT) TFs form a key signalling pathway for the production of cytokines and growth factors, as well as the transcription of key activator genes like inducible nitric oxide synthase (iNOS) and IL-12 (Hu et al., 2021; Shio et al., 2012). *Leishmania* exploits the regulatory mechanisms of this pathway, favoring signalling inhibition to prevent immune cell activation and parasite clearance. This pathway is regulated by PTPs like SHP-1 as previously discussed. and members of the suppressors of cytokine signaling (SOCS) family. Proteins belonging to the suppressors of cytokine signaling (SOCS) family also regulate JAK/STAT signalling, with

evidence of SOCS3 induction following *L. donovani* infection (Bertholet et al., 2003; Chandrakar et al., 2020). Beyond JAK2 dephosphorylation, *Leishmania* may also inhibit the JAK2/STAT-1 pathway through proteasome-mediated degradation of STAT-1. Forget et al. observed STAT-1 $\alpha$  degradation following *Leishmania* infection, which was reversed by proteasome inhibitors. However, JAK/STAT signaling was not fully restored, likely due to JAK2 dephosphorylation (Forget et al., 2005). A complementary mechanism for JAK2/STAT-1 inhibition is the inhibition of STAT-1 $\alpha$  nuclear translocation by blocking its association with Importin- $\alpha$ 5, though the mechanism behind this remains unclear (Matte & Descoteaux, 2010).

### 1.2.3 *Leishmania* virulence factors

As discussed above, *Leishmania* parasites have uniquely evolved to infect macrophages, key components of mammalian immune response, and the success of the parasite depends entirely upon whether its mammalian host mounts the “proper” immune response for parasite clearance. Thus, it is no surprise that *Leishmania* spp. have evolved a veritable arsenal of virulence factors to modulate host immune response. One of the most important and earliest discovered of these, glycoprotein 63 (GP63), is the most abundant surface protein of *Leishmania* promastigotes and comprises approximately 1% of the entire parasite proteome (Bouvier et al., 1985; Yao et al., 2003). Also known as leishmanolysin or major surface protease (MSP), GP63 is a zinc-dependent metalloprotease found on the surface of *Leishmania* parasites. It is attached to the parasite via a glycosphosphatidyl-inositol (GPI) anchor, which can be cleaved by phospholipase C to shed surface-bound GP63 directly into the environment (Mensa-Wilmot et al., 1994). However, as early as 2002, evidence has arisen that GP63 can be directly secreted independent of GPI cleavage (McGwire et al., 2002). The gene encoding GP63 is organized in tandem arrays in the genome, with variations in the C-terminal region and the 3' UTR. These variations in the 3'-UTR contribute to the differential regulation of GP63 expression depending on the developmental stage of the parasite (Yao et al., 2003).

GP63 is known for its broad substrate specificity, with the ability to cleave a wide range of peptides, preferentially cleaving between polar and hydrophobic residues at positions P1 and P'1, with basic residues at positions P'2 and P'3 (Ip et al., 1990). GP63 also acts as its own substrate, functioning as a zymogen that is activated during the later stages of its post-translational processing. While GPI-anchored GP63 is continually shed from the parasite surface, internal reserves of GP63 are released only in response to specific stimuli, such as increased temperature or contact with host molecules (Yao et al., 2007). Mutants of *L. major* lacking GP63 exhibit significantly reduced virulence both *in vivo* and *in vitro* (Joshi et al., 2002; Thiakaki et al., 2006). This supports previous research suggesting that GP63 cleaves the complement protein C3b into inactive iC3b, aiding the parasite in evading complement-mediated lysis (Brittingham et al., 1995). This ability to evade the complement system is correlated with increased GP63 expression in metacyclic promastigotes, reducing their susceptibility to complement-mediated destruction and in fact increasing macrophage attachment through the binding of complement receptor (CR) 3 to GP63 (Russell & Wright, 1988). Additionally, GP63 degrades extracellular matrix (ECM) components such as collagen IV and fibronectin, facilitating the migration of promastigotes through the ECM (McGwire et al., 2003). Another potential GP63-mediated mechanism for immune evasion is the direct inhibition of NK cells (Lieke et al., 2008). The importance of GP63 was further investigated in a recent study, finding that varying GP63 abundance does not impact initial inflammatory cell recruitment nor immune effector function and activation (Chan et al., 2021).

It has been clearly demonstrated that GP63 can also enter the cytoplasm and nucleus of macrophages, giving the zinc metalloprotease access to a wide range of intracellular host proteins (Figure 4). It is well reported that GP63 cleaves a wide range of PTPs, including SHP-1 as discussed above. Along with SHP-1, PTP1B and T-cell protein tyrosine phosphatase (TCPTP) were also demonstrated to be targets of GP63, all being PTPs that interact with JAK2, though their cleavage was dependent on the intact function of lipid rafts (Gomez et al., 2009). The cleavage of these PTPs leads to their activation and the dephosphorylation of key signalling molecules like JAK2, MAPKs, and IRAK1, modulating immune response through their inactivation. GP63 is also responsible for the direct cleavage of p65 RelA, AP-1, and p38

MAPK, the effects of which have been previously discussed (Contreras et al., 2010; Gregory et al., 2008; Halle et al., 2009). Additionally, GP63 has also been shown to cleave mammalian target of rapamycin (mTOR), a component of the mTOR complex 1 (mTORC1). mTORC1 is required for protein translation initiation and is a key component of innate immune response, thus its inactivation through mTOR cleavage favors *Leishmania* survival and propagation (Jaramillo et al., 2011). Interestingly, GP63 has also been found to cleave the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE) vesicle-associated membrane protein 8 (VAMP8), causing a reduction in MHCI antigen presentation and inhibiting phagosome oxidative activity (Matheoud et al., 2013). This further implies that GP63 may interfere with macrophage vesicle function. However, the extent of host protein cleavage by GP63 has recently been called into question (Guay-Vincent et al., 2022).

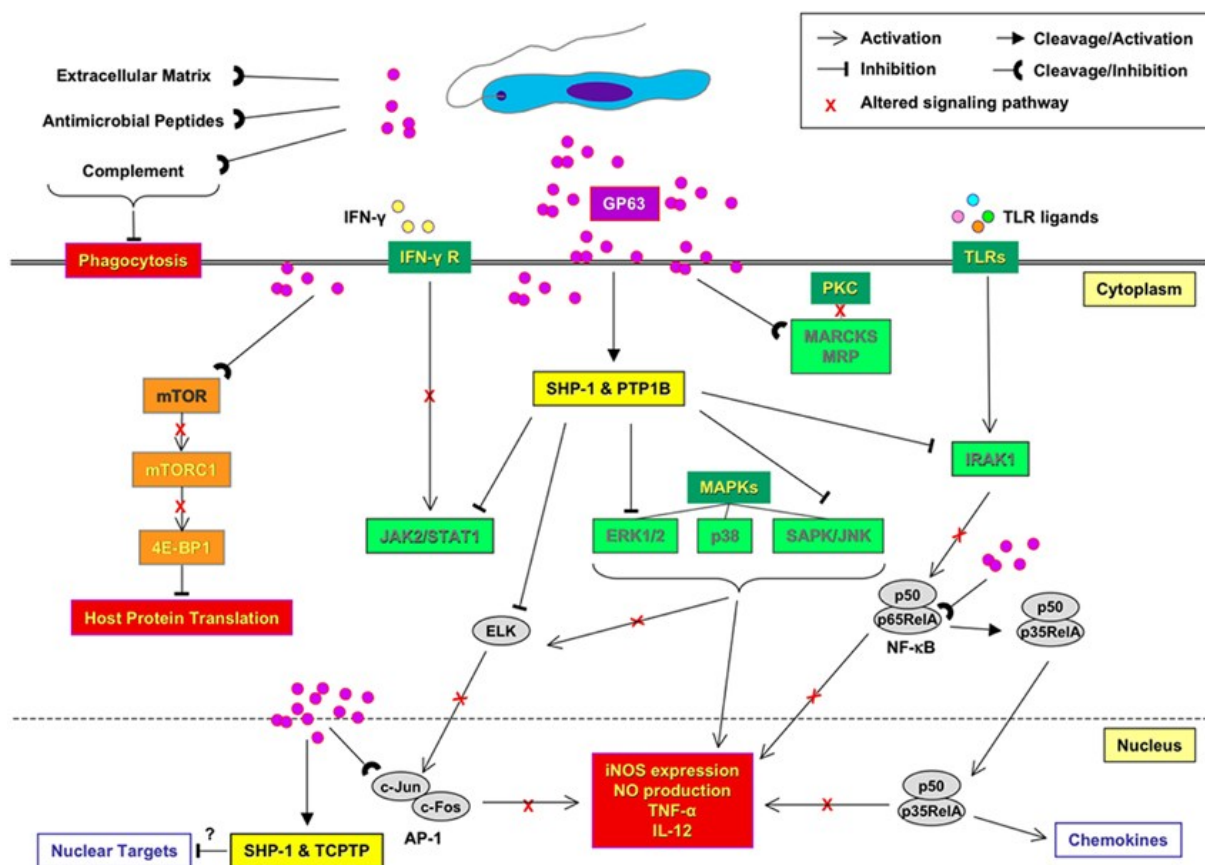


Figure 4. Schematic demonstrating the extensive modulation of innate immune signalling by GP63. Reproduced from (Isnard et al., 2012).

LPG is another prominent *Leishmania* surface molecule and is in fact the most abundant glycoconjugate on the surface of *Leishmania* promastigotes. It consists of a conserved glycan core and a PI anchor, with species-specific variations in the number of repeating units and capping structures. As previously discussed, this is likely one of the key features driving *Leishmania* spp. specificity of restrictive sandfly vectors, as LPG plays a crucial role in attaching the promastigote to the midgut to prevent excretion as well as protecting promastigotes from midgut digestive enzymes (Forestier et al., 2014; Sacks et al., 2000). During metacyclogenesis, LPG elongates by effectively doubling the number of repeating units, preparing the parasite for infection by facilitating detachment from the midgut and C5 complement inactivation (Puentes et al., 1990; Turco & Descoteaux, 1992). Paradoxically, C3-mediated phagocytosis is enhanced by LPG, likely mediated by the binding of mannan-binding protein (MBP) to mannose-terminating oligosaccharides in the cap structure of LPG (Descoteaux & Turco, 2002; Green et al., 1994; Puentes et al., 1989). This in fact works concurrently with GP63-mediated C3b cleavage into C3bi, as C3bi is still bound by CR 1/3 (Da Silva et al., 1989; Wilson & Pearson, 1988).

In the host, LPG inhibits key macrophage signaling pathways, for example MAPK signalling through the inhibition of ERK 1/2 activation (Prive & Descoteaux, 2000). Surprisingly, LPG-deficient *L. donovani* mutants were also able to inhibit ERK 1/2 activation, though the authors hypothesize this is through redundant surface virulence factors no longer being masked by wild type (WT) LPG (Descoteaux & Turco, 2002). *Leishmania* can also inhibit NO synthesis in a dose-dependent manner, though this effect relies on specific domains of LPG (Proudfoot et al., 1996; Proudfoot et al., 1995). Contrastingly, LPG-mediated downregulation of IL-1 $\beta$  gene expression depends on intact LPG (Hatzigeorgiou et al., 1996). Once phagocytosed by host macrophages, LPG and especially its metacyclic form with many repeating units is key to the inhibition of phagolysosome biogenesis and maturation through its transfer and incorporation into host cell membranes, inhibiting fusion (Desjardins & Descoteaux, 1997; Miao et al., 1995). This is additionally accomplished through LPG-mediated inhibition of protein kinase C (PKC), which also serves to inhibit other microbicidal functions like oxidative burst (Giorgione et al., 1996; Miao et al., 1995). Another potential

mechanism for the inhibition of both phagolysosome maturation and PKC activation is the sequestration of lipid rafts by the unique GPI anchors of LPG (Dermine et al., 2001; Descoteaux & Turco, 2002).

*Leishmania* also possess calpain-like cysteine proteases (CPs), key virulence factors found across all pathogenic protozoan parasites (Siqueira-Neto et al., 2018). CPs are enzymes that catalyze the hydrolysis of cysteine peptide bonds. Genome sequencing of *Leishmania* has revealed numerous CPs potentially involved in metabolism and pathogenicity, with the TriTRYP database identifying 60 putative CP genes in *L. mexicana*. The first evidence suggesting CPs, specifically clan CA family C1 CPs, as virulence factors emerged specifically through the study of CPB gene disruption, as CPB mutants displayed reduced pathogenicity and lesion growth in BALB/c mice (Mottram et al., 1996). Since CPB genes are arranged in a multi-copy tandem array (19 copies in *L. mexicana*), the addback of the full gene array rather than a single gene copy is necessary to restore infectivity (Denise et al., 2003). This organization suggests that CPB expression is variable, which has been demonstrated to regulate *Leishmania* stage progression and substrate preference (Mottram et al., 1997). Comparatively, CPA and CPC, 2 other well studied CPs, are present as single copy genes (Bart et al., 1995; Mottram et al., 1992).

CPA and CPB are both cathepsin L-like proteases, suggesting they are functionally redundant. However, they seem to also have complementary functions as the mutation of both CPs results in a greater reduction in infectivity compared to CPB disruption alone (Alexander et al., 1998). CPB virulence is suggested to result from the inhibition of a Th1 response through the degradation of NF- $\kappa$ B as well as the cleavage of JNK and ERK, leading to a reduction in IL-12 production (Cameron et al., 2004). *In vitro* studies have also shown that CPs are crucial for the intracellular survival of *L. amazonensis* amastigotes, catalyzing the degradation of major histocompatibility complex (MHC) class II molecules (De Souza Leao et al., 1995). CPA and CPB have been further implicated in autophagy, metacyclogenesis, and amastigote transformation, presenting an alternative explanation for the observed reduction in virulence (Williams et al., 2006). Other clan CA CPs, Atg4.1 and

4.2, have been found to possess similar functions (Williams et al., 2013). CPB has also been demonstrated to modulate *Leishmania* GP63 expression, with the restoration of GP63 expression being sufficient to restore CPB-deficient *Leishmania* virulence (Casgrain et al., 2016). This calls into question whether CPB directly modulates macrophage gene expression or affects *Leishmania* virulence through GP63 modulation. CPC is instead a cathepsin B-like protease with a hypothesized role in *Leishmania* cell death and secretome remodeling (El-Fadili et al., 2010; Gerbaba & Gedamu, 2013).

### 1.3 The role of EVs in *Leishmania* virulence and survival

*Leishmania* parasites can be considered veritable experts in evading the mammalian immune response and have evolved a sophisticated arsenal of virulence factors to accomplish this. Much of the previous research done on these virulence factors has not only shown the many ways they interact with immune cell signalling, especially regarding macrophage function, but also suggested that these virulence factors can be delivered to their target cells independently from *Leishmania* phagocytosis. Interest in the secretory capabilities of *Leishmania* arose from these observations, with research focused on identifying *Leishmania*-derived secreted immune-modulatory factors (Santarem et al., 2007; Silverman et al., 2008). With already growing interest in EV research, it seemed natural for further research into the relationship between *Leishmania*, its EVs, and the host.

#### 1.3.1 Types and physical characteristics of EVs

EVs are lipid membrane bound secretory vesicles released by all types of cells. Their secretion process is evolutionarily conserved across a wide range of organisms, from bacteria to humans and plants (van Niel et al., 2018). Initially, these constitutively secreted EVs were thought to primarily serve as a mechanism for expelling unwanted cellular components and waste (Johnstone et al., 1987). However, further research has revealed that EVs play a more complex role, being essential for intercellular communication, transferring components such as nucleic acids, lipids, and proteins, and acting as signaling vehicles in both normal cellular processes and pathological conditions (Colombo et al., 2014; Yanez-

Mo et al., 2015). EVs are primarily classified into three subtypes: microvesicles (MVs), exosomes, and apoptotic bodies (Figure 5) (van Niel et al., 2018). These subtypes are distinguished by their biogenesis, release mechanisms, size, content, and function. Although no specific protein markers have been identified to definitively differentiate these types of EVs, each subtype exhibits distinct protein profiles corresponding to their unique formation pathways (Jeppesen et al., 2019; Kalra et al., 2013; Tauro et al., 2012). However, significant overlap in these protein profiles often occurs, likely due to the absence of standardized methods for isolating and analyzing EVs and making it difficult to specify one type of EV as the main focus of any particular study. This has led to collaborative attempts to do exactly that through the minimal information for studies of extracellular vesicles (MISEV) (Welsh et al., 2024).

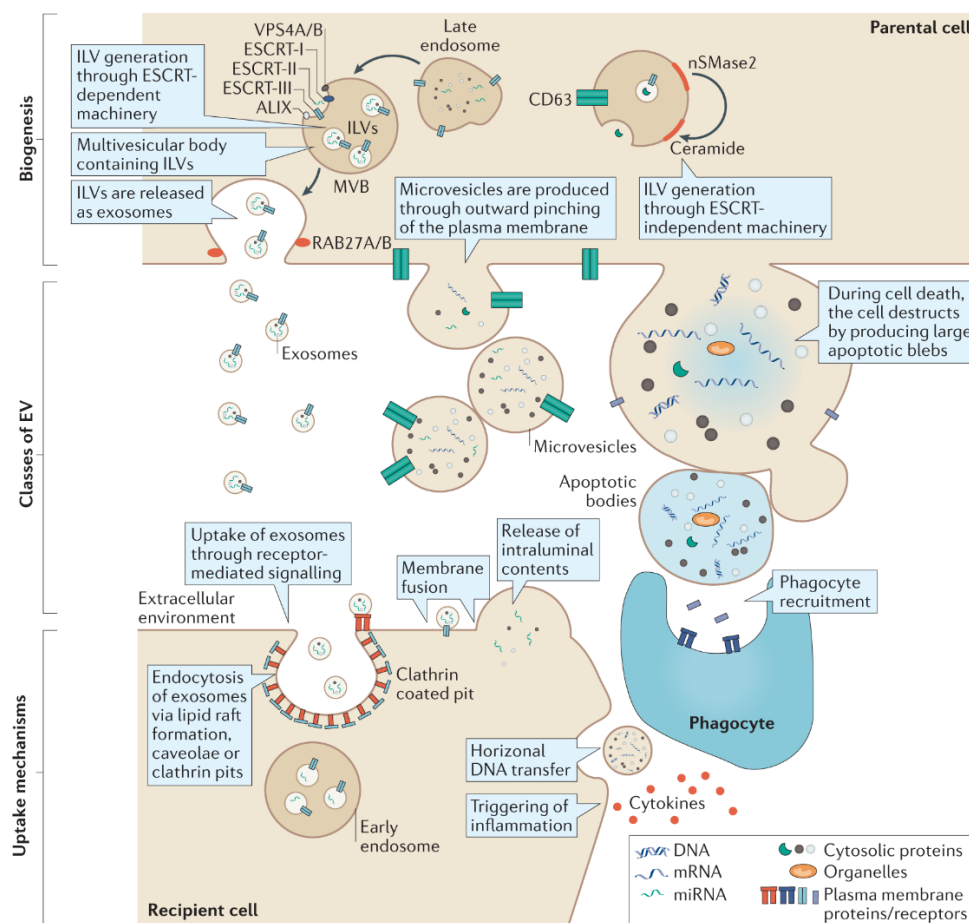


Figure 5. General schematic differentiating the three different types of EVs based on their biogenesis, secretion, and uptake. Reproduced from (Cheng & Hill, 2022).

MVs are a type of EV that form through the direct outward budding or pinching of the cell's plasma membrane, a comparatively simpler mechanism to exosome biogenesis (Tricarico et al., 2017). Their size typically ranges from 100 nm to 1  $\mu$ m in diameter (Clancy et al., 2021). While the proteomic profiles of MVs can vary significantly depending on the isolation methods used, there exists a group of “marker proteins” commonly found in MVs across different cell types due to their unique biogenesis process (Sedgwick & D'Souza-Schorey, 2018). Given their formation from the plasma membrane, MVs are rich in cytosolic and plasma membrane-associated proteins, particularly those that cluster at the membrane surface, such as tetraspanins (Greening et al., 2017). These proteins can be present in MVs at concentrations up to 100 times higher than in the cell lysate (Zoller, 2009). Additionally, MVs frequently contain cytoskeletal proteins, heat shock proteins, integrins, and proteins with PTMs, including glycosylation and phosphorylation (Di Vizio et al., 2012; Heijnen et al., 1999; Morello et al., 2013). It has been suggested through the study of cancer-related MVs (oncosomes) that the presence of glycan-binding proteins on the surface of MVs may play a crucial role in directing MVs to specific cells and facilitating their interactions (Martins et al., 2021). The biogenesis of MVs also explains why proteins from organelles not involved in their formation, such as the mitochondria, Golgi apparatus, nucleus, and endoplasmic reticulum (ER), are generally depleted in MVs compared to the cell lysate (Tricarico et al., 2017; Yanez-Mo et al., 2015). Despite these distinguishing characteristics, specific markers to differentiate MVs from exosomes remain elusive (Jeppesen et al., 2019).

Exosomes, also known as intraluminal vesicles (ILVs), are small, single-membrane vesicles secreted by all cell types and found in nearly all human fluids (Doyle & Wang, 2019). As a subtype of extracellular vesicles (EVs), exosomes are typically 30–150 nm in diameter and are formed through an endosomal pathway. They originate via inward budding of the limiting membrane of early endosomes, which then mature into multivesicular bodies (MVBs) (Raposo & Stoorvogel, 2013; van Niel et al., 2018). The formation and transport of exosomes are largely regulated by the endosomal sorting complex required for transport (ESCRT) proteins, as well as accessory proteins like ALG-2-interacting protein X (Alix), tumor susceptibility gene (TSG) 101, heat shock cognate (HSC) 70, and heat shock protein (HSP)

90β, which are frequently termed “exosomal marker proteins” (Geminard et al., 2004; Morita et al., 2007; Tauro et al., 2012; Thery et al., 2001; van Niel et al., 2006). These proteins are generally found in exosomes regardless of the cell type. There is evidence for an alternative, ESCRT-independent mechanism of exosome release, which may rely on the biogenesis of ceramide by the enzyme sphingomyelinase (Trajkovic et al., 2008). The existence of an ESCRT-independent mechanism of exosome release is further supported by the production of CD63-positive exosomes in cells lacking the ESCRT machinery (Buschow et al., 2010; Stuffers et al., 2009; Theos et al., 2006; van Niel et al., 2011). CD63, along with other tetraspanins like CD9 and CD81, are common markers in exosomes and are often more enriched in these vesicles than in the cell lysate (Witwer et al., 2013).

Although tetraspanins were initially considered specific markers of exosomes, they have also been identified in both MVs and apoptotic bodies, muddying the waters (Crescitelli et al., 2013). Exosomes generally show higher levels of glycoproteins compared to their secreting cells, whereas MVs contain proteins with greater levels of PTMs, such as glycosylation and phosphorylation. This distinction in protein content may help differentiate between these vesicle types (Palmisano et al., 2012; Sinha et al., 2014). Conflictingly, recent studies have also challenged the belief that proteins specifically associated with certain organelles, like mitochondria and the nucleus, are absent from exosomal vesicles, demonstrating interorganelle trafficking between mitochondria and the endolysosomal system (Soto-Herederó et al., 2017). Proteins linked to the Golgi apparatus and endoplasmic reticulum are also present at low levels in exosomes due to early endosomal interactions with these organelles. There is now growing evidence that exosome markers previously considered to be “strongly specific” instead share a large degree of overlap with markers found in other types of EVs, leading to a trend of referring to bioactive/relevant EVs as small EVs (sEVs), relying mainly on size to classify EVs instead (Jia et al., 2022).

Initially thought to serve primarily as a means for cells to dispose of unwanted materials, exosomes are now recognized for their roles in cell–cell communication, cellular maintenance, and tumor progression (Harding et al., 2013). Importantly, they can also

stimulate immune responses by functioning as antigen-presenting vesicles (Rak, 2010). MVs have also been greatly implicated in both local and distant cell-cell communication, and this shared functionality with exosomes/sEVs makes their specific functions difficult to distinguish from exosomes (Ratajczak & Ratajczak, 2020). On the other hand, the function of apoptotic bodies is fairly unambiguous, forming when the cell's plasma membrane separates from the cytoskeleton due to increased hydrostatic pressure following cellular contraction (Wickman et al., 2012). Apoptotic bodies are released by dying cells into the extracellular space and typically range in size from 50 nm to 5000 nm, with most tending to be on the larger side. Their composition differs significantly from that of exosomes and MVs. Unlike exosomes and MVs, apoptotic bodies contain intact organelles, chromatin, and small amounts of glycosylated proteins (Nossing & Ryan, 2023; Thery et al., 2001). The proteomic profiles of apoptotic bodies closely resemble those of the cell lysate, whereas exosomes and MVs show marked differences in their proteomic profiles compared to the cell lysate (Doyle & Wang, 2019).

### 1.3.2 Mechanisms of EV biogenesis and secretion

One of the key differences separating the different types of EVs is their biogenesis and secretion. Comparatively, the secretion of MVs and apoptotic bodies is simple, mostly resulting from membrane blebbing and apoptosis, respectively (van Niel et al., 2018). With apoptotic bodies, their formation truly starts with apoptosis, a form of programmed cell death (Elmore, 2007; Kerr et al., 1972). Apoptotic cells can often be distinguished from viable ones by several morphological features, including prominent nuclear condensation, fragmentation, and the presence of plasma membrane blebs. These blebs are balloon-like protrusions that form when the plasma membrane detaches from the cortical cytoskeleton, creating dynamic blisters that retract and reform across the cell surface (Charras et al., 2008). The formation of blebs is driven by increased hydrostatic pressure following actomyosin-mediated cellular contraction (Charras et al., 2005). Initially, new blebs lack actin and other cytoskeletal proteins, but an actin cortex quickly polymerizes within them, and cytoskeletal bundling proteins and myosin are recruited to facilitate retraction (Charras

et al., 2006). During apoptosis, this cycle of bleb formation and retraction can persist, eventually leading to blebs filled with organelles and condensed chromatin, which form membrane-bound apoptotic bodies (Croft et al., 2005). This actomyosin-mediated process of apoptotic blebbing is regulated by rho-associated coiled-coil-containing protein kinase (ROCK) 1, which itself is atypically activated by caspase-3 cleavage and not rho guanosine triphosphatase (GTPase) (Coleman et al., 2001; Sebbagh et al., 2001). It has also been suggested that ROCK2 may regulate an alternate mechanism of apoptotic blebbing, depending on granzyme B-mediated cleavage instead of caspase (Perl et al., 2012; Sebbagh et al., 2005).

In the past, apoptotic bodies were also considered to be a form of MVs, but further research has shown them to form through separate mechanisms, considering that MVs are released from viable cells (Hristov et al., 2004). MV biogenesis involves the outward blebbing and pinching off of the plasma membrane, releasing newly formed MVs into the extracellular space (Muralidharan-Chari et al., 2010; Tricarico et al., 2017). This process is characterized by specific, localized alterations in membrane proteins and lipids, which help regulate changes in membrane curvature and rigidity, involving multiple molecular rearrangements at the plasma membrane, including changes in lipid composition, protein distribution, and calcium ( $\text{Ca}^{2+}$ ) levels (Kozlov et al., 2014; McMahon & Boucrot, 2015; Minciacchi et al., 2015).  $\text{Ca}^{2+}$ -dependent enzymes, such as aminophospholipid translocases, scramblases, and calpain, facilitate the reorganization of membrane phospholipids in response to elevated  $\text{Ca}^{2+}$ , causing membrane bending and remodeling of the actin cytoskeleton necessary for MV budding (Al-Nedawi et al., 2008; Piccin et al., 2007). PKC activation via phorbol esters can similarly lead to cytoskeleton remodeling (Cocucci et al., 2009). Although the rearrangement of phosphatidylserine seems to be key to MV budding, it is not necessary (Jimenez et al., 2003). This suggests other lipids are likely involved in the process, for example the necessity of cholesterol in neutrophil MV formation (Del Conde et al., 2005).

As is the case with apoptotic bodies, MV biogenesis is tightly controlled by ROCKs, along with the small GTPases belonging to the adenosine diphosphate (ADP)-ribosylation factor

(ARF), ras-associated binding (rab), and rho families (Antonyak et al., 2012; Li et al., 2012; Muralidharan-Chari et al., 2009; Schlienger et al., 2014; Sedgwick et al., 2015; T. Wang et al., 2014). The activation of these is adenosine triphosphate (ATP)-dependent and promotes phosphorylation of the myosin light chain (MLC) and actomyosin contraction, enabling vesicles to bud off from cell membranes (Sedgwick et al., 2015; van Niel et al., 2018). To complicate matters, it has been reported that MV release can occur in an ESCRT-dependent manner as well, specifically requiring TSG101 and vacuolar protein sorting-associated protein (Vps) 4 in the case of MVs containing arrestin domain-containing protein (ARRDC)-1 (Nabhan et al., 2012). This alternate ESCRT-dependent MV release has also been observed in *Caenorhabditis elegans* embryos, occurring when regular MV biogenesis is blocked by TAT-5 flippase KO, though it has been argued if this is due to TAT-5 being an inhibitor of EV release in general (Pitts et al., 2023; Wehman et al., 2011). Cargo targeting to MVs can occur through the affinity of lipids and membrane-associated molecules for lipid rafts or through the anchoring of cytoplasmic proteins to plasma membrane lipids, similar to the budding mechanisms seen in viruses like HIV (Shen et al., 2011; Yang & Gould, 2013). ARF6 and Rab22a have been implicated in this process, though they are surely not the only cargo sorting factors (Muralidharan-Chari et al., 2009; T. Wang et al., 2014).

Compared to the other two types of EVs, exosome research is much more extensive, and our understanding of the mechanisms related to their biogenesis and secretion is much more detailed as a result, necessitating their discussion in a separate section. Exosome biogenesis is closely linked with MVB biogenesis and endocytosis, where various mechanisms drive the inward budding of the plasma membrane to create early endosomes (Doherty & McMahon, 2009). These early endosomes recycle some proteins back to the plasma membrane while incorporating other cargos into ILVs, ultimately forming MVBs. Once matured, MVBs can interact dynamically with other organelles through several routes, such as exchanging vesicles with the trans-Golgi network (TGN) or establishing direct contacts with the ER, mitochondria, or phagosomes (Kaksonen & Roux, 2018). Thus, exosome biogenesis can be generally understood as four main steps: cargo sorting, MVB formation, MVB transport, and finally MVB fusion with the plasma membrane, releasing the

ILVs as exosomes. These steps are regulated by competing or coordinated mechanisms that sort a diverse array of molecular cargos into distinct exosome subpopulations, contributing to their heterogeneity (Han et al., 2022). A large driving force for interest in exosome research is the vast evidence of cancer cells manipulating exosome biogenesis to promote tumor progression (Rak, 2010). As a result, cancer therapies targeting exosome biogenesis are under active investigation. However, EVs have been further shown to play a crucial role in pathogenic and viral infections, demonstrating a need for greater EV research in fields beyond cancer (A et al., 2023; Dong et al., 2021; Xie et al., 2023).

### 1.3.3 Exosome biogenesis, secretion, and ESCRT

MVB formation is central to exosome biogenesis, with membrane budding and ILV generation being crucial steps in this process. This process occurs during clathrin-mediated endocytosis, a classical mechanism of budding (Kaksonen & Roux, 2018). In a seminal study on yeast vacuole formation and release, Vps proteins were identified as key players in the formation of ILVs during endocytosis (Raymond et al., 1992). However, ESCRT itself was only described much later, quite literally putting the puzzle pieces (Vps proteins) together (Katzmann et al., 2001). This study clearly demonstrated how Vps proteins complex together to form ESCRTI and identified ubiquitination as a sorting signal that is recognized by ESCRT, specifically Vps23. From here, ESCRTII and III were quickly identified, producing a much clearer picture of endocytosis and MVB formation (Babst, Katzmann, Estepa-Sabal, et al., 2002; Babst, Katzmann, Snyder, et al., 2002). However, with further research, MVB formation has been found to occur independently of ESCRT, with both ESCRTI and II being dispensable (Babst, Katzmann, Snyder, et al., 2002; Stuffers et al., 2009; Yang et al., 2004). This has led to multiple mechanisms being proposed for MVB biogenesis, generally categorized as ESCRT-dependent or independent (Figure 6), though this section will focus on ESCRT.

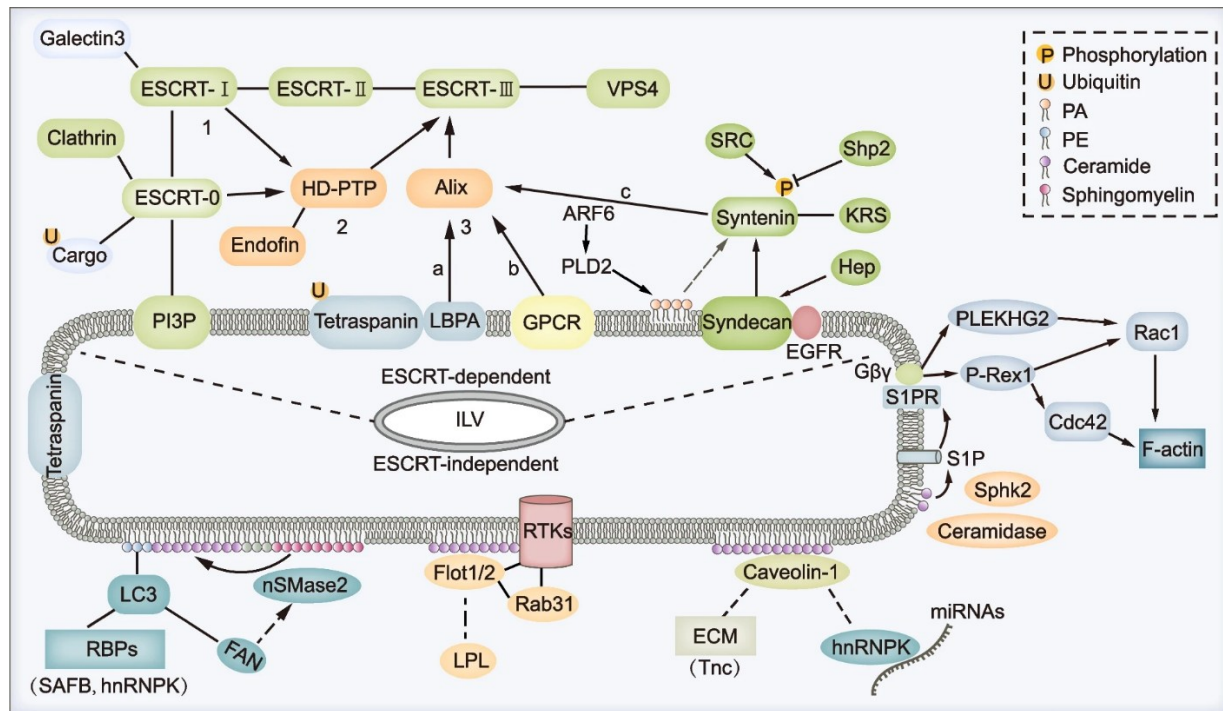


Figure 6. Schematic representing the many ESCRT-dependent and independent mechanisms of MVB formation. Depicted above are the classical ESCRT pathway (1), HD-PTP-dependent pathway (2), and the Alix-dependent pathway (3), the last of which is further subdivided based on LBPA (a), GPCR (b), or Syntenin (c) interaction. Note that tetraspanin is involved in both ESCRT-dependent and independent pathways and most ESCRT-independent pathways involve direct remodeling of the plasma membrane via the manipulation of lipids. Reproduced from (Cheng & Hill, 2022).

ESCRTI was originally characterized in yeast as a heterotrimeric complex of Vps23, 28, and 37, whereas it consists of TSG101, Vps28, and one of four potential isoforms of Vps37 (A-D) in humans (Williams & Urbe, 2007). It is recruited to the endosomal membrane by Vps27, which together with herpes simplex encephalitis type 1 (Hse1) form ESCRT0 (Bilodeau et al., 2003; Katzmman et al., 2003). TSG101 has been described as the most important protein of this complex, responsible for the maintenance of endosome morphology, binding ubiquitinated cargo, and being the literal backbone of the complex (Feng et al., 2000; Razi & Futter, 2006; Sundquist et al., 2004). The recruitment of ESCRTII by Vps28 is diverges between yeast and mammals: in yeast, the Vps28 C-terminal domain binds to a zinc-finger domain of Vps28, forming a strong linkage between ESCRTI and II (Gill et al., 2007; Teo et al.,

2006). In comparison, mammalian Vps36 lacks this zinc finger domain, and it has been suggested that TSG101 recruits the ESCRTII complex rather than Vps28 (Langelier et al., 2006).

Even without a zinc-finger domain, the N-terminal domain of mammalian Vps36 still acts as a glue, literally termed a GRAM-like ubiquitin-binding in Eap45 (ESCRT-associated protein) (GLUE) domain (Slagsvold et al., 2005). This domain extends outside of the ESCRTII core, consisting of Vps22, 25 and 36, and is responsible for binding ubiquitin directly at the pleckstrin homology (PH) fold (Hirano et al., 2006; Teo et al., 2004). Interestingly, the zinc-finger domain is necessary for ubiquitin binding in yeast, showing that Vps36 function is conserved despite a lack of structural conservation between mammals and fungi. This suggests an ancient origin from a common ancestor of plants and animals (Williams & Urbe, 2007). In yeast, ESCRTII transiently associates with endosomes, aiding in the recruitment of ESCRTIII components to the membrane (Babst, Katzmann, Snyder, et al., 2002). The deletion of any ESCRTII leads to the incorrect sorting of vacuolar hydrolases (Howard et al., 2001). However, in mammalian cells, depletion of ESCRTII using siRNA doesn't affect the degradation of ubiquitylated MHC-I complexes but significantly impairs the downregulation of ferroportin, a cell-surface receptor regulated by the hormone hepcidin (Bowers et al., 2006; Langelier et al., 2006). Interestingly, this demonstrates that epidermal growth factor receptor (EGFR) and epidermal growth factor (EGF) are degraded by ESCRTII-dependent and independent mechanisms, respectively, two clearly distinct pathways. These findings suggest that mammalian cells may have developed cargo-specific MVB pathways, with some potentially bypassing ESCRTII altogether.

The recruitment of ESCRTIII to membrane scission sites follows two main pathways: one involving ESCRTI and ESCRTII, and another mediated by ALIX (Schoneberg et al., 2017). Initially, it was believed that ESCRTI and ESCRTII are obligate partners due to their strong mutual affinity as well as evidence from *in vitro* reconstitution studies (Boura et al., 2012; Carlson & Hurley, 2012; Gill et al., 2007). However, knockdown studies showed that ESCRTII subunits had no impact on processes like cytokinesis or HIV1 release (Morita et al., 2007;

Morita et al., 2011). It was then shown that ALIX-mediated ESCRTIII recruitment can compensate for ESCRTII knockdown, providing a redundant/compensatory recruitment mechanism (Christ et al., 2016; Tang et al., 2016). However, depletion of ALIX, either on its own or in combination with ESCRTII, does not affect MHC-I or EGF degradation (Morita et al., 2007). ESCRTI and ESCRTII are found together at the necks of membrane buds, as seen in reconstituted giant unilamellar vesicles (Wollert & Hurley, 2010). These complexes form “strut-like” structures, as observed in electron micrographs (Cashikar et al., 2014). In these cases, the curvature at the membrane neck is described as “saddle-shaped,” meaning any protein complex stabilizing this area must feature both concave and convex surfaces (Mercker & Marciniak-Czochra, 2015; Rozycki et al., 2012).

In yeast, ESCRTIII consists of the proteins Vps2, Vps24, Sucrose Non-Fermentable 7 (Snf7), and Vps20, which are believed to form two subcomplexes (Babst, Katzmann, Estepa-Sabal, et al., 2002). Additionally, two related proteins, Did2 and Vps60, participate in the late MVB pathway (Kranz et al., 2001; Nickerson et al., 2006). Although the functional significance of ESCRTIII lattices in the MVB pathway remains unclear, it has been suggested that these structures may spatially limit membrane curvature factors, initiating budding away from the cytoplasm (Williams & Urbe, 2007). These lattices appear to be a general feature of ESCRTIII subunits, as full-length subunits do not interact freely in the cytosol but assemble on endosomal membranes upon recruitment or when expressed as truncated variants. Crucially, multiple ESCRT-III-related proteins bind to the MIT domain of the AAA+ ATPase Vps4 through their C-terminal MIT-interacting regions (Scott et al., 2005; Zamborlini et al., 2006). For example, Did2 acts as an adaptor by linking Vps4 with Vps2 and Vps24, a function essential for the disassembly of ESCRTIII (Nickerson et al., 2010).

As previously discussed, the deletion of any ESCRTIII-related subunit in yeast leads to the accumulation of endocytic cargo in class E compartments (Howard et al., 2001). Previously, this, along with the dominant-negative effects of chromatin-modifying protein/charged multivesicular body protein (CHMP)-GFP fusions in mammalian cells, suggested that these proteins were crucial for ILV formation (Hori et al., 2006; Skibinski et al., 2005; Ward et al.,

2005; Yorikawa et al., 2005). However, matching previous observations with HRS and TSG101, recent studies indicate that ILV formation can still proceed in the absence of certain ESCRTIII components, though endosomal structure and cargo sorting to lysosomes are notably disrupted (Williams & Urbe, 2007). For instance, in mammalian cells, depletion of the ESCRTIII component Vps24 (CHMP3) strongly inhibits the degradation of EGFR, yet the kinetics of signal termination remain unaffected (Bache et al., 2006). It is suggested that Vps24-depleted cells improperly sort EGFR into ILVs, where it can no longer signal to cytosolic effectors and effectively silencing EGFR. This contrasts with TSG101 depletion, which prevents ILV formation and leads to prolonged EGFR signaling (Bache et al., 2006; Doyotte et al., 2005; Razi & Futter, 2006). Additionally, knockout of CHMP5 in mice results in enlarged late endosomes filled with ILVs, but these MVB-like bodies are unable to degrade fluid-phase markers or receptors like EGFR and TGF $\beta$ IIIR, suggesting that Vps24/CHMP5 are more involved downstream of ILV and MVB formation (Shim et al., 2006; Ward et al., 2005). Similarly, in yeast, the deletion of the peripheral ESCRTIII-related protein Did2 only impaired efficient cargo incorporation into ILVs (Hurley, 2015). Did2 functions as an adaptor linking Vps4 to the disassembly of some ESCRTIII subunits from endosomes, while leaving ESCRTI and ESCRTII unaffected (Nickerson et al., 2006). These findings suggest that ILV formation can occur independently of both cargo sorting and ESCRTIII disassembly, further highlighting the distinct roles of ESCRT components throughout the MVB pathway.

The disassembly of ESCRT components by Vps4 is essential for sorting cargo into MVBs. In yeast, when Vps4 is absent, all ESCRT proteins remain tightly bound to membranes (Babst, Katzmann, Estepa-Sabal, et al., 2002; Nickerson et al., 2010; Nickerson et al., 2006). In mammalian cells, Vps4 is similarly needed to disassemble clathrin-coated regions where cargo clusters (Sachse et al., 2004). This disassembly process relies on the ATPase activity of Vps4, specifically requiring ATP hydrolysis but not binding (Fujita et al., 2003). Vps4 likely functions analogously to other AAA+ ATPases, such as those involved in disassembling SNARE complexes and microtubules, by partially unfolding ESCRT subunits to disrupt the interactions that maintain the structural integrity of ESCRT networks on membranes. Vps4 is recruited to endosomal membranes via its N-terminal MIT domain, which interacts with

the C-terminal regions of ESCRTIII-related proteins (Babst, Katzmann, Estepa-Sabal, et al., 2002; Scott et al., 2005; Tsang et al., 2006). Oligomerization of Vps4 is enhanced by ATP binding, and evidence suggests that this process is further catalyzed by the protein Vta1 (LIP5) (Azmi et al., 2006; Yeo et al., 2003). Meanwhile, the depletion of LIP5 (Vta1) in mammalian cells impairs HIV-1 infectivity and slows the degradation of internalized EGFR, though it does not prevent ILV formation or affect endosomal structure (Ward et al., 2005). Interestingly, LIP5 also interacts with the ESCRTIII-related protein CHMP5, and in yeast, the deletion of Vps60 (CHMP5) results in prolonged retention of Vta1 in class E compartments (Azmi et al., 2006; Shiflett et al., 2004). From this, it is evident that Vps60 (CHMP5) is important for the recycling of Vta1 (LIP5), ensuring the proper disassembly and recycling of ESCRT components.

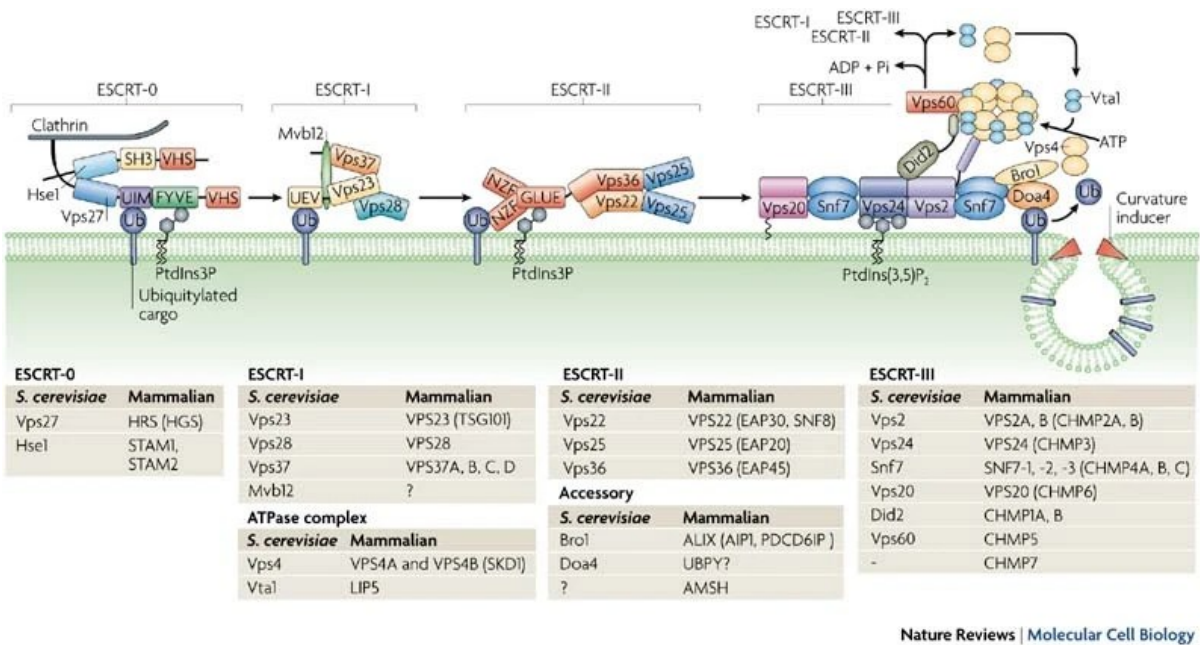


Figure 7. Diagram of the protein components of each ESCRT complex. Their yeast and mammalian counterparts are named and associated with each ESCRT complex. Adapted from (Williams & Urbe, 2007).

While the importance of ESCRT to MVB formation is unarguable, extensive evidence points to the plethora of compensatory mechanisms related to the endosomal pathway, naturally suggesting that ESCRT-independent mechanisms of MVB and ILV formation exist. These

mechanisms largely rely on lipid rafts, which facilitate protein sorting, membrane curvature, and vesicle budding. all without relying on ESCRT (Dawson, 2021; Skotland et al., 2019). The neutral sphingomyelinase 2 (nSMase2)-ceramide pathway is the most studied ESCRT-independent mechanism (Han et al., 2022). nSMase2 is a key enzyme for conversion of sphingomyelin to ceramide, which can induce the formation of large macrodomains on the plasma membrane through hydrogen bonding-dependent self-association. These serve to trap cargo molecules like CD95 and CD40 (Gulbins & Kolesnick, 2003; Savio et al., 2020). Additionally, ceramide can promote the budding and formation of small vesicles in vitro, suggesting that the cone-shaped structure formed by ceramide induces spontaneous negative membrane curvature (Trajkovic et al., 2008). Effectively, ceramide serves as a scaffold that serves to literally shape vesicles.

Similarly, caveolin-1, a hairpin-like integral membrane protein that binds cholesterol, acts as a scaffold that organizes lipids and proteins, initiating the formation of caveolae on the plasma membrane and mediating caveola-dependent endocytosis (Parton et al., 2020). When caveolae assembly is disrupted, caveolin-1 is internalized, sorted into ILVs, and subsequently degraded in the lysosome through a ubiquitin- and ESCRT machinery-dependent mechanism (Albacete-Albacete et al., 2020). Notably, caveola-dependent endocytosis has also been implicated in *Leishmania* internalization by macrophages (Kumar et al., 2019; Rodriguez et al., 2006). Flotillins, another group of membrane scaffolding proteins, are also involved in lipid raft formation and thus play a role in endosome trafficking and protein sorting (Kwiatkowska et al., 2020). In fact, flotillin-1 has been a recognized and utilized exosome marker for nearly 20 years (de Gassart et al., 2003). It has been proposed that the activation of EGFR by EGF stimulation can subsequently activate Rab31, which binds directly to flotillins and facilitates lipid raft-dependent exosome sorting of receptor tyrosine kinases like EGFR (Wei et al., 2021). This process requires ceramide and cholesterol but is independent of ESCRT, demonstrating how ESCRT-independent exosome pathways are interconnected. For example, flotillin-1 knockdown reduces the exosomal release of caveolin-1 in prostate cancer 3 (PC3) cells (Phuyal et al., 2014). However, flotillin-positive microdomains are notably distinct from caveolin-1-positive caveolae on the plasma

membrane (Frick et al., 2007). Therefore, it remains unclear whether caveolin-1 and flotillins function cooperatively to mediate cargo sorting and ILV formation or if they are associated with separate microdomains on MVBs.

Tetraspanins are perhaps the most important family of membrane scaffold proteins that participate in MVB biogenesis, organizing proteins into tetraspanin-enriched microdomains as well as regulating signaling pathways and various cellular activities (Kummer et al., 2020). Evidence increasingly shows that tetraspanins contribute to ILV sorting of multiple cargos. For example, CD63 coupled with Apolipoprotein E promotes ILV formation and facilitates the sorting of the pre-melanosome protein (PMEL) independently of both ESCRT and ceramide (van Niel et al., 2011). Reductions in ILV formation and exosome biogenesis upon CD63 knockdown or knockout underscore its critical role in these processes. Additionally, Tetraspanin 6 (TSPN6) has been found to interact with Syntenin, promoting the sorting of ILVs and exosomal secretion of amyloid precursor protein in human embryonic kidney 293 (HEK293) cells (Guix et al., 2017). Contrastingly, TSPN6 appears to have an opposing role in Michigan Cancer Foundation-7 (MCF-7) cells, where it directly mediates the lysosomal degradation of Syndecan-4, thereby inhibiting exosome generation (Ghossoub et al., 2020). This suggests that TSPN6 may serve distinct functions across different cell types or for specific cargo, with its role potentially dictated by cell context or cargo specificity.

Like its protein counterparts, cholesterol, which is abundant in ILVs/exosomes, is a lipid molecule that is also critical for the localization and trafficking of late endosomes (Han et al., 2022). Cholesterol can be synthesized *de novo* in the ER or acquired externally through endocytosis (Ikonen & Zhou, 2021). To balance these two processes and regulate MVB cholesterol content, interactions between endosomal annexin A1 and ER-localized S100A11 are crucial, facilitating contact site formation by lipid transfer protein StAR related lipid transfer domain containing 3 (STARD3) (Wilhelm et al., 2017). These contact sites enable PTP1B interaction with MVB cargos like HRS and EGFR and also enhance the transfer of cholesterol from the ER to MVBs via the lipid transfer protein oxysterol-binding protein-related protein 1L (ORP1L). Altogether, this further enhances MVB formation and the

sequestration of cargos such as EGFR into ILVs (Di Mattia et al., 2020; Eden et al., 2016). Beyond its role in ILV formation, cholesterol has also been demonstrated to influence exosome biogenesis. In cancer cells, cholesterol promotes the exosomal secretion of flotillin-2 and miR-122-5p (Strauss et al., 2010; Zhao et al., 2020). Conversely, in astrocytes, cholesterol negatively regulates exosome release, both *in vitro* and *in vivo*, indicating that cholesterol's effect on exosome biogenesis can vary depending on cell type (Abdullah et al., 2021). It should be evident by this point that this is a common trend with almost all participants in MVB/exosome biogenesis, indicating that this entire ancestral process is finely regulated by numerous compensatory and redundant processes and resulting in a highly complex and nuanced system.

#### 1.3.4 Isolation, characterization, and quantification of EVs

As discussed, with how much different classes of EVs tend to overlap based on cargo, morphology, function, and size, the methods used to isolate and differentiate EVs become greatly important. Considering the popularity of EV research, there is a veritable ocean of protocols and methodologies to choose from, making the establishment of standard practice exceptionally important (Welsh et al., 2024). The Olivier lab primarily uses differential ultracentrifugation (UC) to isolate EVs, and as such this section will centre around ultracentrifugation. UC remains the most widely employed technique for sEV isolation due to its "gold standard" reputation for yielding high purity sEVs (Coughlan et al., 2020; Thery et al., 2006). This process separates particles based on their density and size differences through sequential centrifugation at increasing speeds, selectively removing cells, debris, and larger vesicles before isolating sEVs at higher speeds. The isolated pellet is often resuspended in PBS, followed by a final UC step to eliminate any residual protein contamination, ensuring high purity. Standardized centrifugation speeds are applied based on experimental needs, and the procedure is kept at 4°C to inhibit protease, DNase, and RNase activity (Thery et al., 2018; Welsh et al., 2024). UC is both economical and straightforward, requiring minimal specialized equipment and allowing for reproducibility in processing large sample volumes (Zerlinger et al., 2015). In a comparative study, UC yielded

superior isolation purity when separating sEVs from plasma, particularly over size-based or precipitation methods (Tian et al., 2020). However, UC's performance can be inconsistent due to variations in biological material, rotor type, or centrifugation time, potentially leading to reproducibility issues if these parameters are not carefully recorded (Zhu et al., 2020). There are several further limitations to UC: it is time-intensive, subject to high output variability, and may affect sEV structural and functional integrity due to the high-speed rotations (Nordin et al., 2015; Stam et al., 2021). Furthermore, low recovery rates limit its utility for smaller sample volumes (Guo et al., 2021). Although UC generally provides high-purity isolations, co-isolation of contaminants remains a concern due to overlapping densities and sizes, especially regarding the separation of EV classes (van Niel et al., 2018). To address these limitations, recent adaptations have been made, such as serum dilution with PBS, extended initial UC cycles, and the use of sucrose cushions (An et al., 2018; Langevin et al., 2019). These significantly reduce protein contaminants with minimal sEV loss compared to size-exclusion chromatography (SEC), making UC a robust choice for high-purity sEV isolation. However, further characterization/enrichment is required to differentiate different classes of EVs present in the resulting samples.

Once EV samples have been obtained, they must be qualitatively and quantitatively assessed. One of the first assays performed on EV samples is nanoparticle tracking analysis (NTA), an advanced technique for the direct, real-time visualization and characterization of nanoparticles in liquids. NTA determines particle size by analyzing the rate of Brownian motion (Dragovic et al., 2011). Currently, the most commonly used methods to perform NTA are with two systems manufactured by competing companies: NanoSight from Malvern Panalytical and ZetaView from Particle Metrix. NanoSight is the first NTA system and introduced NTA as an EV analytical method. It uses light scattering and a light microscope to visualize and record particles in a liquid sample (Filipe et al., 2010; Gross et al., 2016; Soo et al., 2012). The accompanying software then tracks the Brownian motion of individual vesicles, allowing for precise calculations of their size and overall concentration. In comparison, ZetaView additionally uses micro-electrophoresis to calculate the zeta potential of particles, indicating the surface charge of analyzed particles (Muturi et al.,

2013). In a direct comparison of the two systems, it was found that the NanoSight NS300 typically overestimates nanosphere concentrations but can provide highly detailed particle size distribution profiles (Bachurski et al., 2019). In contrast, the ZetaView system delivers more consistent particle concentration measurements but exhibits higher standard deviations in size distribution, suggesting lower precision in single-particle size determination. ZetaView's advantages in particle quantification were further highlighted by its more accurate assessments of serial dilutions and its ability to track freeze-thaw-cycle-dependent EV concentration losses. Both systems tended to overestimate EV size compared to TEM estimations, which the authors attribute to the lower resolution of optical microscopy causing EVs smaller than 60nm to not be counted.

Before the advent of NTA, EV visualization and quantification was performed solely through microscopy alone. Optical microscopy is typically used for *in vivo* imaging to investigate the biodistribution of EVs, providing valuable insights into their pharmacokinetic properties as potential drug carriers or theranostic vehicles (Chuo et al., 2018). However, tracking and visualizing EVs is inherently challenging due to their small size, often necessitating labeling techniques to enable effective visualization. For the quantification and characterization of EVs alone, electron microscopy (EM) was and still remains the gold standard (Welsh et al., 2024). With a resolution of approximately 0.5 nm, EM is well-suited for providing detailed structural information about EVs (Hirsova et al., 2016). However, this technique cannot capture EVs in their native state, as samples must undergo fixation and processing before imaging (Pascucci & Scattini, 2021). Transmission electron microscopy (TEM) is the most commonly used EM technique for EV imaging (Cizmar & Yuana, 2017). To prepare samples for TEM, EVs are initially fixed, dehydrated, mounted onto a carbon-coated grid, and stained for imaging, often using negative staining (Jung & Mun, 2018). For whole cells, the samples are also embedded and sectioned before staining. TEM employs electron beams that either transmit through or are diffracted by the specimens. Transmitted electrons are collected on a charge-coupled device (CCD) to produce bright-field images, commonly used for structural verification. Scattered electrons, on the other hand, are utilized to create dark-field images, offering higher contrast to enhance structural detail.

Due to dehydration during sample preparation, EVs often appear cup-shaped under TEM (Chernyshev et al., 2015; Lobb et al., 2015). EVs also tend to shrink by up to 21% compared to their original size (van der Pol et al., 2014). Cryo-EM avoids these artifacts by using cryo-immobilization to preserve samples in their native hydrated state by vitrifying water through rapid cooling with liquid ethane (Cizmar & Yuana, 2017). Samples are imaged at extremely low temperatures (below  $-175^{\circ}\text{C}$ ), allowing EVs to maintain their natural spherical shape (Koifman et al., 2017). As a result, EVs observed under cryo-EM often appear larger compared to measurements from other electron microscopy techniques (Chernyshev et al., 2015). After cryo-immobilization, samples can also undergo freeze substitution with fixation and embedding reagents, enabling imaging under traditional TEM at room temperature. Immunogold labeling can be used in conjunction with TEM to identify EV-associated proteins and provide stronger evidence of EV identity, whether that be their type or source (Lobb et al., 2015; Park et al., 2016). With the power of AI and computational tools, TEM and Cryo-EM can also be used quantitatively through software support (Kotrbova et al., 2019).

Scanning electron microscopy (SEM), another form of EM, employs an electron beam to scan the surface of a specimen, providing detailed topographical information (Noble et al., 2020). SEM sample preparation involves either chemical or cryogenic fixation, followed by dehydration. The immobilized samples are then sputter-coated with a thin conductive layer, typically gold or carbon, to prevent electron accumulation and enhance secondary electron generation. While some studies describe EVs as round-shaped under SEM, others report a saucer-shaped appearance, likely due to EV collapse during the dehydration process. Compared to TEM, SEM preparation of cell samples is simpler, as it does not require embedding or sectioning. However, while TEM allows imaging of the cellular interior, SEM only allows the visualization of the cell surface, though SEM does prove useful for the observation of EV budding (Elsner et al., 2023; Larson et al., 2005). Additionally, while the conductive coating is unlikely to significantly affect imaging results, it may alter the surface structure of EVs due to their small size. To address this, low-voltage SEM can reduce electron charging and radiation damage, eliminating the need for sputter coating (Kondratov et al., 2017). Additionally, phenomena such as the "coffee ring effect," caused by capillary flow

during sample dehydration, can introduce biases in EV size and quantity measurements (Chernyshev et al., 2015). To mitigate this, the entire specimen surface must be systematically imaged and analyzed to ensure accurate results.

### 1.3.5 EV-mediated *Leishmania*-host and *Leishmania*-*Leishmania* interactions

EV release appears to be the primary mode of protein secretion in *Leishmania* and other trypanosomes (Corrales et al., 2010; Dong et al., 2021). This is a relatively new perspective on *Leishmania* protein secretion, as vesicle-mediated secretion is still commonly referred to as an “unconventional secretory pathway” (Balmer & Faso, 2021). Combined with the fact that GP63 exhibits its immunomodulatory functions even in the absence of the *Leishmania* parasite, it stands to reason that *Leishmania* EVs play a crucial role during the early stages of infection to initiate the immunomodulation of macrophages and to ensure parasite survival after phagocytosis. Indeed, it has been robustly demonstrated that *Leishmania* constitutively secrete EVs (exosomes) within the sandfly midgut and are then co-egested by the sandfly during a blood meal as part of the inoculum (Atayde et al., 2015). Through TEM imaging, MVB formation and vesicle secretion were observed in *Leishmania* parasites obtained from sandfly midgut dissection. Proteomic analysis and western blotting confirmed that these vesicles from the midgut are enriched with several virulence factors, including GP63, as well as other proteins typically found in *Leishmania* EVs like HSP83 and HSP70. Co-inoculation of mouse footpads with both *Leishmania* parasites and EVs significantly increased infection severity, corroborating previous *in vitro* findings (Hassani et al., 2011; Silverman & Reiner, 2011). Another potential role for *Leishmania* EVs secreted in the sandfly midgut may be to support *Leishmania* survival within the midgut, though this hypothesis requires further investigation.

Importantly, the previously observed requirement of GP63 expression by *L. major* for virulence also applies to EVs. It has been demonstrated that EVs from GP63<sup>-/-</sup> *L. major* were less effective at modulating PTPs and TFs compared to EVs from GP63<sup>+/+</sup> *L. major*, confirming that GP63 largely mediates the immunomodulatory functions of *Leishmania* EVs (da Silva Lira Filho et al., 2021; Hassani et al., 2014). Notably, the absence of GP63 also altered the

protein content of *L. major* EVs, with proteomic analysis showing a significantly different protein profile in GP63<sup>-/-</sup> EVs compared to GP63<sup>+/+</sup> EVs, suggesting that GP63 may play a role in sorting the cargo of *Leishmania* EVs. Contrastingly, it was also found that GP63 abundance in *Leishmania* EVs does not impact EV protein content, suggesting that the observed effect on protein sorting only occurs in the complete absence of GP63 (da Silva Lira Filho et al., 2021). These findings align with previous research by Silverman et al., who found that *L. donovani* EVs are predominantly immunosuppressive, promoting parasite survival by suppressing monocyte response to IFN- $\gamma$ , inducing IL-10 production, and inhibiting TNF- $\alpha$  production. Mice pre-inoculated with *L. donovani* EVs exhibited higher levels of IL-4-producing CD4<sup>+</sup> T cells, leading to disease exacerbation upon *L. donovani* challenge (Silverman & Reiner, 2011).

Surprisingly, *Leishmania* EVs are not just carriers of virulence factors, but can be carriers of actual viruses as well. Just as other viruses, such as HIV, HCV, HSV, and EBV, exploit host EV biogenesis to release viral EVs containing viral proteins, nucleic acids, or entire virions, the *Leishmania* RNA Virus 1 (LRV1) also exploits *Leishmania* EVs (Alenquer & Amorim, 2015; Atayde et al., 2019; Meckes & Raab-Traub, 2011; Teow et al., 2017). LRV1 is a key indicator of MCL severity caused by *L. (V.) guyanensis*, and its small size (50 nm) raised the possibility that it could be packaged within EVs. Furthermore, *L. donovani* and *L. braziliensis* EVs are enriched in small non-coding RNAs, demonstrating a high propensity for RNA transport within *Leishmania* EVs (Lambertz et al., 2015). Eventually, TEM was used to confirm the presence of LRV1 viral particles in up to 30% of all LRV1<sup>+</sup> *L. (V.) guyanensis* (Lg21<sup>+</sup>) EVs, characterized as exosomes through TEM, NTA, and western blotting (Atayde et al., 2019). These Lg21<sup>+</sup> exosomes facilitated rapid infection of *L. mexicana* and *L. (V.) panamensis*, though only *L. (V.) panamensis* could sustain the infection, suggesting that LRV1 can infect only *Leishmania Viannia* species. The LRV<sup>+</sup> *L. (V.) panamensis* exhibited greater virulence and a unique protein profile compared to WT *L. (V.) panamensis*. Notably, Lg21<sup>+</sup> EVs significantly increased the severity of *L. (V.) panamensis* and *L. mexicana* infections, while naked LRV1 did not induce the same level of virulence, indicating that LRV1 requires exosome envelopment to trigger host immunopathology. Supporting these findings, it was

reported that LRV1<sup>+</sup> EVs alone are sufficient to induce TLR3 activation and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inhibition (de Carvalho et al., 2019). Although LRV1 can induce TLR3-mediated NLRP3 inflammasome inhibition on its own, its packaging into EVs is necessary for this process to occur effectively.

The studies above reinforce the idea that EVs play a crucial role in *Leishmania* virulence and host immune modulation, with their ability to package and transport LRV1 sparking interest in their potential applications for molecule delivery. It has also been hypothesized that *Leishmania* EVs can deliver bioactive nucleic acids to recipient cells, including *Leishmania* itself. Through the investigation of EVs purified from several drug-resistant *L. infantum* strains, Douanne et al. identified numerous enriched proteins, including virulence factors and TFs. Notably, EVs from an antimony-resistant strain were found to be enriched in proteins such as SPRY domain-containing ubiquitin ligase and proteasome regulatory units, which are vital for parasite survival within the intracellular environment (Douanne et al., 2022). Interestingly, drug resistance has also been shown to cause alterations in the size and morphology of EVs released by the parasite. Further research is needed to better understand the role of EVs in the transmission of drug resistance, both in laboratory settings and in the field.

## 1.4 Objectives and rationale

Despite extensive efforts by global health organizations and research institutions, leishmaniasis continues to pose a significant public health challenge, particularly in impoverished regions and among immunocompromised individuals. Gaining a deeper insight into how the parasite modulates the immune system could open new avenues for therapeutic and preventive measures. EV research represents one of the most promising of these potential new avenues of study, yet research into the role of EVs in parasite-host interactions falls far behind the immense popularity of EV research in other therapeutic and human health fields like cancer. As is abundantly evident from this research, the regulation of and by EVs in the context of cell-cell communication is highly nuanced even within the same organism. Thus, it is of great importance to translate our understanding of EVs to other

eukaryotes, particularly pathogenic eukaryotic organisms. In this case, the study of EVs secreted by the protozoan parasite *Leishmania* serves dual purpose: the verification of our knowledge and understanding of EV biosynthesis in higher eukaryotes (and yeast) in parasites that cause significant global health burden, as well as a deeper understanding of how this parasite modulates its mammalian host to evade immune response and establish infection.

Building upon previous work with other eukaryotes as well as with *Leishmania*, research from the Olivier lab has further cemented the key role of parasite EVs in *Leishmania* infection and disease progression. *Leishmania* EVs act as vehicles for virulence factors that modulate the host immune response. Their secretion is not only stimulated by 37°C TS, simulating entry into the human host, but also proceeds constitutively within the sandfly midgut and forming a part of the initial co-inoculum. These EVs can even act as a vehicle for LRV1, facilitating a viral infection that also benefits *Leishmania* infection and enhances leishmaniasis severity. While this body of work has established the importance of *Leishmania* EVs to infection, the deeper mechanisms remain mysterious. Though it is impossible to directly apply our knowledge about EV biogenesis from other eukaryotes, this previous work serves as a great base to build from. One key challenge to this translation is the inherent heterogeneity of EVs, both between different EV types as well as within those types. It has been repeatedly demonstrated that EV regulation and function depends greatly upon the cell type and cell state, making the study of EV release from a homogenous cell population or even single cells an important facet to EV research. With *Leishmania*, the culturing of *Leishmania* clones could prove to be a unique method to study EV heterogeneity and the resulting insights may identify key components of *Leishmania* EV biogenesis and secretion. In fact, previous proteomic analysis from our lab has identified ESCRT analogs in *Leishmania* beyond those belonging to ESCRTIII, making the validation of ESCRT function in *Leishmania* another priority. These form the main objectives of my PhD thesis:

- i. Investigate *Leishmania* EV heterogeneity of EV populations secreted by different clonal colonies of *Leishmania mexicana*, comparing their physical properties, contents, and infectious capabilities
- ii. Investigate the role of the ESCRTII protein Vps36 in *Leishmania major* EV biogenesis as well as any resulting effects on parasite viability and infectivity due to *Vps36* disruption

Chapter 2. Unique *Leishmania mexicana* clones secrete populations of extracellular vesicles with unique protein profile and variable infectious capability

## Preface and rationale

EVs have emerged as the main facilitators of cell-cell communication, being the literal vehicles of cellular interaction. Therefore, further our understanding of the biogenesis and effect of EVs will greatly improve our knowledge on human disease, which is often the outcome of improper cellular signalling. This can result from either an internal (cancer or autoimmune diseases) or external (infectious diseases) source. However, the regulation of EV biogenesis is highly complex and unique for each cell type. Even the effector role of EVs depends on the context of their secretion and recipient cell. This results in highly heterogeneous populations of EVs being secreted by cell populations, even those of the same cell type. This poses a great challenge to the study of EVs in not only multicellular eukaryotes, but also in pathogenic unicellular eukaryotes like *Leishmania*, the causative agent of the NTD leishmaniasis.

Despite being relatively “simpler,” the unicellular protozoan parasite *Leishmania* also demonstrates a high degree of population heterogeneity due to their genetic plasticity. Thus, it stands to reason that the resulting secreted EVs will be highly heterogeneous as well. Thankfully, due to their unicellular nature, there is a fairly simple method to “homogenize” *Leishmania*: culturing clonal colonies on solid media. By generating several “clonal” cultures of *L. mexicana* through this method, we can investigate the population of EVs secreted by a homogenous culture of *L. mexicana* and compare them to EVs secreted by other clonal *L. mexicana* cultures. This may lead to phenotypic differences in the clonal EV populations that lead to altered infection and disease progression.

*L. mexicana* was cultured on solid SDM agar plates, yielding eight distinct clonal colonies from which EVs were collected after expansion in liquid culture. These EVs were subsequently characterized using NTA, TEM, and proteomic analysis. The eight clonal *L. mexicana* cultures appeared visually identical, exhibiting similar growth rates, with these similarities extending to the physical characteristics of their EVs. However, proteomic analysis revealed distinct protein profiles among the EVs from each clone, setting them apart from one another and from EVs derived from a heterogeneous (Ht) *L. mexicana* culture.

Furthermore, we observed that EVs from clone CL7 consistently led to reduced footpad swelling in C57BL/6 mice compared to EVs from clones CL1, CL8, and the Ht *L. mexicana*. Interestingly, the clonal parasites alone did not exhibit significantly different infectious properties in C57BL/6 mice, though CL1 *L. mexicana* alone exhibited increased infectivity in BALB/C mice. These findings indicate that EVs from different clonal colonies of *L. mexicana* contain unique protein cargoes, which can distinctly influence infection outcomes.

## Unique *Leishmania mexicana* clones secrete populations of extracellular vesicles with unique protein profile and variable infectious capability

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## Abstract

The study of extracellular vesicles has become an incredibly important field of study, but the inherent heterogeneity of these vesicles continues to make their study challenging. The genetic variability and well-documented protocols for the growth and vesicle isolation from *Leishmania* parasites provide a unique opportunity to compare the heterogeneity of different populations secreted by *Leishmania* clones. *Leishmania mexicana* was cultured on solid SDM agar plates and 8 clonal colonies were selected. The EVs collected from the liquid cultures of these 8 clones were assessed by NTA, TEM, and proteomic analysis. We found that all 8 clonal *L. mexicana* cultures were visually indistinguishable from each other and had similar growth rate, and these physical similarities extended to their EVs. However, proteomic analysis reveals that the EVs collected have unique protein profiles compared to each other and EVs isolated from a heterogeneous liquid culture of *L. mexicana*. We selected 3 clonal EVs for further mouse infection experiments and found that EVs from CL7 *L. mexicana* consistently caused reduced footpad swelling in C57BL6 mice footpads compared to EVs from CL1, CL8, and heterogenous *L. mexicana*. This trend was not observed when infecting Balb/c mice with the parasites alone, with only CL1 *L. mexicana* causing significantly increased infection. Our results together show that EVs isolated from different clonal colonies of *L. mexicana* have distinct differences in protein cargo which can lead to varying outcomes on *Leishmania* infection. Further evaluation will be needed to determine the underlying mechanisms behind this and verify that differences observed in infectivity are directly caused by variations between our *L. mexicana* clones, especially genetic sequencing and immunoblotting to validate our results.

## Introduction

Extracellular vesicles (EVs) are important vehicles for cell-cell communication and are constitutively secreted by both bacteria and eukaryotes (1). They carry many biologically active macromolecules, ranging from nucleic acids to proteins, from their cell of origin and deliver these to a recipient cell to induce the desired physiological change (2). EVs have been of great interest due to their importance in intercellular communication, especially in the context of immune and tumor cell research and have recently been established to play an important role in pathogen-host interaction (3). Understanding the mechanisms of EV biogenesis and release/uptake have already been proven to be key to understanding human disease progression and diagnosis, but the study of EVs in pathogens and parasites is still a developing field of research.

*Leishmania* spp. are protozoan parasites that are the causative agents of the neglected tropical disease Leishmaniasis (4). These parasites specifically infect the macrophages of mammalian hosts and have evolved several mechanisms to evade the host immune response involving a variety of immune-modulating virulence factors (5). Our lab has extensively studied these immunomodulatory virulence factors, especially GP63, and our research along with that of others in the field has revealed the important role of *Leishmania* EVs as vehicles for the delivery of *Leishmania* virulence factors to target mammalian host cells. *Leishmania* EVs are constitutively secreted and can also be induced by 37°C temperature shock (6-8). These EVs are loaded with common virulence factors like GP63 and EF1a and have profound immunomodulatory effects on mammalian macrophages. *Leishmania* EVs are also constitutively secreted within the midgut of its Sandfly vector and are therefore present during initial infection (9). This co-inoculation causes heightened infection severity.

There are many distinct classes of EVs, including ectosomes, microvesicles, apoptotic bodies, exosomes, exomeres, and more (10). Most recent EV research has focused on exosomes, a class of EVs ranging from 30 to 140nm in diameter. Exosomes originate from the endocytic pathway and first form as intraluminal vesicles within multivesicular bodies

(MVB). Eventually, these vesicles are released once the MVB fuses with the plasma membrane. It was this distinct biogenesis pathway with directed cargo sorting that made exosomes particularly attractive to study as the main vehicles for intercellular communication. However, it has been shown that exosomes cannot be defined by consistent biomarkers or a conveniently distinct cellular origin and have instead begun to be classified based on their small size (sEVs) (11). This heterogeneity of EVs extends further to the subpopulations of EVs that can be secreted by not only different cell types but even individual cells within a single population (12).

The study of EV heterogeneity at the single cell level is a relatively recent development and often involve the use of expensive and/or custom-made microfluidic systems (13). However, *Leishmania* spp. present an alternative novel method of shedding light on the variance of EVs. *Leishmania* can be grown in liquid cultures and can also be plated onto solid media. When plated, *Leishmania* forms clonal colonies like many bacteria cultures (14). By selecting clonal colonies of *Leishmania*, genetically identical liquid cultures of *Leishmania* can be expanded and EVs can be collected from these cultures. Following this methodology, 8 different clones of *Leishmania mexicana*, a species with great genetic heterogeneity (15), were selected and the EVs released by these clones were collected and compared based on their characteristics and effects on *Leishmania* infection.

## Methods

### Parasite culture and plating

*L. mexicana* m379 was cultured in 26C liquid Schneider's Drosophila Medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µl/ml streptomycin (complete Schneider's). To make solid SDM agar, un-supplemented SDM was mixed with 2.5% agar solution and heat inactivated FBS in a ratio of 5:4:1, then this mixture was heated to 56C and poured into 100mm petri dishes to a thickness of 5mm and cooled.  $2 \times 10^6$  log phase (3-4 day old liquid

culture) *L. mexicana* promastigotes were then spread onto the SDM-agar plates and incubated at 26°C for 10 days. Individual colonies were then carefully picked under magnification and cultured in liquid SDM. The resulting liquid cultures were then frozen after 3-4 days. Subsequent culturing of frozen *L. mexicana* clones was limited to a maximum of 2 passages.

#### EV purification

Purification of *Leishmania* exosomes was performed according to standard methods for mammalian cells (2, 6). *L. mexicana* stationary phase promastigotes (day 6-8) were washed twice with PBS, resuspended in RPMI 1640 medium (Life Technologies, Rockville, MD) without FBS and phenol red at a concentration of  $2-4 \times 10^8$  promastigotes/ml, and incubated for 4 hours at 37°C for the release of EVs in the culture medium. At the end of a 4-hour incubation, the sample was centrifuged at 4000 RPM to remove parasites and large debris and the supernatant was filtered through a 0.45 µm syringe filter, followed by a 0.20 µm syringe filter. Next, EVs were pelleted by 2 rounds of 1-hour centrifugation at 100 000 g at 4°C and resuspended in exosome buffer (137 mM NaCl, 20 mM Hepes, pH 7.5). Resulting sEV samples were aliquoted and frozen at -80°C, with each aliquot being thawed no more than twice. sEV protein content was determined using Thermo Fisher MicroBCA kit according to manufacturer instructions.

#### Transmission electron microscopy (TEM)

In vitro purified *L. mexicana* clone EVs were coated directly onto carbon-coated copper grids by dropping 5 µl on to the grid and allowing to dry for 2 minutes. The grids were then dried using the edge of a Kimwipe and fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 minute, washed 3 times in milliQ water, and stained with 1% uranyl acetate for 1 minute. After drying, grids were visualized in the FEI Tecnai G2 Spirit Twin 120 kV Cryo-TEM and images were taken with the Gatan Ultrascan 4000 4k x 4k CCD Camera System Model 895. Microscopy and sample preparation was performed at the Facility for Electron Microscopy Research at McGill University.

#### Nanoparticle tracking analysis (NTA)

sEV samples were initially diluted by 1:100 in exosome buffer and NTA was performed using the NanoSight NS500 (Malvern Panalytical). Samples were loaded into the machine at a minimum initial volume of 500  $\mu$ l. Focus and camera level were then adjusted based on the first sample before standard script was run (camera level was unchanged for subsequent samples). Three 30 second videos were taken using the standard camera and analysis was performed using the NTA software to determine particle quantity and size as well as to perform statistical analysis.

#### LC/MS-MS

LC-MS/MS was performed as previously described at the Institute de Recherches Cliniques de Montréal (IRCM, University of Montreal, Canada) (5, 9, 16). EVs were diluted in ultrapure water and protein content was determined using the microBCA protein assay kit (Thermo Scientific) to account for the high dilution ratio (1:20). Protein concentration was determined using an Infinite 200 Pro spectrophotometer (Tecan) and interpolation with a BSA standard curve. EV protein was then precipitated with 15% trichloroacetic acid (TCA)/acetone and processed for LC-MS/MS analysis, utilizing 3 $\mu$ g of protein from each sample of clonal EVs and Ht clonal EVs. After precipitation, in solution digestion was performed by the addition of trypsin at a ratio of 1:25 protease: protein. After an overnight incubation at 37°C, the reactions were quenched by the addition of formic acid to a final concentration of 0.2% and cleaned with C18 Zip Tip pipette tips (Millipore, Billerica, MA), before mass spectrometry analysis. Extracted peptides were injected onto a Zorbax Extended-C18 desalting column (Agilent, Santa Clara, CA) and subsequently chromatographically separated on a Biobasic 18 Integrafrit capillary column (Thermo Scientific, Rockford, IL) on a Nano High-Performance liquid chromatography system (1100 series unit; Agilent, Santa Clara, CA). Eluted peptides were electrosprayed as they exited the capillary column and were analyzed on a QTRAP 4000 linear ion trap mass spectrometer (SCIEX/ABI, Framingham, MA).

#### Proteomic analysis

Individual sample tandem mass spectrometry spectra were peak listed using the Distiller version 2.1.0.0 software ([www.matrixscience.com/distiller](http://www.matrixscience.com/distiller)) with the following parameters:

minimum mass set to 500 Da, maximum mass set to 6,000 Da, no grouping of MS/MS spectra, precursor charge set to auto, minimum number of fragment ions set to 5, and peak picking parameters set at 1 for signal noise ratio and at 0.3 for correlation threshold. The peak-listed data was then searched against the NCBI database with the Mascot software version 2.3.02 (Matrix Science, London, UK). Mascot was set up to search the *L. mexicana* database (txid5665, 17 265 proteins) with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 1.5 Da. Carbamidomethyl was specified in both search engines as a fixed modification. Oxidation of methionine residues was specified in Mascot as a variable modification. Scaffold software version 4.0.6.1 (Proteome Software Inc., Portland, OR) was used to validate MS/MS peptide and protein identifications. Identification of peptides was accepted if it could be established at greater than 95.0% probability. Identification of proteins was accepted if it could be established at greater than 95.0% probability and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The final number of peptides per protein was represented by the average of the biological replicas after normalization to the total number of peptides. Unidentified proteins were removed from analysis. Unique and shared proteins were compared between different *L. mexicana* clones using UpSet (17). Gene ontology (GO) annotations were attributed using Panther by converting *L. mexicana* uniprot accession IDs to *Leishmania major* to functionally classify each protein (18). *L. mexicana* interacting EV proteins were mapped using STRING, searching against *L. major* as the STRING database lacks *L. mexicana* (19). The full STRING network was used with interactions determined based on evidence and a medium minimum interaction score (0.400). Clusters were generated using k-means clustering with 4 clusters and interactions were determined based on textmining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence.

### Mice infections

All experiments with mice were carried out in pathogen-free housing and in accordance with the regulations of the Canadian Council of Animal Care, approved by the McGill University

Animal Care Committee. Female C57BL6 mice (6 to 8 weeks old) were infected in the right hind footpad with  $5 \times 10^6$  stationary phase *L. mexicana* Ht promastigotes and 5  $\mu$ g of in vitro purified *L. mexicana* Ht, CL1, 7, and 8 sEVs. Female C57BL6 mice were also infected in the right hind footpad with  $5 \times 10^6$  *L. mexicana* Ht, CL1, 7, and 8 promastigotes without vesicle co-inoculation. This infection was also performed using male BALB/c mice (6 to 8 weeks old). Disease progression was monitored by measuring footpad swelling weekly with a metric caliper, up to 10 weeks post-infection. At the end of each experiment, footpads were imaged. Statistical analysis was performed using Graphpad Prism 8.

## Results

### *L. mexicana* clone EVs have similar size and morphology

NTA was performed on EVs isolated from 8 different *L. mexicana* cultures grown from clonal colonies, as well as an Ht *L. mexicana* culture, using the NS500. The average concentration, mean particle diameter, and mode particle diameter were recorded (Table 1) and the particle size distribution of each EV population was graphed using the NanoSight NTA software (Figure 1). Particle concentration of each *L. mexicana* clone EV and the Ht EVs were compared, with CL2 having the lowest particle concentration and Ht having the highest (Figure 2A). However, no significant difference in particle concentration was found between the clones or Ht. Mean particle size (Figure 2B) and proportional size distribution (Figure 2C) were also compared, with no significant difference found in either comparison, though CL2 EVs had much lower mean and median particle size. TEM imaging was also performed to confirm successful EV isolation (Figure 1). Well defined EVs were identified in all samples, demonstrating a circular shape with the signature cupped form due to sample drying. The EVs were visually indistinguishable between samples.

| <b><i>L. mexicana</i><br/>culture</b> | <b>Mean Particle<br/>Concentration<br/>(particles/ml)</b> | <b>Mean Particle<br/>Diameter (nm)</b> | <b>Mode Particle<br/>Diameter (nm)</b> |
|---------------------------------------|-----------------------------------------------------------|----------------------------------------|----------------------------------------|
| CL1                                   | 1.76E11                                                   | 185                                    | 159                                    |
| CL2                                   | 5.47E10                                                   | 171                                    | 149                                    |
| CL3                                   | 1.29E11                                                   | 183                                    | 137                                    |
| CL4                                   | 3.11E11                                                   | 194                                    | 148                                    |
| CL5                                   | 3.25E11                                                   | 188                                    | 153                                    |
| CL6                                   | 1.39E11                                                   | 201                                    | 148                                    |
| CL7                                   | 1.56E11                                                   | 199                                    | 168                                    |
| CL8                                   | 3.43E11                                                   | 197                                    | 156                                    |
| Ht                                    | 3.72E11                                                   | 203                                    | 151                                    |

*Table 1. Mean concentration, mean particle diameter, and mode particle diameter of EVs isolated from L. mexicana clones and Ht. Stats were determined by the NS500 and NTA software from Malvern Panalytical from 3 separate videos for each sample.*

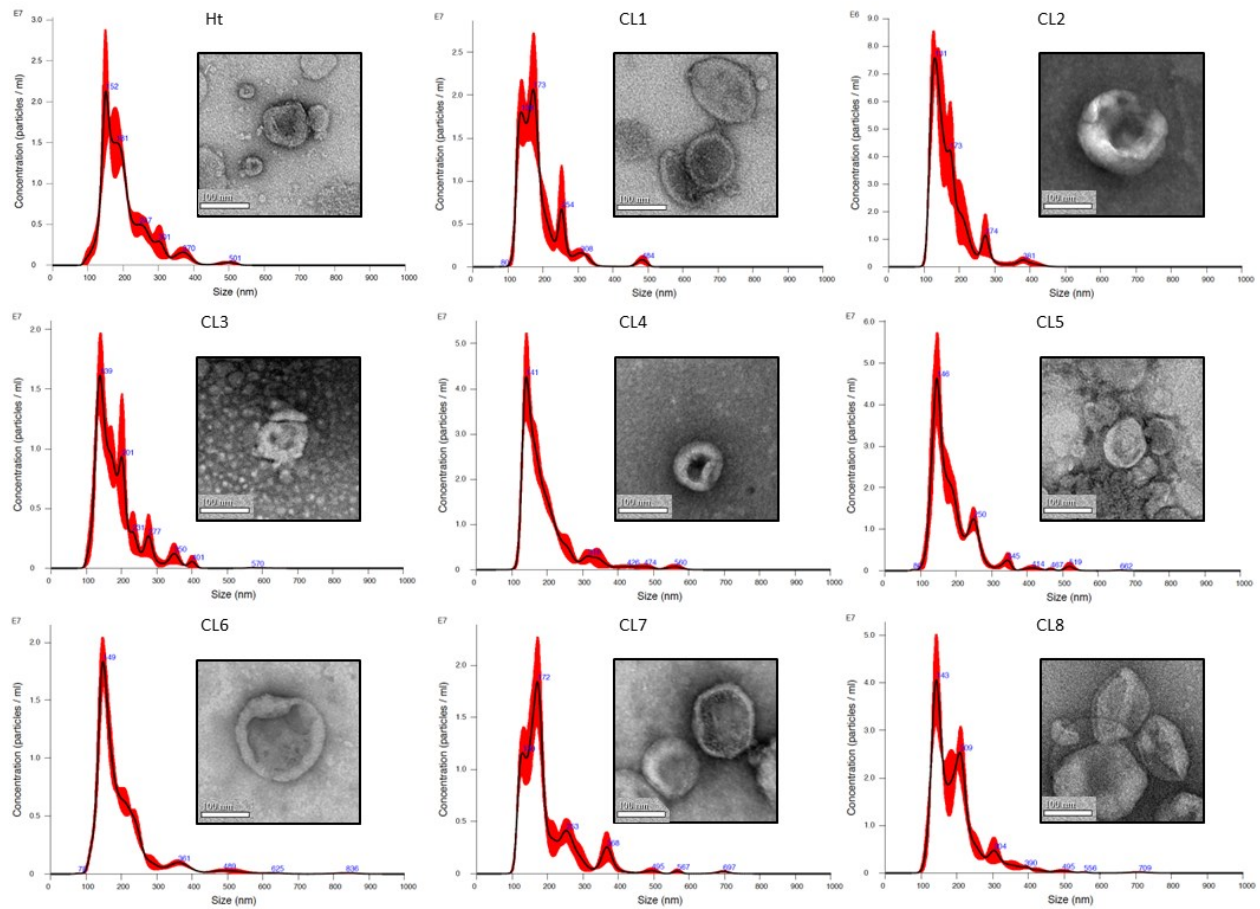


Figure 1. Particle diameter distribution of EVs isolated from each *L. mexicana* clone and Ht graphed by Malvern Panalytical NTA software. Example TEM images of the EVs are also provided, each having been taken at 30 000x magnification, with the scale bar representing 100nm.

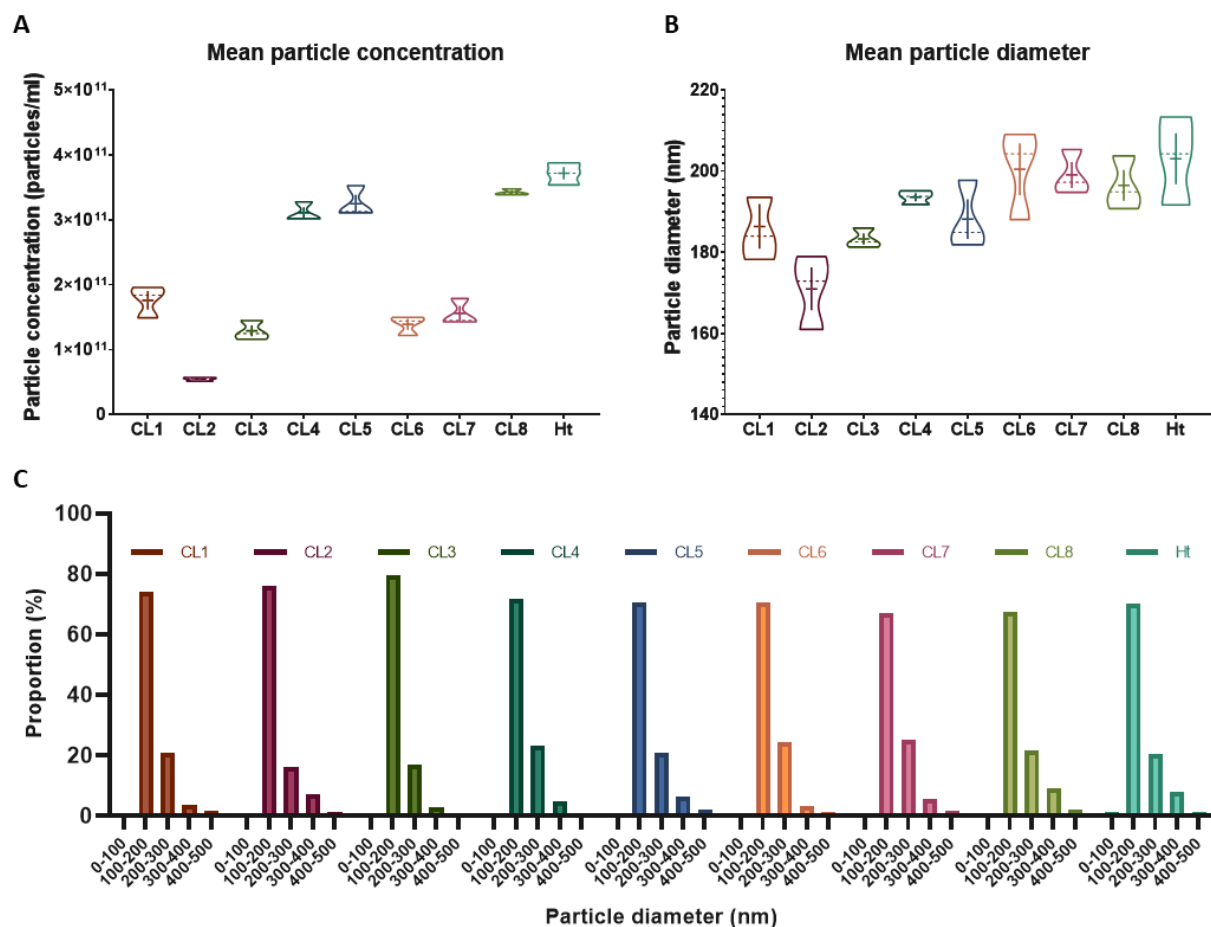


Figure 2. Mean particle concentration and size were compared between the *L. mexicana* clones and Ht *L. mexicana* using violin plots (A and B). The solid line represents the mean while the dotted line represents the median. Proportional size distribution of EVs isolated from each *L. mexicana* clone and Ht was also graphed and to compare overall variations between the EV populations (C). Particle concentration is represented as proportional to the total EV concentration of each sample.

### EV protein content varies between different *L. mexicana* clones

A total of 253 proteins were identified across all 8 *L. mexicana* clonal EVs and the Ht EVs. These proteins were analyzed using Scaffold 4 (Supp. Table 1). Unique and shared proteins between the 8 *L. mexicana* clones and Ht *L. mexicana* was visualized using an UpSet plot (Figure 3). Surprisingly few proteins were identified in sEVs collected from the Ht *L. mexicana* culture, having the lowest number of individual proteins identified, followed by CL3 and 7.

Ht *L. mexicana* and CL3 also had the most proteins uniquely absent, at 11 and 9 respectively, as well as sharing 15 proteins absent from all other groups (Table 2). This was unexpected considering that the Ht *L. mexicana* was the original progenitor strain, and thus we expected its EVs to contain most if not all proteins found in other clonal EVs. Surprisingly, despite CL7 having the next fewest individual proteins identified, there were only 3 proteins, long-chain-fatty-acid-CoA ligase, surface protein amastin, and amastin-like protein, uniquely absent from CL7 compared to all other groups, showing that there was strong overlap between CL7 and other clones altogether. CL8 EVs also uniquely lacked a cytochrome C protein, which is an important cell respiratory component.

| Unique to | Protein Name                                                                 | Accession number | Molecular Weight | Function                  |
|-----------|------------------------------------------------------------------------------|------------------|------------------|---------------------------|
| CL1       | Protein disulfide-isomerase                                                  | E9AUD1_LEIMU     | 52 kDa           | Catalyses protein folding |
|           | Putative carboxypeptidase                                                    | E9B493_LEIMU     | 57 kDa           | Metalloprotease           |
|           | Fructose-bisphosphate aldolase                                               | E9ASP6_LEIMU     | 41 kDa           | Glycolysis                |
|           | Putative ATP-dependent Clp protease subunit, heat shock protein 100 (HSP100) | E9ALU6_LEIMU     | 97 kDa           | ATP binding               |
|           | Putative short chain dehydrogenase                                           | E9B601_LEIMU     | 25 kDa           | Oxidoreductase            |
|           | Putative lipophosphoglycan biosynthetic protein                              | E9AM02_LEIMU     | 87 kDa           | Chaperone                 |

|            |                                                                      |              |         |                                           |
|------------|----------------------------------------------------------------------|--------------|---------|-------------------------------------------|
| <b>CL3</b> | S-methyl-5'-thioadenosine phosphorylase                              | E9AKI9_LEIMU | 33 kDa  | L-methionine biosynthesis salvage pathway |
| <b>CL4</b> | Conserved TPR domain protein                                         | E9B146_LEIMU | 45 kDa  | HSP-binding                               |
|            | Kinesin-like protein                                                 | E9AR52_LEIMU | 105 kDa | ATP-dependent microtubule motor           |
| <b>CL8</b> | Putative asparagine synthetase a (Putative aspartate-ammonia ligase) | E9AY61_LEIMU | 40 kDa  | Asparagine biosynthesis                   |
|            | Putative serine/threonine-protein kinase                             | E9AVE6_LEIMU | 90 kDa  | Protein kinase                            |
| <b>Ht</b>  | Amastin-like surface protein-like protein                            | E9B0L6_LEIMU | 25 kDa  | Integral membrane protein                 |
|            | Putative zinc transporter                                            | E9B012_LEIMU | 47 kDa  | Transmembrane zinc transporter            |
|            | GTP-binding nuclear protein                                          | E9AXM1_LEIMU | 24 kDa  | Protein transport                         |
|            | Transmembrane 9 superfamily member                                   | E9ALR2_LEIMU | 77 kDa  | Signal transmembrane helix                |

| Missing from | Protein Name                                 | Accession number | Molecular Weight | Function                                      |
|--------------|----------------------------------------------|------------------|------------------|-----------------------------------------------|
| CL1          | Putative 3'-nucleotidase/nuclease            | E9ANW7_LEIMU     | 41 kDa           | Endonuclease                                  |
| CL3          | Putative myosin heavy chain                  | E9B3G5_LEIMU     | 119 kDa          | Actin binding motor                           |
|              | Putative ecotin                              | E9AQ49_LEIMU     | 41 kDa           | Serine-type endopeptidase inhibitor           |
|              | Putative casein kinase                       | E9B5Y1_LEIMU     | 40 kDa           | Serine/threonine kinase                       |
|              | Putative calpain-like cysteine peptidase     | E9AK26_LEIMU     | 89 kDa           | Calcium-dependent cysteine-type endopeptidase |
|              | Proteasome regulatory ATPase subunit         | E9ATM0_LEIMU     | 46 kDa           | Proteasome-activating                         |
|              | Glyceraldehyde-3-phosphate dehydrogenase     | E9B170_LEIMU     | 39 kDa           | Glycolysis                                    |
|              | Putative proteophosphoglycan ppg3 (Fragment) | E9B5T5_LEIMU     | 120 kDa          | Extracellular matrix                          |
|              | GDP-mannose pyrophosphorylase                | Q9BLW4_LEIME     | 41 kDa           | Mannose-1-phosphate guanylyltransferase       |
|              | Putative small GTP-binding protein Rab1      | E9AYX8_LEIMU     | 22 kDa           | GTPase                                        |
|              |                                              |                  |                  |                                               |
| CL5          | Clathrin heavy chain                         | E9AST4_LEIMU     | 191 kDa          | Intracellular/vesicle-mediated transport      |
|              | Putative small GTP-binding protein Rab11     | E9ANA1_LEIMU     | 23 kDa           | GTP binding                                   |

|            |                                                             |              |         |                                          |
|------------|-------------------------------------------------------------|--------------|---------|------------------------------------------|
| <b>CL6</b> | Putative c2 domain protein                                  | E9B1N1_LEIMU | 30 kDa  | Calcium/lipid-binding domain             |
| <b>CL7</b> | Putative long-chain-fatty-acid-CoA ligase                   | E9AJD9_LEIMU | 78 kDa  | Long-chain fatty acid-CoA ligase         |
|            | Amastin-like protein                                        | E9B4S0_LEIMU | 21 kDa  | Integral membrane protein                |
|            | Putative surface protein amastin                            | E9B0L4_LEIMU | 21 kDa  | Integral membrane protein                |
| <b>CL8</b> | Putative cytochrome c                                       | E9AQU2_LEIMU | 12 kDa  | Electron transport                       |
| <b>Ht</b>  | Pyruvate kinase                                             | E9B5P1_LEIMU | 54 kDa  | Glycolysis                               |
|            | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | E9AUA1_LEIMU | 61 kDa  | Glycolysis                               |
|            | Heat shock protein 70-related protein                       | E9AYA3_LEIMU | 71 kDa  | ATP binding                              |
|            | Putative ABC transporter                                    | E9AYZ8_LEIMU | 203 kDa | ATPase-coupled transmembrane transporter |
|            | Putative seryl-tRNA synthetase                              | E9ANF6_LEIMU | 53 kDa  | Seryl-tRNA aminoacylation                |
|            | Trypanothione reductase                                     | E9AKE1_LEIMU | 53 kDa  | Glutathione reductase equivalent         |
|            | Serine/threonine-protein phosphatase                        | E9AKB4_LEIMU | 72 kDa  | Serine/threonine phosphatase             |
|            | Putative adenosine kinase                                   | E9B0L7_LEIMU | 37 kDa  | AMP biosynthesis salvage pathway         |
|            |                                                             |              |         |                                          |

|  |                               |              |        |                               |
|--|-------------------------------|--------------|--------|-------------------------------|
|  | Kinesin-like protein          | E9ARX7_LEIMU | 94 kDa | Microtubule-based movement    |
|  | Proteasome subunit alpha type | E9AVH9_LEIMU | 27 kDa | Ubiquitin-dependent hydrolase |
|  | Putative Qc-SNARE protein     | E9AM81_LEIMU | 24 kDa | Vesicle-mediated transport    |

Table 2. Proteins categorized based on being present in only a single *L. mexicana* clone (Blue) or missing from only a single *L. mexicana* clone (Yellow)

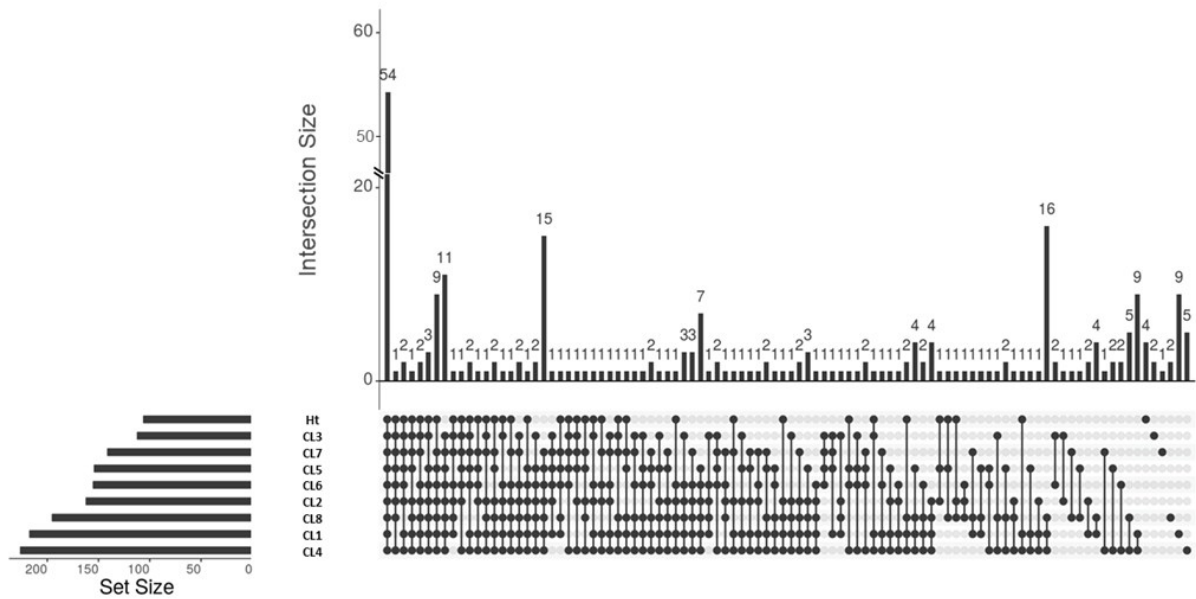


Figure 3. UpSet plot representing the shared and unique proteins present in *L. mexicana* clones and *Ht*. The legend is found below the plot, where each dot represents the presence of the proteins in that sample and the line representing that these proteins are shared between all samples with a dot. Samples are ordered from least unique proteins to most.

### EV protein abundance varies between different *L. mexicana* clones

Overall protein abundance was measured in total spectra score using scaffold and compared between the 8 clones (Figure 4). From this comparison, it is evident that CL1, 4, and 8 sEVs generally contain more proteins compared to other clones, with the blue line representing average total spectrum count of all clones. This higher protein abundance is especially clear when representing this data using a Z-score heatmap with all 253 identified proteins (Figure 5). To better display typically high abundance proteins found in *Leishmania*, a heatmap was also generated with the top 50 most abundant proteins across all clonal and Ht EVs, with this limited scope also allowing visualization of total spectra rather than Z-score (Figure 6). CL7 EVs contained the lowest amount of GP63, HSP70, HSP83-1, plasma membrane ATPase, EF1a, and EF2 (Figure 6, Supp. Table 1). CL3 EVs had lower overall protein abundance but a significantly higher quantity of clathrin heavy chain. CL1 EVs contained the highest amount of actin, GP63, EF1a, and EF2 amongst the clones. Although Ht EVs contained fewer unique proteins, overall protein abundance was higher than EVs from other *L. mexicana* clones, having the highest amount of HSP70, GP63, EF1a, and tubulin beta. CL8 EVs contained the highest amount of receptor-type adenylate cyclase A. The combined low abundance and low number of proteins identified in CL3 EVs suggested that there was material loss during the processing or analysis of this sample, resulting in its exclusion from further analysis. However, CL3 EVs contained by far the most clathrin heavy chain despite their low overall protein abundance.

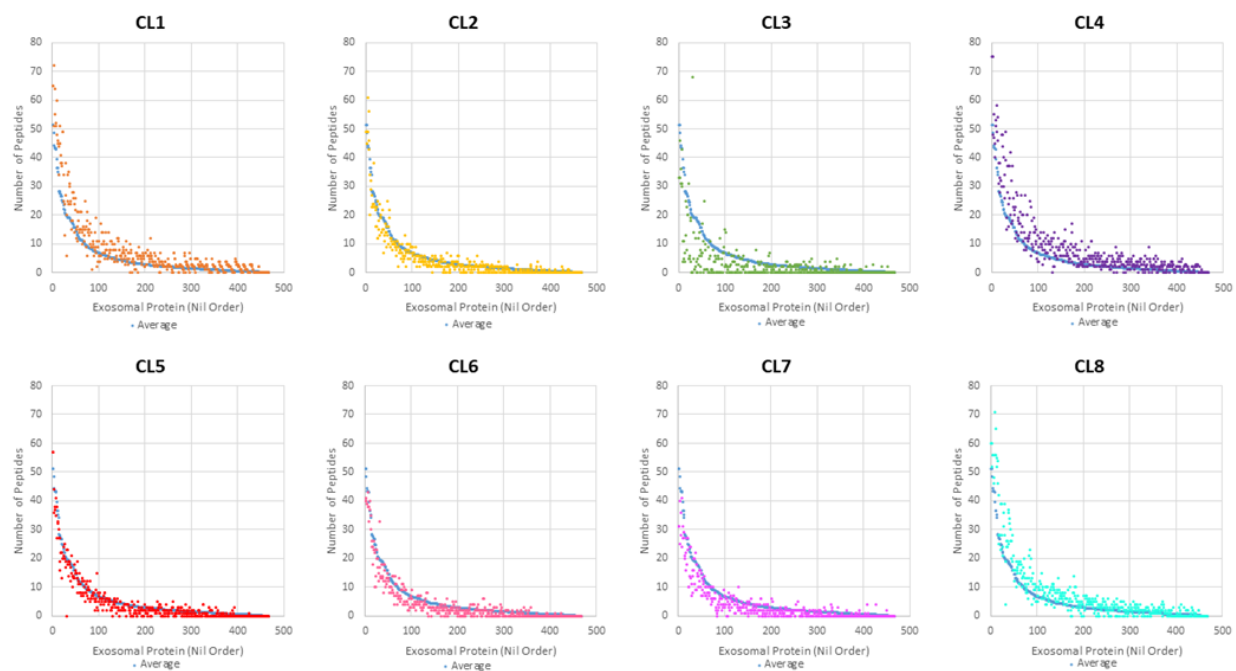


Figure 4. Overall protein abundance of each *L. mexicana* clone was plotted and compared, with the blue dots representing the baseline. Each dot represents an individual protein and the spectrum count associated with that protein.

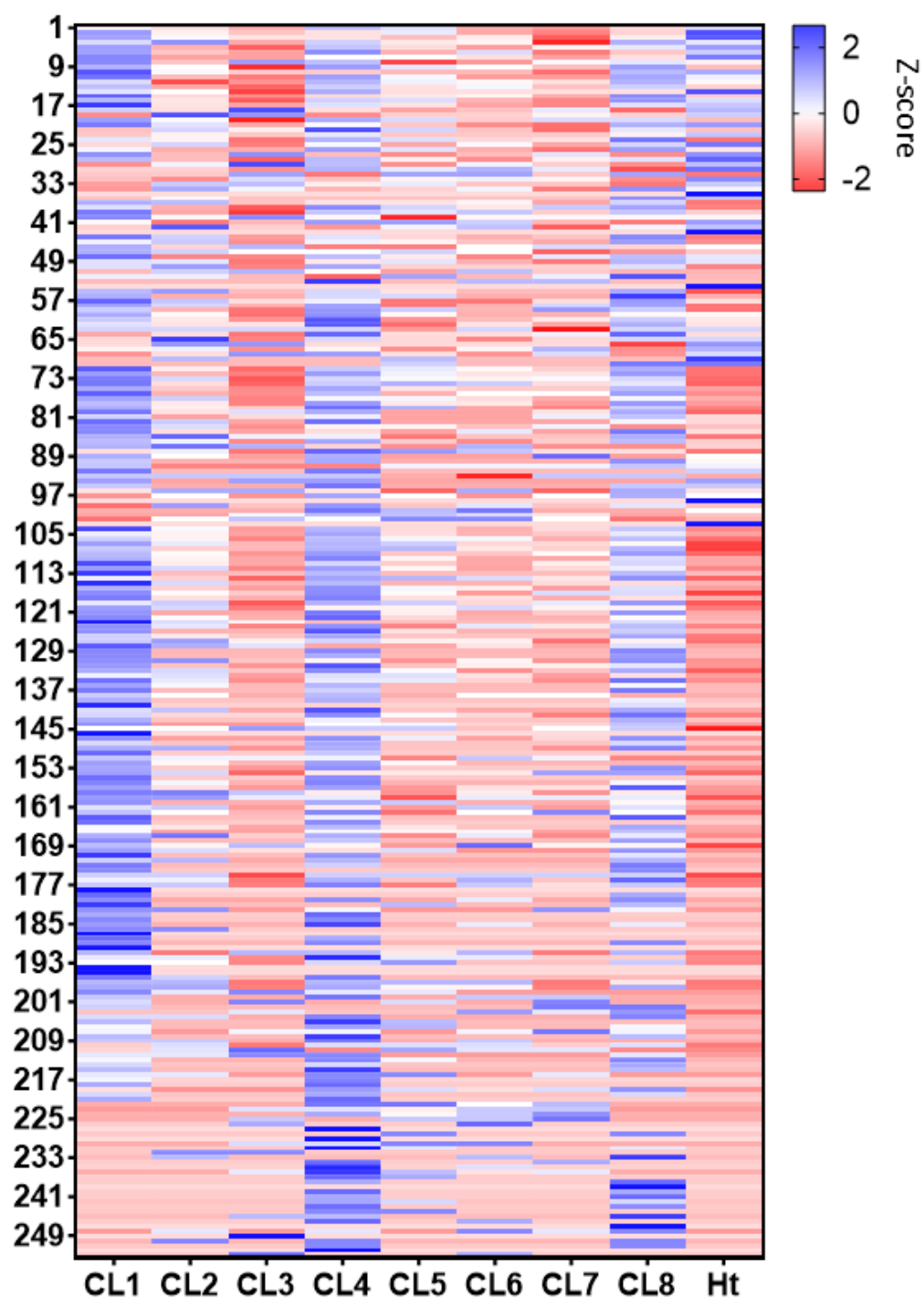


Figure 5. Heatmap generated representing all proteins identified (253) in the *L. mexicana* clones and *Ht* using Z-score. For protein identities, see supplementary materials (S.Table 1)



Figure 6. Heatmap generated representing the top 50 most abundant proteins identified in the *L. mexicana* clones and Ht using total spectrum count and ordered by Ht.

### Overall protein function does not vary between clone EVs

Gene ontology (GO) annotations were attributed using Panther and represented using percentages for each clone and Ht *L. mexicana* EVs (Figure 7). Annotations for biological processes are visualized on the left and molecular function on the right. Cellular and metabolic processes were the most common biological processes annotated to *L. mexicana* EVs. Although localization and establishment of localization appeared as distinct categories during the GO search, these categories overlapped entirely. When searching by molecular function, there was strong overlap with the very broad molecular function category which encompassed almost all proteins with a hypothesized mechanism of action. There was no significant variation in EV protein content when looking at these broader GO terms between the different clones compared. However, proteins related to enzyme regulation were entirely absent in CL7 EVs. The 51 shared proteins between all groups are visualized in a STRING diagram to show hypothesized protein interactions (Figure 8, Table 3). We found 4 functional protein clusters shared between all *L. mexicana* clones and Ht *L. mexicana*: purine ribonucleoside triphosphate metabolism (9), GTP-binding (7), one-carbon metabolism (4), and stress response (3). Several *Leishmania* virulence factors, like GP63, EF1 $\alpha$ , and enolase, fall under the purine ribonucleoside triphosphate metabolism cluster, while important cytoskeletal proteins, like actin and tubulin, fall under the GTP-binding cluster.

| Protein Name                         | Accession number | Function                                |
|--------------------------------------|------------------|-----------------------------------------|
| Putative heat-shock protein<br>hsp70 | E9B099_LEIMU     | Heat/stress response                    |
| Elongation factor 1-alpha            | E9ARD0_LEIMU     | Translation elongation<br>factor GTPase |
| Plasma membrane ATPase               | E9ART6_LEIMU     | Plasma membrane proton<br>transport     |
| GP63, leishmanolysin                 | E9AN57_LEIMU     | Virulence and cell survival             |
| Receptor-type adenylate cyclase<br>a | E9ARD7_LEIMU     | Cell signalling and survival            |

|                                               |              |                                               |
|-----------------------------------------------|--------------|-----------------------------------------------|
| Elongation factor 2                           | E9ASD6_LEIMU | Translation elongation factor GTPase          |
| Surface antigen-like protein                  | E9AK01_LEIMU | EGF-like domain                               |
| Actin                                         | C6KJD5_LEIME | Cytoskeletal structure                        |
| Heat shock protein 83-1                       | E9B3L2_LEIMU | Heat/stress response                          |
| Calpain-like cysteine peptidase               | E9AUQ7_LEIMU | Calcium-dependent cysteine-type endopeptidase |
| Tubulin beta chain                            | E9AMJ9_LEIMU | Cytoskeletal structure                        |
| p-glycoprotein                                | E9B4S1_LEIMU | Drug resistance                               |
| ATP synthase subunit beta                     | E9AXJ6_LEIMU | Mitochondrial ATP synthesis                   |
| Enolase                                       | E9APW3_LEIMU | Glycolysis                                    |
| Tubulin alpha chain                           | E9AP62_LEIMU | Cytoskeletal structure                        |
| Surface antigen-like protein                  | E9AKJ6_LEIMU | EGF-like domain                               |
| Putative calcium motive P-type ATPase         | E9B686_LEIMU | Cellular ion homeostasis                      |
| Putative histidine secretory acid phosphatase | E9AU82_LEIMU | Acid phosphatase                              |
| Kinesin-like protein                          | E9AKI2_LEIMU | Microtubule motor activity                    |
| Putative heat shock protein                   | E9ARS1_LEIMU | Heat/stress response                          |
| Glucose transporter, lmg2                     | E9AU63_LEIMU | Cellular glucose homeostasis                  |
| Surface antigen-like protein                  | E9AKM8_LEIMU | EGF-like domain                               |
| Tryparedoxin peroxidase                       | E9AQA6_LEIMU | Hydroperoxide resistance                      |
| Putative nucleoside transporter 1             | E9ASW8_LEIMU | Transmembrane nucleoside transport            |

|                                                                    |              |                                               |
|--------------------------------------------------------------------|--------------|-----------------------------------------------|
| Putative calpain-like cysteine peptidase                           | E9AUS1_LEIMU | Calcium-dependent cysteine-type endopeptidase |
| Putative cystathione gamma lyase                                   | E9B6K2_LEIMU | Cysteine biosynthesis                         |
| Peptidyl-prolyl cis-trans isomerase                                | E9AXG9_LEIMU | Protein folding                               |
| Putative ATPase alpha subunit                                      | E8NHQ7_LEIMU | Mitochondrial ATP synthesis                   |
| Putative vacuolar-type proton translocating pyrophosphatase 1      | E9B1S0_LEIMU | Plasma membrane proton transport              |
| Putative amino acid permease                                       | E9AYW8_LEIMU | Plasma membrane amino acid transport          |
| Putative ADP ribosylation factor 3                                 | E9ALY6_LEIMU | Initiates microtubule polymerization          |
| Nucleoside diphosphate kinase                                      | E9B376_LEIMU | Triphosphate biosynthesis                     |
| Putative NADP-dependent alcohol dehydrogenase                      | E9AW40_LEIMU | Alcohol dehydrogenase                         |
| Putative membrane-bound acid phosphatase 2                         | E9AT36_LEIMU | Acid phosphatase                              |
| Putative calpain-like cysteine peptidase                           | E9AUR8_LEIMU | Calcium-dependent cysteine-type endopeptidase |
| Putative serine/threonine-protein kinase (Putative protein kinase) | E9AXW8_LEIMU | Protein kinase                                |
| Surface antigen-like protein                                       | E9AK03_LEIMU | EGF-like domain                               |
| Putative ADP,ATP carrier protein 1, mitochondrial                  | E9ARX1_LEIMU | Mitochondrial ATP/ADP transport               |

|                                                                                                    |              |                                               |
|----------------------------------------------------------------------------------------------------|--------------|-----------------------------------------------|
| Putative tyrosine aminotransferase                                                                 | E9AT13_LEIMU | Amino acid metabolism                         |
| Mitochondrial processing peptidase, beta subunit, putative, metallo-peptidase, Clan ME, Family M16 | E9B617_LEIMU | Mitochondrial matrix                          |
| Adenosylhomocysteinase                                                                             | E9ATH3_LEIMU | Homocysteine biosynthesis                     |
| Putative ADP-ribosylation factor                                                                   | E9B278_LEIMU | GTPase                                        |
| Putative polyubiquitin                                                                             | E9AMU1_LEIMU | Proteosome recruitment                        |
| Superoxide dismutase                                                                               | E9B2V9_LEIMU | Free radical resistance                       |
| S-adenosylmethionine synthase                                                                      | E9B1C6_LEIMU | Adenosylmethionine biosynthesis               |
| Putative calpain-like cysteine peptidase (Putative cysteine peptidase, clan ca, family c2)         | E9APT0_LEIMU | Calcium-dependent cysteine-type endopeptidase |
| Putative surface antigen (Fragment)                                                                | E9ANZ9_LEIMU | EGF-like domain                               |
| Histone H4                                                                                         | E9ASB9_LEIMU | Nucleosome core component                     |
| Histone H4                                                                                         | E9AKM9_LEIMU | Nucleosome core component                     |

*Table 3. All proteins shared between all L. mexicana clones and Ht. Proteins were present with at least 2 peptides and 95% confidence, as determined by Scaffold4.*

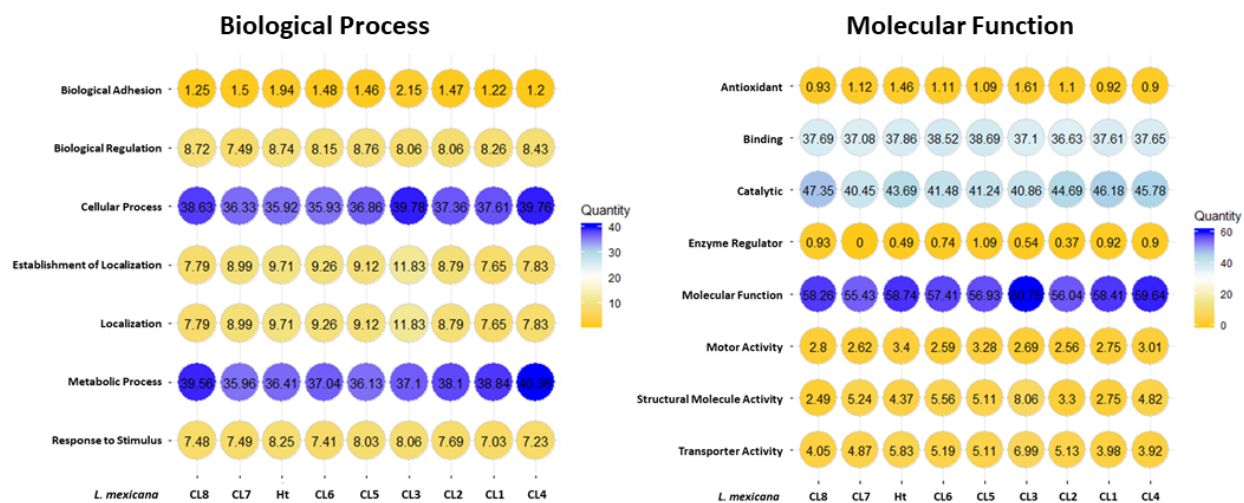


Figure 7. Gene ontology performed for searching for terms related to biological processes and molecular functions. Number present in circles represents the percentage of proteins of that specific category (Eg. Biological adhesion) compared to all proteins identified in the sample. The color legend indicates the spectrum count.

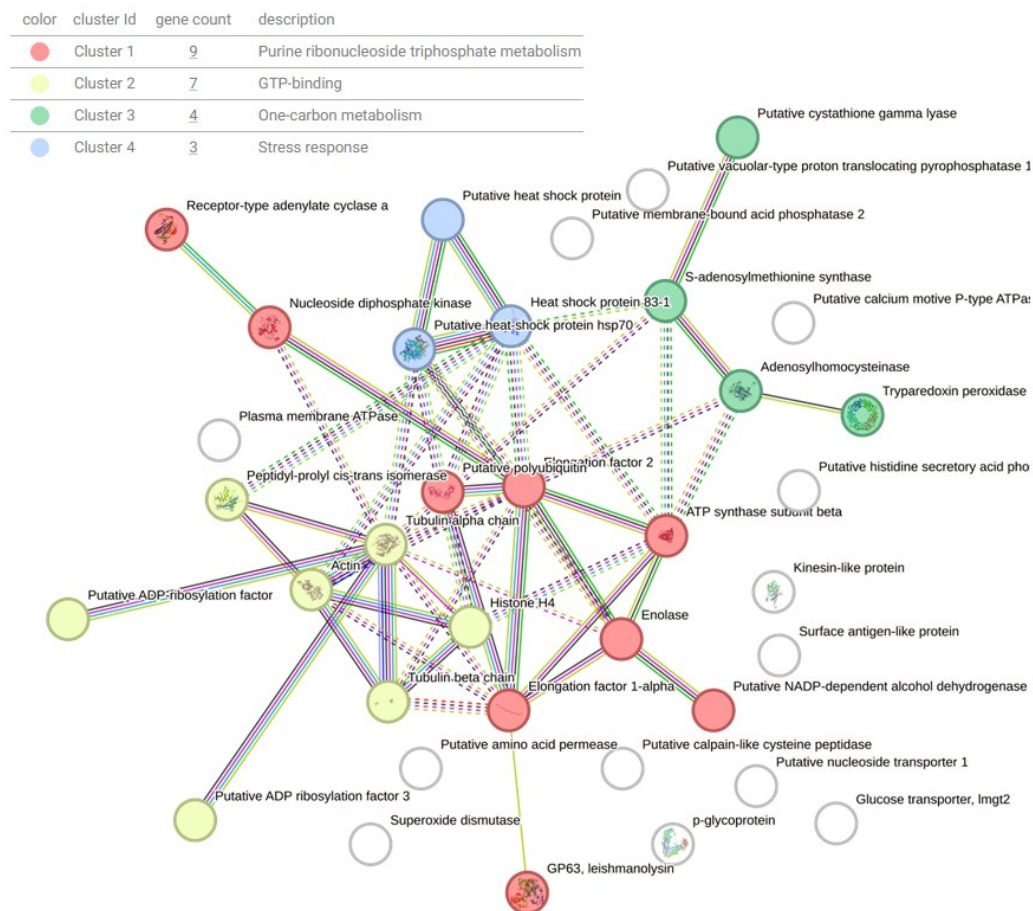


Figure 8. STRING diagram representing interactions between shared proteins present in all EVs isolated from *L. mexicana* clones and Ht. Proteins are clustered based on function using *k*-means clustering. Lines represent a direct relationship between proteins, with dotted lines representing the edge of a cluster.

Vesicles isolated from selected clones exhibit varied infectious capability compared to parasite virulence alone

The infectious capability of *L. mexicana* clone EVs was tested using a C57BL6 murine footpad infection model, measuring the subsequent footpad swelling to determine infection severity (Figure 9A). The co-inoculation of Ht *L. mexicana* along with Ht and CL1 EVs significantly worsened the footpad infection compared to Ht *L. mexicana* alone at all time

points measured, consistently causing a 0.2mm increase in footpad swelling at the start of infection and increasing to a 0.3mm difference by week 6 of the infection. CL8 EVs co-inoculation actually reduced footpad swelling at the start of infection compared to Ht and CL1 EVs, even showing an inhibitory effect at week 2. However, the infection proceeded as expected from week 3 onwards, causing around a 0.1mm increase in swelling compared to Ht alone. Strikingly, CL7 EVs did not cause significantly increased footpad swelling compared to Ht alone. The infectious capability of the *L. mexicana* clone parasites was tested as well, omitting EV co-inoculation (Figure 9B). Surprisingly, the infectious capability of the parasites themselves differed from what was seen with the EVs. Although CL1 EVs did not augment infection more than Ht EVs, CL1 parasites alone caused more severe infection during the first 4 weeks compared to Ht parasites, although footpad swelling was only significantly greater at weeks 1 and 3. The other selected clones did not cause significantly altered infection overall compared to Ht. We also assessed parasite infectious capability using Balb/c mice (Figure 9C). The resulting footpad swelling was significantly greater compared to C57BL6 infection. With the Balb/c model, we found that all *L. mexicana* clones caused similar infection to Ht *L. mexicana* with the exception of CL1, which caused significantly more footpad swelling at almost all timepoints measured (weeks 1, 4, 6, and 8 post-infection). CL7 only caused reduced footpad swelling at week 4.

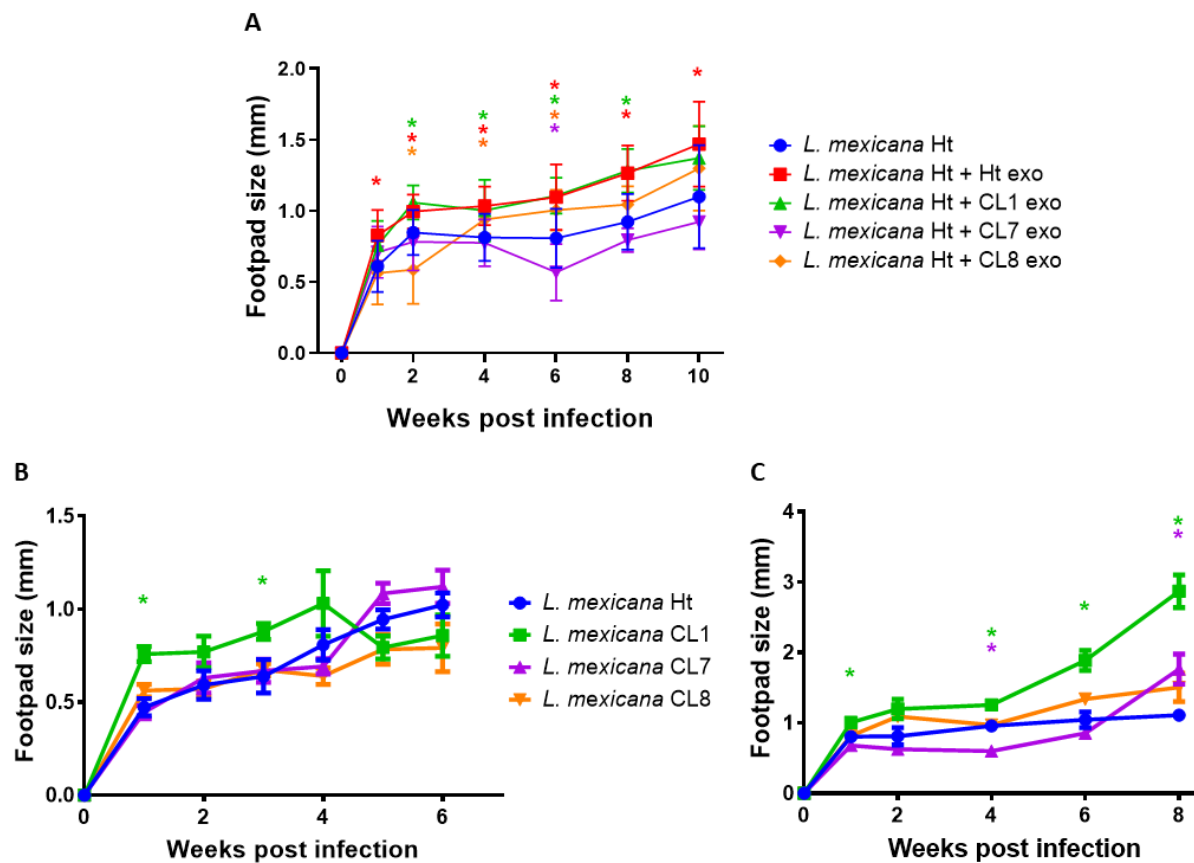


Figure 9. Graphs depicting murine footpad infections. C57BL6 (A) and BALB/C (C) mice footpad infections were performed with Ht *L. mexicana* supplemented with CL1, 7 and 8 EVs infecting C57BL6 mice (A) or Ht, CL1, 7, and 8 *L. mexicana* parasite alone infecting C57BL6 mice (B) or Balb/c mice (C).

## Discussion

The heterogeneity of EVs is a source of great discussion within the field of EVs, leading to great interest in isolating and comparing subpopulations of EVs secreted by a single population of cells or organisms. By isolating clonal colonies of *L. mexicana*, a highly heterogeneous species of *Leishmania*, we are attempting to similarly isolate different populations of EVs from a single progenitor strain of *L. mexicana*. When comparing clonal *L. mexicana* EVs based on their physical properties, they are indistinguishable from one another. There are slight variations in mean diameter and size distribution, but such

variations are common between different preparations of EVs rather than being indicative of an overall trend. The parasites themselves are also indistinguishable in terms of morphology and growth rate.

However, proteomic analysis reveals that there is significant variation in the EV protein content of each *L. mexicana* clone. With over 200 individual proteins identified across all groups, only 51 were shared between all groups. As evident from the STRING diagram, these are all important *Leishmania* proteins that are typically found in *Leishmania* EVs, relating to stress response, virulence, cytoskeleton, and metabolism (20-22). Surprisingly, Ht EVs had the fewest individual proteins identified, despite having comparable protein abundance to other groups. This may be due to the inherent heterogeneity of the Ht EVs, with the large amount of unique low-abundance proteins causing the equivalent of noise and preventing their detection by mass spectrometry, especially when compounded with the relatively low starting protein quantity. Such difficulties are commonly reported when attempting proteomic analysis on samples with only a few highly abundant proteins (23, 24).

On the other hand, CL3 EVs had fewer identified proteins as well as low protein abundance, which could suggest material loss during sample preparation and processing for mass spectrometry. Interestingly, despite the low overall protein abundance, CL3 EVs had the greatest abundance of clathrin heavy chain, which has been implicated in EV uptake by cells (25). However, many other such uptake mechanisms have been reported that are clathrin-independent (26, 27), and the high abundance of clathrin may even suggest CL3 EVs originate from a unique pathway compared to EVs from the other clones which contain much less clathrin, explaining the lower quantity of collected material. Regardless, due to the low amount of material, CL3 EVs were not used in further experiments.

CL1, 4, and 8 EVs had higher overall protein abundance compared to other clones, suggesting potential enrichment of protein cargo in the EVs of these clones. Of particular interest were CL1 EVs, which contained the highest amount of GP63, EF1 $\alpha$ , and EF2, all important *Leishmania* virulence factors (28-30). These differences are important due to the limited size of EVs, which means cargo sorted into the EVs must be selected by some

mechanism and such minute differences can heavily impact EV physiological function (31), specifically their ability to enhance infection in the case of these *Leishmania* EVs. However, these differences are relatively small when considering the variance that is inherent to mass spectrometry-based proteomic analysis. Indeed, analysis using gene ontology did not reveal any significant broad functional differences between *L. mexicana* clonal EVs.

The most striking differences between the *L. mexicana* clones were apparent when testing the infectious capabilities of the EVs. Ht *L. mexicana* EVs were able to augment infection as expected, *L. mexicana* CL7 EVs completely lost their infection-enhancing properties and failed to significantly augment infection at any time point. This reduced virulence was not observed when comparing the infectious capabilities of the clonal *L. mexicana* alone without EV co-inoculation in both C57BL6 and Balb/c mice, with Balb/c infection resulting in greater footpad swelling as expected (32). This is consistent with what was observed in *L. amazonensis*, where EVs containing high amounts of GP63 did not significantly enhance infection compared to WT *L. amazonensis* EVs, but GP63<sup>low</sup> EVs did not significantly augment infection compared to *L. amazonensis* alone (33). However, the difference in GP63 quantity observed in this study is much lower comparatively, so this explanation alone is not sufficient in explaining the loss of infectious capability of CL1 EVs. Of particular interest, CL7 EVs were also deficient in surface protein amastin and CL8 EVs were deficient in cytochrome C. Cytochrome C is an important oxidase that allows *Leishmania* to adapt to human macrophage environment and amastin is a classical *Leishmania* surface virulence factor, important for parasite survival in the sandfly vector as well as infection (34, 35). However, as we observed, the lack of cytochrome C did not result in any reduced infectivity in either CL8 parasites or its EVs.

In conclusion, although the EVs secreted by different *L. mexicana* clones are physically indistinguishable from each other, proteomic analysis reveals that they each have unique protein content when considering the overall presence/absence of individual proteins as well as protein abundance. These differences may lead to varying infectious capabilities of the clonal *L. mexicana* EVs, as we observed when infecting C57BL6 footpads with CL1, 7,

and 8 EVs, comparing to Ht *L. mexicana*, with CL7 EVs losing their infection-augmenting properties. Comparatively, the clonal parasites alone caused mostly consistent levels of footpad swelling.. This shows that EVs have unique properties compared to the parasite that secreted them. Despite each clonal *L. mexicana* being derived from the same *L. mexicana* Ht culture, their EVs have notable differences in protein content and infectious capability. To validate this work, we would like to sequence the *L. mexicana* clones used in this study, especially CL1, CL7, and CL8, in order to verify the genetic homogeneity of each clone as well as identify the genetic variations that may lead to the observed differences in infectious capability. This would open the up further gene manipulation studies. It will also be prudent to perform further immunoblotting to validate the proteomic data.

## Conflict of interest

MO is supported by the Canadian Institutes of Health Research (CIHR, grant number PJT-159765) and The Natural Sciences and Engineering Research Council of Canada (NSERC). GD was supported by a MSc studentship from Fonds de Recherche du Québec en Santé (FRQS) and the Canada Graduate Scholarships Doctoral Award (CGS-D) provided by the CIHR. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author contributions

The project was conceptualized by MO and GD. All experiments and analysis, including the EV purifications, NTA analysis, microBCA, TEM imaging, proteomic analysis, and in vivo mice infections, were performed by GD. Mice footpad measurements were performed with support from MO. All figures were generated by GD except Figure 7, which was generated by ND, who also processed the GO data necessary for the figure. Manuscript was written by GD and reviewed/edited by GD and MO.

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## Supplemental figures and tables

|   | Identified Protein                                            | Accession Number | Molecular Weight (kDa) | CL1 | CL2 | CL3 | CL4 | CL5 | CL6 | CL7 | CL8 | Ht |
|---|---------------------------------------------------------------|------------------|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|----|
| 1 | Putative heat-shock protein hsp70                             | E9B099_LEIMU [2] | 71                     | 36  | 33  | 26  | 51  | 44  | 25  | 22  | 32  | 76 |
| 2 | GP63, leishmanolysin                                          | E9AN57_LEIMU [5] | 64                     | 45  | 35  | 32  | 34  | 38  | 30  | 27  | 33  | 52 |
| 3 | Elongation factor 1-<br>alpha                                 | E9ARD0_LEIMU     | 49                     | 35  | 26  | 23  | 23  | 20  | 24  | 11  | 24  | 44 |
| 4 | Plasma membrane ATPase                                        | E9ART6_LEIMU [2] | 108                    | 32  | 39  | 22  | 33  | 27  | 28  | 11  | 36  | 31 |
| 5 | Tubulin beta chain                                            | E9AMJ9_LEIMU [2] | 50                     | 26  | 17  | 13  | 24  | 19  | 16  | 20  | 22  | 29 |
| 6 | Heat shock protein 83-1                                       | E9B3L2_LEIMU [2] | 81                     | 30  | 18  | 16  | 28  | 17  | 23  | 14  | 18  | 24 |
| 7 | Putative vacuolar-type proton translocating pyrophosphatase 1 | E9B1S0_LEIMU     | 84                     | 17  | 2   | 2   | 8   | 8   | 6   | 2   | 6   | 21 |
| 8 | ATP synthase subunit beta                                     | E9AXJ6_LEIMU     | 56                     | 27  | 18  | 26  | 24  | 12  | 16  | 21  | 22  | 20 |
| 9 | Surface antigen-like protein                                  | E9AK01_LEIMU [2] | 74                     | 36  | 26  | 2   | 39  | 29  | 26  | 19  | 39  | 19 |

|           |                                          |                   |     |    |    |    |    |    |    |    |    |    |
|-----------|------------------------------------------|-------------------|-----|----|----|----|----|----|----|----|----|----|
| <b>10</b> | Glucose transporter, lmg2                | E9AU63_LEIMU      | 61  | 24 | 14 | 11 | 11 | 10 | 10 | 6  | 18 | 19 |
| <b>11</b> | Calpain-like cysteine peptidase          | E9AUQ7_LEIMU [2]  | 104 | 31 | 10 | 4  | 27 | 17 | 7  | 7  | 26 | 18 |
| <b>12</b> | Tubulin alpha chain                      | E9AP62_LEIMU      | 60  | 19 | 10 | 13 | 25 | 18 | 17 | 16 | 21 | 17 |
| <b>13</b> | Actin                                    | C6KJD5_LEIME (+2) | 42  | 42 | 11 | 2  | 29 | 18 | 23 | 14 | 41 | 16 |
| <b>14</b> | Putative calpain-like cysteine peptidase | E9AUS1_LEIMU      | 15  | 11 | 10 | 4  | 13 | 9  | 9  | 9  | 9  | 16 |
| <b>15</b> | Elongation factor 2                      | E9ASD6_LEIMU      | 94  | 33 | 16 | 11 | 16 | 17 | 18 | 13 | 28 | 15 |
| <b>16</b> | Enolase                                  | E9APW3_LEIMU      | 46  | 18 | 11 | 2  | 16 | 15 | 8  | 5  | 23 | 15 |
| <b>17</b> | Tryparedoxin peroxidase                  | E9AQA6_LEIMU [2]  | 22  | 18 | 10 | 8  | 12 | 10 | 8  | 7  | 12 | 13 |
| <b>18</b> | Putative ATPase alpha subunit            | E8NHQ7_LEIMU (+1) | 63  | 12 | 9  | 17 | 9  | 7  | 8  | 11 | 5  | 13 |
| <b>19</b> | Putative nucleoside transporter 1        | E9ASW8_LEIMU      | 54  | 8  | 17 | 15 | 8  | 10 | 10 | 11 | 10 | 13 |
| <b>20</b> | Surface antigen-like protein             | E9AKM8_LEIMU      | 30  | 21 | 15 | 2  | 20 | 17 | 12 | 17 | 17 | 11 |

|    |                                                                                                    |                       |     |    |    |    |    |    |    |    |    |    |
|----|----------------------------------------------------------------------------------------------------|-----------------------|-----|----|----|----|----|----|----|----|----|----|
| 21 | Peptidyl-prolyl cis-trans isomerase                                                                | E9AXG9_LEIMU          | 19  | 12 | 8  | 4  | 7  | 5  | 3  | 2  | 10 | 11 |
| 22 | Putative histidine secretory acid phosphatase                                                      | E9AU82_LEIMU (+2)     | 128 | 11 | 16 | 13 | 24 | 16 | 11 | 7  | 15 | 11 |
| 23 | Putative polyubiquitin                                                                             | E9AMU1_LEIMU (+2)     | 15  | 2  | 3  | 2  | 4  | 2  | 2  | 2  | 3  | 10 |
| 24 | Receptor-type adenylate cyclase a                                                                  | E9ARD7_LEIMU [4]      | 151 | 29 | 24 | 9  | 36 | 30 | 21 | 22 | 49 | 9  |
| 25 | Nucleoside diphosphate kinase                                                                      | A0A1Y0DDB3_LEIAM (+1) | 17  | 3  | 5  | 1  | 4  | 2  | 4  | 2  | 5  | 9  |
| 26 | Putative cystathione gamma lyase                                                                   | E9B6K2_LEIMU          | 44  | 14 | 6  | 6  | 13 | 10 | 6  | 4  | 13 | 8  |
| 27 | Mitochondrial processing peptidase, beta subunit, putative, metallo-peptidase, Clan ME, Family M16 | E9B617_LEIMU          | 55  | 7  | 3  | 7  | 3  | 2  | 3  | 5  | 2  | 7  |
| 28 | Glyceraldehyde-3-phosphate dehydrogenase                                                           | E9B170_LEIMU          | 39  | 5  | 2  | 0  | 5  | 3  | 1  | 3  | 4  | 7  |
| 29 | Putative membrane-bound acid phosphatase 2                                                         | E9AT36_LEIMU          | 63  | 4  | 6  | 9  | 7  | 4  | 6  | 5  | 4  | 7  |
| 30 | Histone H4                                                                                         | E9ASB9_LEIMU          | 11  | 3  | 3  | 2  | 5  | 4  | 5  | 4  | 1  | 6  |

|    |                                                         |                   |     |    |    |   |    |    |    |    |    |   |
|----|---------------------------------------------------------|-------------------|-----|----|----|---|----|----|----|----|----|---|
| 31 | Putative amino acid permease                            | E9AYW8_LEIMU [3]  | 56  | 6  | 6  | 8 | 5  | 8  | 8  | 6  | 8  | 5 |
| 32 | Histone H2B                                             | E9AR94_LEIMU [2]  | 12  | 1  | 0  | 3 | 1  | 2  | 6  | 2  | 0  | 5 |
| 33 | WGS<br>CADB000000000 data,<br>contig 84                 | E8NHQ5_LEIMU [2]  | 76  | 1  | 5  | 9 | 3  | 4  | 2  | 2  | 0  | 5 |
| 34 | Putative long-chain-<br>fatty-acid-CoA ligase           | E9AJD9_LEIMU      | 78  | 1  | 8  | 5 | 2  | 3  | 5  | 0  | 12 | 5 |
| 35 | Amastin-like surface<br>protein-like protein            | E9B0L6_LEIMU      | 25  | 0  | 0  | 0 | 0  | 0  | 0  | 0  | 0  | 5 |
| 36 | I 6 autoantigen-like<br>protein                         | E9AVX8_LEIMU      | 23  | 0  | 1  | 0 | 0  | 0  | 0  | 0  | 4  | 5 |
| 37 | Putative calcium<br>motive P-type ATPase                | E9B686_LEIMU      | 134 | 27 | 18 | 9 | 11 | 11 | 12 | 7  | 16 | 4 |
| 38 | Surface antigen-like<br>protein                         | E9AKJ6_LEIMU      | 31  | 15 | 7  | 1 | 22 | 16 | 11 | 17 | 20 | 4 |
| 39 | Putative casein<br>kinase                               | E9B5Y1_LEIMU      | 40  | 9  | 3  | 0 | 7  | 6  | 7  | 4  | 7  | 4 |
| 40 | Putative ADP,ATP<br>carrier protein 1,<br>mitochondrial | E9ARX1_LEIMU (+1) | 35  | 6  | 4  | 6 | 4  | 1  | 4  | 4  | 4  | 4 |
| 41 | Histone H2A                                             | E9AV77_LEIMU [2]  | 14  | 2  | 0  | 1 | 4  | 4  | 3  | 1  | 0  | 4 |

|    |                                                        |                   |     |    |    |    |    |    |    |    |    |   |
|----|--------------------------------------------------------|-------------------|-----|----|----|----|----|----|----|----|----|---|
| 42 | Putative surface protein amastin                       | E9B0L4_LEIMU (+1) | 21  | 2  | 6  | 2  | 1  | 3  | 4  | 0  | 3  | 4 |
| 43 | Putative zinc transporter                              | E9B012_LEIMU      | 47  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 4 |
| 44 | p-glycoprotein                                         | E9B4S1_LEIMU [2]  | 147 | 32 | 21 | 6  | 19 | 12 | 8  | 10 | 30 | 3 |
| 45 | Putative heat shock protein                            | E9ARS1_LEIMU      | 92  | 8  | 10 | 3  | 15 | 7  | 5  | 4  | 12 | 3 |
| 46 | Ribonucleoprotein p18, mitochondrial, putative         | E9AQ29_LEIMU      | 21  | 8  | 2  | 5  | 0  | 0  | 3  | 4  | 2  | 3 |
| 47 | Superoxide dismutase                                   | E9B2V9_LEIMU      | 22  | 8  | 5  | 4  | 4  | 5  | 4  | 1  | 2  | 3 |
| 48 | Glycosomal phosphoenolpyruvate carboxykinase, putative | E9AZ81_LEIMU (+1) | 58  | 6  | 0  | 0  | 4  | 2  | 0  | 3  | 4  | 3 |
| 49 | Putative long-chain-fatty-acid-CoA ligase              | E9AJD6_LEIMU      | 77  | 6  | 3  | 0  | 2  | 3  | 1  | 0  | 4  | 3 |
| 50 | Adenosylhomocysteinase                                 | E9ATH3_LEIMU      | 48  | 5  | 6  | 3  | 7  | 4  | 4  | 4  | 5  | 3 |
| 51 | Clathrin heavy chain                                   | E9AST4_LEIMU      | 191 | 4  | 22 | 49 | 13 | 0  | 25 | 6  | 3  | 3 |

|    |                                                               |                  |     |   |    |   |   |   |   |   |    |   |
|----|---------------------------------------------------------------|------------------|-----|---|----|---|---|---|---|---|----|---|
| 52 | Putative calpain-like cysteine peptidase                      | E9AUR8_LEIMU     | 17  | 4 | 4  | 3 | 2 | 5 | 3 | 4 | 6  | 3 |
| 53 | Histone H4                                                    | E9AKM9_LEIMU     | 11  | 3 | 3  | 3 | 5 | 3 | 4 | 4 | 3  | 3 |
| 54 | Putative surface antigen (Fragment)                           | E9ANZ9_LEIMU [3] | 74  | 2 | 2  | 2 | 2 | 2 | 2 | 2 | 2  | 3 |
| 55 | Putative NADP-dependent alcohol dehydrogenase                 | E9AW40_LEIMU     | 38  | 9 | 10 | 5 | 8 | 6 | 5 | 5 | 11 | 2 |
| 56 | Surface antigen-like protein                                  | E9AK03_LEIMU     | 37  | 9 | 2  | 2 | 7 | 7 | 2 | 2 | 14 | 2 |
| 57 | Putative kinesin K39                                          | E9APV6_LEIMU     | 536 | 6 | 4  | 2 | 3 | 0 | 0 | 2 | 5  | 2 |
| 58 | WGS CADB000000000 data, contig 52 (Fragment)                  | E8NHK6_LEIMU     | 209 | 6 | 5  | 3 | 6 | 2 | 3 | 5 | 6  | 2 |
| 59 | WGS CADB000000000 data, contig 72 (Fragment)                  | E8NHN7_LEIMU [2] | 96  | 5 | 3  | 2 | 6 | 5 | 3 | 6 | 5  | 2 |
| 60 | Putative small GTP-binding protein Rab1                       | E9AYX8_LEIMU     | 22  | 5 | 2  | 0 | 4 | 2 | 1 | 1 | 2  | 2 |
| 61 | Putative ADP-ribosylation factor-like (Putative small gtpase) | E9AQU9_LEIMU     | 20  | 4 | 2  | 0 | 8 | 0 | 1 | 2 | 4  | 2 |
| 62 | Putative small GTP-binding protein Rab11                      | E9ANA1_LEIMU     | 23  | 3 | 2  | 2 | 6 | 0 | 2 | 1 | 4  | 2 |

|    |                                                               |                  |     |    |    |   |    |    |    |    |    |   |
|----|---------------------------------------------------------------|------------------|-----|----|----|---|----|----|----|----|----|---|
| 63 | Amastin-like protein                                          | E9B4S0_LEIMU     | 21  | 2  | 2  | 2 | 2  | 1  | 2  | 0  | 2  | 2 |
| 64 | Putative<br>proteophosphoglyca<br>n ppg3 (Fragment)           | E9B5T5_LEIMU [2] | 120 | 1  | 2  | 0 | 6  | 2  | 2  | 3  | 6  | 2 |
| 65 | Protein tyrosine<br>phosphatase-like<br>protein               | E9AQI0_LEIMU     | 19  | 1  | 4  | 0 | 2  | 1  | 0  | 1  | 2  | 2 |
| 66 | Putative cytochrome<br>c                                      | E9AQU2_LEIMU     | 12  | 1  | 2  | 2 | 1  | 1  | 1  | 1  | 0  | 2 |
| 67 | Putative rab1 small<br>GTP-binding protein                    | E9ANC6_LEIMU     | 25  | 1  | 3  | 0 | 1  | 0  | 1  | 2  | 0  | 2 |
| 68 | 3'-nucleotidase<br>nuclease                                   | Q9GNZ4_LEIME     | 42  | 0  | 1  | 4 | 3  | 1  | 0  | 2  | 0  | 2 |
| 69 | Putative<br>transmembrane<br>amino acid<br>transporter        | E9ALD5_LEIMU     | 54  | 0  | 1  | 0 | 0  | 1  | 0  | 0  | 0  | 2 |
| 70 | Universal minicircle<br>sequence binding<br>protein, putative | E9AST2_LEIMU     | 13  | 0  | 0  | 0 | 0  | 1  | 0  | 0  | 2  | 2 |
| 71 | Putative myosin<br>heavy chain                                | E9B3G5_LEIMU     | 119 | 33 | 13 | 0 | 21 | 17 | 14 | 10 | 24 | 1 |
| 72 | Kinesin-like protein                                          | E9AKI2_LEIMU     | 154 | 19 | 6  | 2 | 19 | 12 | 9  | 9  | 21 | 1 |
| 73 | Putative ecotin                                               | E9AQ49_LEIMU     | 41  | 18 | 11 | 0 | 11 | 9  | 8  | 9  | 11 | 1 |

|           |                                                                                                        |                  |    |    |   |   |    |   |   |   |    |   |
|-----------|--------------------------------------------------------------------------------------------------------|------------------|----|----|---|---|----|---|---|---|----|---|
| <b>74</b> | Putative ADP<br>ribosylation factor 3                                                                  | E9ALY6_LEIMU     | 20 | 11 | 5 | 1 | 8  | 9 | 8 | 5 | 7  | 1 |
| <b>75</b> | Cluster of<br>Proteasome<br>regulatory ATPase<br>subunit                                               | E9ATM0_LEIMU [5] | 46 | 10 | 3 | 0 | 15 | 4 | 3 | 3 | 9  | 1 |
| <b>76</b> | Putative calpain-like<br>cysteine peptidase<br>(Putative cysteine<br>peptidase, clan ca,<br>family c2) | E9APT0_LEIMU     | 13 | 9  | 2 | 1 | 6  | 2 | 4 | 4 | 7  | 1 |
| <b>77</b> | GDP-mannose<br>pyrophosphorylase                                                                       | Q9BLW4_LEIME     | 41 | 8  | 4 | 0 | 3  | 3 | 2 | 1 | 3  | 1 |
| <b>78</b> | Putative calpain-like<br>cysteine peptidase                                                            | E9AK26_LEIMU     | 89 | 8  | 4 | 0 | 11 | 4 | 4 | 1 | 11 | 1 |
| <b>79</b> | Putative<br>serine/threonine-<br>protein kinase<br>(Putative protein<br>kinase)                        | E9AXW8_LEIMU     | 44 | 7  | 4 | 3 | 9  | 7 | 2 | 1 | 6  | 1 |
| <b>80</b> | Putative ADP-<br>ribosylation factor                                                                   | E9B278_LEIMU     | 20 | 6  | 3 | 4 | 5  | 2 | 2 | 3 | 5  | 1 |
| <b>81</b> | Putative<br>aminopeptidase P                                                                           | E9B6B5_LEIMU     | 54 | 6  | 0 | 1 | 2  | 0 | 0 | 2 | 3  | 1 |
| <b>82</b> | Acetyl-coenzyme A<br>synthetase                                                                        | E9AW86_LEIMU     | 77 | 5  | 3 | 0 | 5  | 0 | 0 | 0 | 2  | 1 |
| <b>83</b> | Putative glucose-<br>regulated protein 78                                                              | E9AZT9_LEIMU     | 72 | 5  | 3 | 0 | 2  | 2 | 1 | 6 | 0  | 1 |

|           |                                                                                                     |                  |    |   |   |   |   |   |   |   |   |   |
|-----------|-----------------------------------------------------------------------------------------------------|------------------|----|---|---|---|---|---|---|---|---|---|
| <b>84</b> | Cluster of Phosphoglycerate kinase                                                                  | E9AUF1_LEIMU [2] | 45 | 4 | 1 | 0 | 1 | 0 | 2 | 0 | 5 | 1 |
| <b>85</b> | S-adenosylmethionine synthase                                                                       | E9B1C6_LEIMU     | 43 | 4 | 5 | 3 | 3 | 1 | 2 | 2 | 4 | 1 |
| <b>86</b> | Phosphodiesterase                                                                                   | E9ALI0_LEIMU     | 80 | 3 | 2 | 0 | 5 | 1 | 0 | 1 | 3 | 1 |
| <b>87</b> | Putative major vault protein                                                                        | E9AKB0_LEIMU     | 93 | 3 | 4 | 3 | 1 | 0 | 3 | 0 | 0 | 1 |
| <b>88</b> | Putative tyrosine aminotransferase                                                                  | E9AT13_LEIMU     | 50 | 3 | 4 | 1 | 7 | 6 | 5 | 3 | 3 | 1 |
| <b>89</b> | Mitochondrial processing peptidase alpha subunit, putative (Metallo-peptidase, clan me, family m16) | E9APB1_LEIMU     | 58 | 2 | 1 | 0 | 2 | 0 | 0 | 3 | 0 | 1 |
| <b>90</b> | Proteasome endopeptidase complex                                                                    | E9AYS1_LEIMU     | 26 | 2 | 0 | 0 | 4 | 0 | 0 | 0 | 3 | 1 |
| <b>91</b> | Putative adenylate kinase                                                                           | E9AVA9_LEIMU     | 30 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 1 |
| <b>92</b> | Putative ATP-binding cassette protein subfamily G, member 2                                         | E9AKN7_LEIMU     | 74 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 |
| <b>93</b> | Putative c2 domain protein                                                                          | E9B1N1_LEIMU     | 30 | 2 | 2 | 2 | 2 | 1 | 0 | 2 | 2 | 1 |

|            |                                    |                   |     |   |   |   |    |   |   |   |   |   |
|------------|------------------------------------|-------------------|-----|---|---|---|----|---|---|---|---|---|
| <b>94</b>  | Phospholipid-transporting ATPase   | E9APH7_LEIMU      | 125 | 1 | 0 | 1 | 1  | 0 | 0 | 0 | 0 | 1 |
| <b>95</b>  | Putative cysteine synthase         | E9ATD8_LEIMU      | 35  | 1 | 0 | 0 | 2  | 0 | 0 | 0 | 1 | 1 |
| <b>96</b>  | Putative isoleucyl-tRNA synthetase | E9ATZ6_LEIMU      | 126 | 1 | 2 | 2 | 1  | 0 | 2 | 0 | 2 | 1 |
| <b>97</b>  | Autophagy-related protein          | E9AS34_LEIMU (+2) | 12  | 0 | 1 | 0 | 2  | 1 | 0 | 1 | 3 | 1 |
| <b>98</b>  | GTP-binding nuclear protein        | E9AXM1_LEIMU      | 24  | 0 | 0 | 0 | 0  | 0 | 0 | 0 | 0 | 1 |
| <b>99</b>  | Putative 3'-nucleotidase/nuclease  | E9ANW7_LEIMU      | 41  | 0 | 3 | 1 | 4  | 1 | 2 | 1 | 2 | 1 |
| <b>100</b> | Putative 40S ribosomal protein L14 | E9AVY4_LEIMU      | 20  | 0 | 0 | 0 | 3  | 2 | 3 | 0 | 0 | 1 |
| <b>101</b> | Putative 40S ribosomal protein S4  | E9APE6_LEIMU      | 47  | 0 | 0 | 3 | 10 | 5 | 4 | 2 | 0 | 1 |
| <b>102</b> | Putative 60S ribosomal protein L21 | E9AQK4_LEIMU      | 18  | 0 | 2 | 3 | 2  | 4 | 4 | 2 | 0 | 1 |
| <b>103</b> | Transmembrane 9 superfamily member | E9ALR2_LEIMU      | 77  | 0 | 0 | 0 | 0  | 0 | 0 | 0 | 0 | 1 |

|     |                                                                        |                   |     |    |   |   |    |    |   |   |    |   |
|-----|------------------------------------------------------------------------|-------------------|-----|----|---|---|----|----|---|---|----|---|
| 104 | Cluster of 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | E9AUA1_LEIMU [2]  | 61  | 16 | 6 | 1 | 10 | 4  | 2 | 4 | 9  | 0 |
| 105 | Transketolase                                                          | E9AX52_LEIMU (+1) | 72  | 16 | 4 | 0 | 10 | 3  | 1 | 2 | 6  | 0 |
| 106 | Heat shock protein 70-related protein                                  | E9AYA3_LEIMU      | 71  | 15 | 6 | 1 | 10 | 7  | 7 | 3 | 10 | 0 |
| 107 | Cluster of Pyruvate kinase                                             | E9B5P1_LEIMU [2]  | 54  | 12 | 7 | 4 | 9  | 8  | 5 | 5 | 6  | 0 |
| 108 | Putative ABC transporter                                               | E9AYZ8_LEIMU      | 203 | 12 | 7 | 6 | 16 | 13 | 8 | 7 | 13 | 0 |
| 109 | Putative seryl-tRNA synthetase                                         | E9ANF6_LEIMU      | 53  | 12 | 6 | 2 | 11 | 4  | 4 | 6 | 11 | 0 |
| 110 | Transitional endoplasmic reticulum ATPase, putative                    | E9ASQ6_LEIMU      | 87  | 11 | 3 | 0 | 9  | 3  | 0 | 0 | 5  | 0 |
| 111 | Cluster of 6-phosphogluconate dehydrogenase, decarboxylating           | E9B6L3_LEIMU [3]  | 52  | 10 | 2 | 0 | 4  | 1  | 0 | 2 | 4  | 0 |
| 112 | Putative casein kinase I                                               | E9AXN8_LEIMU      | 59  | 10 | 6 | 0 | 10 | 4  | 1 | 3 | 5  | 0 |
| 113 | Phosphotransferase                                                     | E9AUZ2_LEIMU      | 52  | 8  | 0 | 0 | 4  | 0  | 0 | 0 | 4  | 0 |

|            |                                                                           |                  |     |   |   |   |    |    |   |   |    |   |
|------------|---------------------------------------------------------------------------|------------------|-----|---|---|---|----|----|---|---|----|---|
| <b>114</b> | Putative calpain-like cysteine peptidase                                  | E9AUQ9_LEIMU     | 78  | 8 | 5 | 0 | 12 | 11 | 9 | 6 | 13 | 0 |
| <b>115</b> | Putative ecotin                                                           | E9AQ32_LEIMU     | 16  | 8 | 3 | 0 | 4  | 0  | 0 | 1 | 1  | 0 |
| <b>116</b> | Cluster of Putative ABC transporter                                       | E9ANR6_LEIMU [2] | 211 | 7 | 2 | 0 | 12 | 4  | 3 | 0 | 5  | 0 |
| <b>117</b> | Serine/threonine-protein phosphatase                                      | E9AKB4_LEIMU     | 72  | 7 | 4 | 3 | 7  | 4  | 2 | 5 | 5  | 0 |
| <b>118</b> | Malic enzyme                                                              | E9AWR7_LEIMU     | 63  | 6 | 1 | 0 | 5  | 1  | 1 | 0 | 1  | 0 |
| <b>119</b> | Phosphodiesterase                                                         | E9ARP6_LEIMU     | 71  | 6 | 7 | 0 | 8  | 7  | 4 | 6 | 9  | 0 |
| <b>120</b> | Proteasome regulatory non-ATPase subunit 6, putative                      | E9AJK0_LEIMU     | 59  | 6 | 3 | 0 | 2  | 2  | 3 | 2 | 2  | 0 |
| <b>121</b> | Putative ABC transporter                                                  | E9ANR4_LEIMU     | 200 | 6 | 0 | 0 | 7  | 2  | 0 | 1 | 6  | 0 |
| <b>122</b> | Putative thimet oligopeptidase (Metallo-peptidase, clan ma(E), family m3) | E9AYD6_LEIMU     | 77  | 6 | 2 | 0 | 8  | 1  | 0 | 0 | 2  | 0 |
| <b>123</b> | Transmembrane 9 superfamily member                                        | E9B5F7_LEIMU     | 71  | 6 | 2 | 1 | 0  | 0  | 0 | 0 | 2  | 0 |

|     |                                                                  |              |     |   |   |   |   |   |   |   |   |   |
|-----|------------------------------------------------------------------|--------------|-----|---|---|---|---|---|---|---|---|---|
| 124 | Dipeptidyl peptidase 3                                           | E9AKK2_LEIMU | 76  | 5 | 3 | 0 | 5 | 0 | 3 | 2 | 4 | 0 |
| 125 | Proteasome regulatory non-ATP-ase subunit 2,putative             | E9AZZ2_LEIMU | 108 | 5 | 0 | 0 | 6 | 1 | 0 | 0 | 3 | 0 |
| 126 | Proteasome subunit alpha type                                    | E9AVH9_LEIMU | 27  | 5 | 3 | 2 | 8 | 2 | 2 | 2 | 8 | 0 |
| 127 | Proteasome subunit beta                                          | E9B5M9_LEIMU | 25  | 5 | 4 | 2 | 3 | 2 | 2 | 0 | 2 | 0 |
| 128 | Putative aminopeptidase (Metallo-peptidase, clan mf, family m17) | E9ANK8_LEIMU | 57  | 5 | 3 | 2 | 0 | 0 | 1 | 0 | 2 | 0 |
| 129 | Putative dual specificity protein phosphatase                    | E9B505_LEIMU | 153 | 5 | 0 | 0 | 5 | 0 | 0 | 0 | 5 | 0 |
| 130 | Rab GDP dissociation inhibitor                                   | E9ALK7_LEIMU | 50  | 5 | 0 | 0 | 4 | 0 | 0 | 0 | 6 | 0 |
| 131 | Serine/threonine-protein phosphatase                             | E9ANZ3_LEIMU | 109 | 5 | 5 | 1 | 2 | 0 | 2 | 0 | 5 | 0 |
| 132 | Transaldolase                                                    | E9AQN5_LEIMU | 37  | 5 | 1 | 0 | 7 | 1 | 2 | 2 | 3 | 0 |
| 133 | Trypanothione reductase                                          | E9AKE1_LEIMU | 53  | 5 | 4 | 2 | 6 | 3 | 1 | 2 | 5 | 0 |
| 134 | Tryparedoxin                                                     | E9ALV7_LEIMU | 17  | 5 | 2 | 0 | 2 | 2 | 1 | 0 | 1 | 0 |

|     |                                                                                                |                  |     |   |   |   |   |   |   |   |   |   |
|-----|------------------------------------------------------------------------------------------------|------------------|-----|---|---|---|---|---|---|---|---|---|
| 135 | Aminopeptidase                                                                                 | E9ALJ9_LEIMU     | 96  | 4 | 2 | 0 | 2 | 2 | 1 | 0 | 4 | 0 |
| 136 | Biotin/lipoate protein<br>ligase-like protein                                                  | E9B1Q4_LEIMU     | 28  | 4 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| 137 | Cluster of Putative<br>NADH-dependent<br>fumarate reductase                                    | E9B5Z7_LEIMU [2] | 123 | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 4 | 0 |
| 138 | D-lactate<br>dehydrogenase-like<br>protein                                                     | E9AZA6_LEIMU     | 53  | 4 | 1 | 0 | 2 | 0 | 1 | 1 | 0 | 0 |
| 139 | Dihydroorotate<br>dehydrogenase<br>(fumarate)                                                  | E9AQL1_LEIMU     | 34  | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| 140 | Palmitoyltransferase                                                                           | E9AWG3_LEIMU     | 49  | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 141 | Proteasome<br>regulatory non-ATP-<br>ase subunit<br>5,putative                                 | E9AV44_LEIMU     | 54  | 4 | 5 | 0 | 9 | 1 | 0 | 0 | 6 | 0 |
| 142 | Proteasome subunit<br>alpha type                                                               | E9AVG6_LEIMU     | 25  | 4 | 2 | 2 | 6 | 3 | 2 | 0 | 7 | 0 |
| 143 | Proteasome subunit<br>alpha type                                                               | E9AST1_LEIMU     | 30  | 4 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 |
| 144 | Putative 5-<br>methyltetrahydropter<br>oyltriglutamate--<br>homocyst ei<br>nemethyltransferase | E9B1E9_LEIMU     | 86  | 4 | 0 | 0 | 5 | 2 | 1 | 1 | 3 | 0 |

|            |                                                                              |              |     |   |   |   |    |   |   |   |   |   |
|------------|------------------------------------------------------------------------------|--------------|-----|---|---|---|----|---|---|---|---|---|
| <b>145</b> | Putative adenosine kinase                                                    | E9B0L7_LEIMU | 37  | 4 | 4 | 6 | 5  | 5 | 3 | 4 | 5 | 0 |
| <b>146</b> | Putative ATP-dependent Clp protease subunit, heat shock protein 100 (HSP100) | E9ALU6_LEIMU | 97  | 4 | 0 | 0 | 0  | 0 | 0 | 0 | 0 | 0 |
| <b>147</b> | Putative dynein heavy chain                                                  | E9API9_LEIMU | 530 | 4 | 0 | 0 | 4  | 2 | 0 | 1 | 4 | 0 |
| <b>148</b> | Putative dynein heavy chain                                                  | E9AXH6_LEIMU | 537 | 4 | 0 | 0 | 12 | 0 | 0 | 0 | 4 | 0 |
| <b>149</b> | Putative ef-hand protein 5                                                   | E9AMU2_LEIMU | 21  | 4 | 4 | 0 | 4  | 1 | 1 | 0 | 5 | 0 |
| <b>150</b> | Putative Gim5A protein (Glycosomal membrane protein)                         | E9B6P8_LEIMU | 25  | 4 | 0 | 0 | 3  | 0 | 0 | 0 | 0 | 0 |
| <b>151</b> | Putative GTP-binding protein                                                 | E9ALK3_LEIMU | 77  | 4 | 2 | 2 | 2  | 0 | 4 | 2 | 0 | 0 |
| <b>152</b> | Putative sarcoplasmic reticulum glycoprotein                                 | E9ARX9_LEIMU | 69  | 4 | 1 | 0 | 4  | 1 | 0 | 0 | 0 | 0 |
| <b>153</b> | Putative serine/threonine-protein kinase                                     | E9B296_LEIMU | 50  | 4 | 1 | 0 | 5  | 1 | 1 | 1 | 5 | 0 |
| <b>154</b> | Surface antigen-like protein                                                 | E9AK02_LEIMU | 18  | 4 | 3 | 0 | 2  | 2 | 2 | 4 | 4 | 0 |

|     |                                      |                       |     |   |   |   |    |   |   |   |   |   |
|-----|--------------------------------------|-----------------------|-----|---|---|---|----|---|---|---|---|---|
| 155 | 6-phosphogluconolactonase            | E9AYQ1_LEIMU          | 28  | 3 | 0 | 0 | 2  | 0 | 0 | 0 | 0 | 0 |
| 156 | ADP-ribosylation factor-like protein | E9B110_LEIMU          | 20  | 3 | 1 | 0 | 3  | 0 | 0 | 0 | 1 | 0 |
| 157 | Alanine--tRNA ligase                 | E9AVY6_LEIMU          | 106 | 3 | 1 | 0 | 3  | 0 | 1 | 1 | 4 | 0 |
| 158 | ATP-dependent 6-phosphofructokinase  | E9ALH3_LEIMU          | 54  | 3 | 3 | 2 | 1  | 0 | 1 | 0 | 1 | 0 |
| 159 | Miltefosine transporter beta subunit | A0A075DN10_LEIAM (+1) | 40  | 3 | 3 | 2 | 2  | 0 | 2 | 2 | 2 | 0 |
| 160 | Obg-like ATPase 1                    | E9AZE0_LEIMU          | 44  | 3 | 0 | 0 | 4  | 2 | 0 | 0 | 1 | 0 |
| 161 | Putative 60S ribosomal protein L13a  | E9AQ19_LEIMU          | 26  | 3 | 2 | 0 | 1  | 0 | 2 | 0 | 3 | 0 |
| 162 | Putative adenylate kinase            | E9B4H8_LEIMU          | 25  | 3 | 2 | 1 | 4  | 0 | 2 | 4 | 2 | 0 |
| 163 | Putative aminoacylase                | E9AUV8_LEIMU          | 43  | 3 | 0 | 0 | 1  | 0 | 0 | 0 | 3 | 0 |
| 164 | Putative arginyl-tRNA synthetase     | E9AZ37_LEIMU          | 78  | 3 | 0 | 0 | 2  | 0 | 0 | 0 | 0 | 0 |
| 165 | Putative paraflagellar rod protein   | E9AKJ8_LEIMU          | 117 | 3 | 2 | 0 | 10 | 2 | 1 | 1 | 6 | 0 |

|     |                                                               |                   |    |   |   |   |   |   |   |   |   |   |
|-----|---------------------------------------------------------------|-------------------|----|---|---|---|---|---|---|---|---|---|
| 166 | Putative peptidyl dipeptidase                                 | E9AJN6_LEIMU      | 77 | 3 | 0 | 0 | 3 | 1 | 1 | 0 | 2 | 0 |
| 167 | Putative peptidyl-prolyl cis-trans isomerase (Cyclophilin-40) | E9B705_LEIMU      | 39 | 3 | 4 | 1 | 3 | 0 | 2 | 0 | 2 | 0 |
| 168 | Putative proteasome regulatory ATPase subunit 2               | E9APD3_LEIMU      | 49 | 3 | 0 | 0 | 2 | 0 | 0 | 0 | 4 | 0 |
| 169 | Putative Qc-SNARE protein                                     | E9AM81_LEIMU      | 24 | 3 | 2 | 3 | 2 | 1 | 4 | 2 | 2 | 0 |
| 170 | Putative trypanothione synthetase                             | E9AZ89_LEIMU      | 74 | 3 | 2 | 1 | 1 | 2 | 0 | 0 | 4 | 0 |
| 171 | Putative tyrosyl-tRNA synthetase                              | E9APY5_LEIMU      | 75 | 3 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| 172 | Stress-inducible protein STI1 homolog                         | E9ASC5_LEIMU      | 29 | 3 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| 173 | 14-3-3 protein-like protein                                   | E9AT99_LEIMU      | 30 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 |
| 174 | Aconitate hydratase                                           | E9ARI8_LEIMU      | 97 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| 175 | Autophagy-related protein                                     | E9AML6_LEIMU (+1) | 13 | 2 | 2 | 0 | 2 | 2 | 2 | 2 | 2 | 0 |
| 176 | Autophagy-related protein                                     | E9AS40_LEIMU      | 15 | 2 | 2 | 0 | 3 | 1 | 3 | 1 | 4 | 0 |

|            |                                                                                                                                    |              |    |   |   |   |   |   |   |   |   |   |
|------------|------------------------------------------------------------------------------------------------------------------------------------|--------------|----|---|---|---|---|---|---|---|---|---|
| <b>177</b> | D-3-phosphoglycerate dehydrogenase-like protein                                                                                    | E9AJN9_LEIMU | 44 | 2 | 2 | 0 | 3 | 0 | 2 | 1 | 2 | 0 |
| <b>178</b> | Fructose-bisphosphate aldolase                                                                                                     | E9ASP6_LEIMU | 41 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>179</b> | Glyceraldehyde-3-phosphate dehydrogenase-like protein                                                                              | E9B703_LEIMU | 36 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| <b>180</b> | NADH:flavin oxidoreductase/NAD H oxidase, putative                                                                                 | E9AP13_LEIMU | 41 | 2 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 0 |
| <b>181</b> | Phosphatidylinositol-4-phosphate 5-kinase-like protein                                                                             | E9ASF4_LEIMU | 51 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| <b>182</b> | Phosphomannomutase                                                                                                                 | E9ASX1_LEIMU | 28 | 2 | 2 | 0 | 1 | 0 | 0 | 2 | 1 | 0 |
| <b>183</b> | Proteasome regulatory non-ATP-ase subunit 11, putative (19s proteasome regulatory subunit, metallo-peptidase, clan mp, family m67) | E9B4N4_LEIMU | 35 | 2 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| <b>184</b> | Proteasome subunit beta type                                                                                                       | E9AKP2_LEIMU | 28 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| <b>185</b> | Proteasome subunit beta type                                                                                                       | E9ANS8_LEIMU | 31 | 2 | 0 | 0 | 3 | 0 | 1 | 0 | 1 | 0 |

|            |                                                 |              |     |   |   |   |   |   |   |   |   |   |
|------------|-------------------------------------------------|--------------|-----|---|---|---|---|---|---|---|---|---|
| <b>186</b> | Proteasome subunit beta type                    | E9AST7_LEIMU | 34  | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>187</b> | Protein disulfide-isomerase                     | E9AUD1_LEIMU | 52  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>188</b> | Putative aspartyl-tRNA synthetase               | E9B0H1_LEIMU | 62  | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| <b>189</b> | Putative cAMP-specific phosphodiesterase        | E9AJY5_LEIMU | 73  | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| <b>190</b> | Putative carboxypeptidase                       | E9B493_LEIMU | 57  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>191</b> | Putative endoribonuclease L-PSP (Pb5)           | E9AW21_LEIMU | 17  | 2 | 0 | 2 | 2 | 1 | 2 | 0 | 2 | 0 |
| <b>192</b> | Putative fatty acyl CoA syntetase 1             | E9AJD4_LEIMU | 77  | 2 | 2 | 0 | 5 | 2 | 2 | 1 | 1 | 0 |
| <b>193</b> | Putative heat shock protein DNAJ                | E9AZE9_LEIMU | 44  | 2 | 2 | 0 | 5 | 4 | 1 | 1 | 3 | 0 |
| <b>194</b> | Putative lipophosphoglycan biosynthetic protein | E9AM02_LEIMU | 87  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>195</b> | Putative short chain dehydrogenase              | E9B601_LEIMU | 25  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>196</b> | Putative ubiquitin-activating enzyme e1         | E9B6I5_LEIMU | 127 | 2 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |

|     |                                                    |                   |     |   |   |   |   |   |   |   |   |   |
|-----|----------------------------------------------------|-------------------|-----|---|---|---|---|---|---|---|---|---|
| 197 | Putative ubiquitin-conjugating enzyme e2           | E9B609_LEIMU      | 17  | 2 | 2 | 0 | 2 | 2 | 2 | 0 | 1 | 0 |
| 198 | Reticulon-like protein                             | E9B128_LEIMU      | 22  | 2 | 2 | 0 | 2 | 1 | 1 | 0 | 2 | 0 |
| 199 | Serine hydroxymethyltransferase                    | E9APY0_LEIMU      | 51  | 2 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 |
| 200 | 40S ribosomal protein S2                           | E9ARV7_LEIMU (+2) | 33  | 1 | 0 | 0 | 2 | 0 | 1 | 1 | 0 | 0 |
| 201 | Cluster of Putative surface antigen protein 2      | E9AP03_LEIMU [4]  | 41  | 1 | 0 | 2 | 0 | 1 | 0 | 2 | 0 | 0 |
| 202 | Glutathione peroxidase                             | E9AY59_LEIMU (+1) | 20  | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 |
| 203 | Kinesin-like protein                               | E9ARX7_LEIMU      | 94  | 1 | 1 | 2 | 1 | 1 | 3 | 2 | 4 | 0 |
| 204 | Peptidylprolyl isomerase                           | E9ASA6_LEIMU      | 48  | 1 | 1 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| 205 | Phosphodiesterase                                  | E9AQE4_LEIMU      | 103 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| 206 | Proteasome regulatory non-ATPase subunit, putative | E9AM67_LEIMU      | 99  | 1 | 0 | 0 | 4 | 2 | 0 | 0 | 1 | 0 |
| 207 | Putative 40S ribosomal protein S15A                | E9ALP4_LEIMU      | 15  | 1 | 0 | 1 | 1 | 0 | 1 | 3 | 1 | 0 |

|            |                                                 |                   |     |   |   |   |   |   |   |   |   |   |
|------------|-------------------------------------------------|-------------------|-----|---|---|---|---|---|---|---|---|---|
| <b>208</b> | Putative glycogen synthase kinase 3 beta        | Q0PKV3_LEIME      | 41  | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| <b>209</b> | Putative glycyl tRNA synthetase                 | E9ATG6_LEIMU      | 70  | 1 | 1 | 0 | 2 | 0 | 1 | 0 | 0 | 0 |
| <b>210</b> | Putative long chain fatty Acyl CoA synthetase   | E9AJQ8_LEIMU      | 74  | 1 | 2 | 0 | 2 | 2 | 2 | 1 | 4 | 0 |
| <b>211</b> | Putative nucleoside transporter 1               | E9AQB6_LEIMU      | 54  | 1 | 2 | 4 | 0 | 3 | 2 | 2 | 0 | 0 |
| <b>212</b> | Putative oxidoreductase                         | E9ATK1_LEIMU      | 36  | 1 | 1 | 2 | 2 | 0 | 0 | 0 | 1 | 0 |
| <b>213</b> | Putative paraflagellar rod protein 1D           | E9ALP7_LEIMU (+1) | 69  | 1 | 0 | 0 | 3 | 1 | 0 | 0 | 3 | 0 |
| <b>214</b> | Putative peptidase M20/M25/M40                  | E9B3Z8_LEIMU      | 52  | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 3 | 0 |
| <b>215</b> | Putative phosphatase 2C                         | E9B0G2_LEIMU      | 42  | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| <b>216</b> | Putative proteasome regulatory ATPase subunit 5 | E9AVP4_LEIMU      | 49  | 1 | 1 | 0 | 2 | 2 | 0 | 1 | 0 | 0 |
| <b>217</b> | Putative serine/threonine protein kinase        | E9B2K5_LEIMU      | 149 | 1 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| <b>218</b> | Putative serine/threonine-protein kinase        | E9ALG7_LEIMU      | 56  | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |

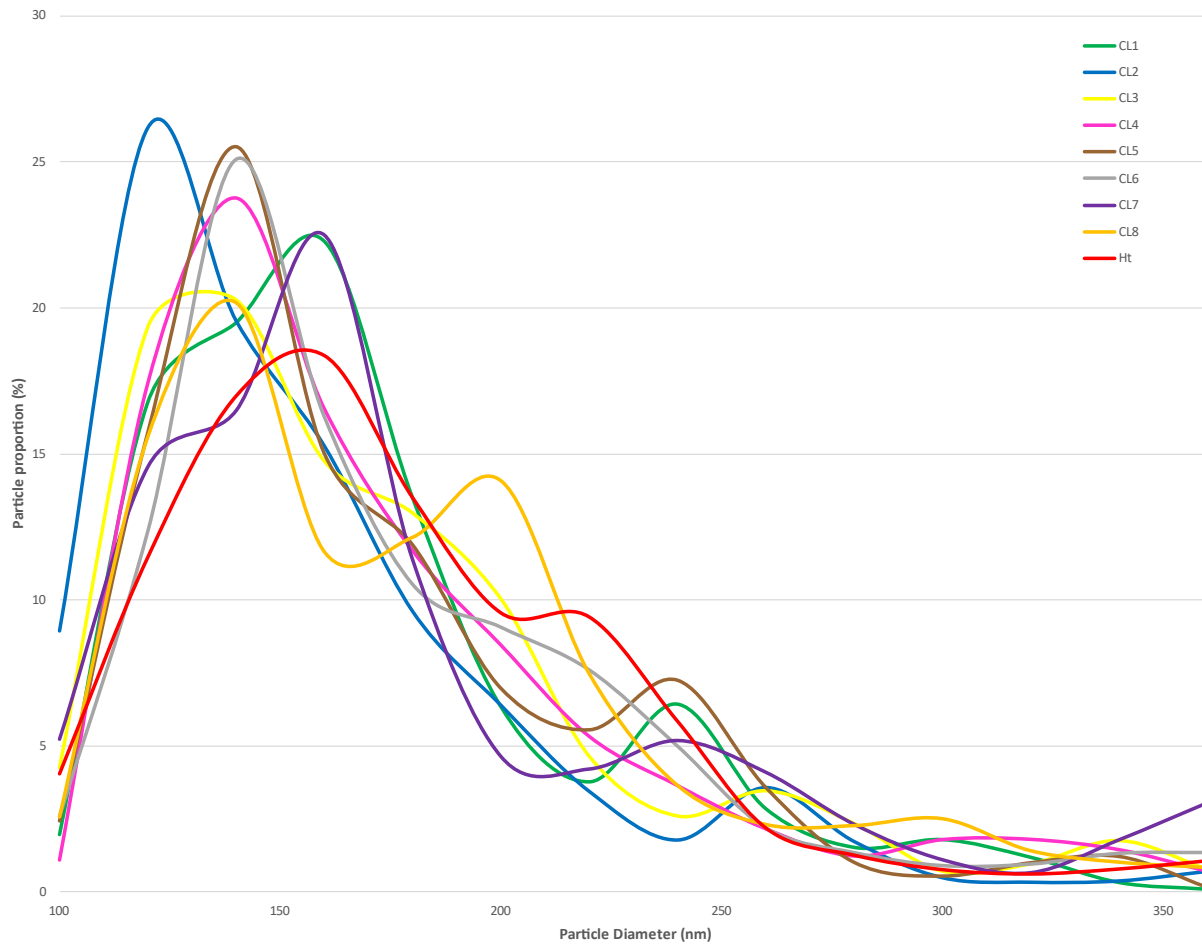
|            |                                          |                   |     |   |   |   |   |   |   |   |   |   |
|------------|------------------------------------------|-------------------|-----|---|---|---|---|---|---|---|---|---|
| <b>219</b> | Putative small GTP-binding protein Rab28 | E9B0U3_LEIMU      | 26  | 1 | 0 | 0 | 3 | 2 | 1 | 2 | 4 | 0 |
| <b>220</b> | Ubiquitinyl hydrolase 1                  | E9ALJ4_LEIMU      | 81  | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 1 | 0 |
| <b>221</b> | WGS CADB000000000 data, contig 73        | E8NHP1_LEIMU (+1) | 94  | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| <b>222</b> | 40S ribosomal protein S24                | E9AT64_LEIMU      | 16  | 0 | 0 | 0 | 3 | 3 | 1 | 2 | 0 | 0 |
| <b>223</b> | 40S ribosomal protein S6                 | E9AVH4_LEIMU      | 28  | 0 | 0 | 3 | 3 | 1 | 2 | 2 | 0 | 0 |
| <b>224</b> | 40S ribosomal protein S8                 | E9AX53_LEIMU      | 25  | 0 | 0 | 0 | 4 | 1 | 2 | 3 | 0 | 0 |
| <b>225</b> | 60S ribosomal protein L11 (L5, L16)      | E9AK29_LEIMU      | 22  | 0 | 0 | 1 | 0 | 1 | 1 | 2 | 0 | 0 |
| <b>226</b> | Adenine phosphoribosyltransferase        | E9AXZ5_LEIMU      | 26  | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 |
| <b>227</b> | Cluster of Kinesin-like protein          | E9AR52_LEIMU [2]  | 105 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| <b>228</b> | Cofilin-like protein                     | E9AM28_LEIMU      | 16  | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 |
| <b>229</b> | Conserved TPR domain protein             | E9B146_LEIMU      | 45  | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |

|            |                                                                                                        |              |     |   |   |   |   |   |   |   |   |   |
|------------|--------------------------------------------------------------------------------------------------------|--------------|-----|---|---|---|---|---|---|---|---|---|
| <b>230</b> | Eukaryotic translation initiation factor 5A                                                            | IF5A_LEIMU   | 18  | 0 | 0 | 1 | 1 | 2 | 2 | 0 | 0 | 0 |
| <b>231</b> | p-glycoprotein-like protein (Abc transporter-like protein) (Multidrug resistance protein-like protein) | E9AYP8_LEIMU | 140 | 0 | 0 | 0 | 4 | 1 | 0 | 0 | 1 | 0 |
| <b>232</b> | Phosphate transporter                                                                                  | E9ANE4_LEIMU | 52  | 0 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| <b>233</b> | Proteasome subunit alpha type                                                                          | E9APM4_LEIMU | 32  | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 0 |
| <b>234</b> | Putative 40S ribosomal protein S3                                                                      | E9AQ94_LEIMU | 24  | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| <b>235</b> | Putative 60S ribosomal protein L10                                                                     | E9AK58_LEIMU | 25  | 0 | 0 | 0 | 4 | 0 | 1 | 0 | 0 | 0 |
| <b>236</b> | Putative 60S ribosomal protein L13                                                                     | E9ALH7_LEIMU | 25  | 0 | 0 | 2 | 6 | 3 | 1 | 2 | 0 | 0 |
| <b>237</b> | Putative 60S ribosomal protein L19                                                                     | E9AKR8_LEIMU | 28  | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| <b>238</b> | Putative adenylate kinase                                                                              | E9AK77_LEIMU | 23  | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 |
| <b>239</b> | Putative asparagine synthetase a (Putative aspartate-- ammonia ligase)                                 | E9AY61_LEIMU | 40  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |

|            |                                                            |              |     |   |   |   |   |   |   |   |   |   |
|------------|------------------------------------------------------------|--------------|-----|---|---|---|---|---|---|---|---|---|
| <b>240</b> | Putative ATP-dependent DEAD-box RNA helicase               | E9B5S5_LEIMU | 46  | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| <b>241</b> | Putative calpain-like cysteine peptidase (Fragment)        | E9AYV1_LEIMU | 269 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 |
| <b>242</b> | Putative calpain-like cysteine peptidase                   | E9B1J0_LEIMU | 89  | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 1 | 0 |
| <b>243</b> | Putative glucosamine-fructose-6-phosphate aminotransferase | E9AKX3_LEIMU | 73  | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 0 |
| <b>244</b> | Putative mitotubule-associated protein Gb4                 | E9AYH5_LEIMU | 321 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| <b>245</b> | Putative Pyridoxal kinase                                  | E9B0Q4_LEIMU | 33  | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 | 0 |
| <b>246</b> | Putative ribosomal protein L3                              | E9B396_LEIMU | 48  | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 0 |
| <b>247</b> | Putative serine/threonine-protein kinase                   | E9AVE6_LEIMU | 90  | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| <b>248</b> | Receptor-type adenylate cyclase a-like protein             | E9AT96_LEIMU | 152 | 0 | 1 | 0 | 1 | 0 | 2 | 1 | 2 | 0 |
| <b>249</b> | S-methyl-5'-thioadenosine phosphorylase                    | E9AKI9_LEIMU | 33  | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

|            |                                                     |                   |    |   |   |   |   |   |   |   |   |   |
|------------|-----------------------------------------------------|-------------------|----|---|---|---|---|---|---|---|---|---|
| <b>250</b> | Succinyl-diaminopimelate desuccinylase-like protein | E9B201_LEIMU      | 51 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| <b>251</b> | Sucrose-phosphate synthase-like protein             | A0A0R6XPE8_LEIME  | 52 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| <b>252</b> | WGS CADB000000000 data, contig 24 (Fragment)        | E8NHG9_LEIMU      | 80 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| <b>253</b> | WGS CADB000000000 data, contig 99                   | E8NHS7_LEIMU (+1) | 63 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 |

*Supplemental Table 1. All proteins identified from L. Mexicana Clones and Ht EVs represented using total spectrum count. Each protein was identified with 95% confidence and at least 2 detected peptides. Accession numbers are given in Uniprot format.*



*Supplemental Figure 1. Proportional size distribution profile of all EVs isolated from L. mexicana clones and Ht L. mexicana superimposed to directly compare their size distribution. Proportion was calculated based on the total particle concentration of each separate clone/Ht.*

Chapter 3. Disruption of *Leishmania major* *Vps36* gene  
using CRISPR/Cas9 severely abrogates vesicle secretion  
and subsequent parasite infectivity and disease  
progression

## Preface and rationale

Previous studies from the Olivier lab have shown that *Leishmania* EVs are perfectly capable of modulating macrophage immune response and their co-inoculation with *Leishmania* parasites augments infection and disease severity. Additionally, our previous manuscript supports the growing body of evidence that even minute differences in the secreted EV populations can have great physiological impacts, as demonstrated by the varying infectious capabilities of the *L. mexicana* clonal EVs. Thus, investigating the deeper machinations of *Leishmania* EV biogenesis is a top priority, and ESCRT proved to be a great starting point. Previous proteomic analysis from our lab identified a Vps36 analog in *Leishmania*, notably not an ESCRTIII component, making the logical next step the validation of Vps36 as a key component of *Leishmania* EV biogenesis. Previously, Vps4 had been identified as an important component of *L. major* autophagy and Vps36 had been identified as necessary for *T. brucei* EV secretion and social motility, but Vps36 had not been specifically investigated in *Leishmania*.

To investigate the role of Vps36 in *L. major*, the *Vps36* gene, an ESCRTII component protein, was disrupted in *L. major* using CRISPR/Cas9. The resulting *Vps36*<sup>(-/-)</sup> *L. major* mutants were then compared to WT *L. major* using nanoparticle tracking analysis, transmission electron microscopy, and LC-MS/MS proteomic analysis. Our initial findings showed that *Vps36*<sup>(-/-)</sup> *L. major* secrete significantly fewer sEVs compared to WT *L. major* and the protein content of these *Vps36*<sup>(-/-)</sup> EVs is significantly altered. Additionally, *Vps36*<sup>(-/-)</sup> *L. major* failed to induce a strong infection in susceptible Balb/c mice, even when co-injected with WT *L. major* EVs. Upon Vps36 addback, both sEV production and infectivity were restored. However, the reduced infectivity in *Vps36*<sup>(-/-)</sup> *L. major* may be due to a defect in parasite amastigote and metacyclic transformation as well as defective metabolism shift upon macrophage phagocytosis rather than being caused by impaired EV biogenesis. These findings indicate that although Vps36 plays an important role in *L. major* EV biogenesis, its impairment of parasite transformation and metabolic shift are the real cause of reduced parasite infectivity and intramacrophage survival.

Disruption of *Leishmania major* *Vps36* gene using CRISPR/Cas9 severely abrogates vesicle secretion and subsequent parasite infectivity and disease progression

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## Abstract

Leishmaniasis is a widespread neglected infectious disease with 0.9-1.6million new cases every year, resulting in up to 40 000 deaths annually. Its causative agent, the *Leishmania* parasite, is a protozoan parasite carried by sandflies that infects host macrophages to propagate by evolving various virulence factors, the metalloprotease GP63 in particular, to evade the host immune response. Our lab has shown that EVs play a key roll in *Leishmania* infection. To further confirm these findings, the gene Vps36, an important member of the ESCRTII complex key to EV production in eukaryotic cells, was disrupted using CRISPR/Cas9 and the resulting Vps36<sup>(-/-)</sup> *L. major* EV production was compared to WT *L. major* using nanoparticle tracking analysis, transmission electron microscopy, and LC-MS/MS proteomic analysis. Our results show that Vps36<sup>(-/-)</sup> *L. major* secreted significantly less sEVs compared to WT and these Vps36<sup>(-/-)</sup> *L. major* EVs contained significantly altered protein cargo. Vps36<sup>(-/-)</sup> *L. major* failed to induce a high level of infection in the susceptible Balb/c mice even when co-injected with WT *L. major* EVs. Vps36 addback successfully restored both sEV production and infectivity of *L. major*. However, the reduced infectivity observed in Vps36<sup>(-/-)</sup> *L. major* may stem from defects in amastigote and metacyclic transformation, as well as disruptions in the parasite's metabolic shift following macrophage phagocytosis, rather than from impaired EV biogenesis alone. These findings suggest that while Vps36 is important for EV production in *L. major*, the true drivers of reduced infectivity and survival within macrophages are the disruptions in parasite transformation and metabolic adaptation caused by Vps36 deficiency.

## Introduction

Leishmaniasis is a neglected tropical disease that impacts up to a million people every year (1). There are 3 forms of leishmaniasis: visceral, mucocutaneous, and cutaneous. While visceral leishmaniasis is by far the most fatal form of the disease, cutaneous leishmaniasis is much more widespread, representing more than 95% of all cases of leishmaniasis (2-4). *Leishmania* spp. are the causative agent of leishmaniasis; they are protozoan parasites carried by sandflies that infect the macrophages of mammalian hosts. Leishmaniasis is an old-world disease, infecting humans since ancient times and evolving alongside them (5, 6). The resulting relationship between parasite, vector, and host is quite complex, involving over 20 *Leishmania* species, over 90 sandfly species, and several mammalian hosts (7).

*Leishmania* parasites have evolved several mechanisms to evade the host immune response, developing a veritable arsenal of immune-modulating virulence factors (8-10). Our lab has previously studied these virulence factors, especially GP63, and our research along with that of others in the field has revealed the important role of *Leishmania* extracellular vesicles (EVs) as vehicles for the delivery of *Leishmania* virulence factors to target mammalian host cells (11-13). *Leishmania* EVs are constitutively secreted and can also be induced by 37°C temperature shock (TS). These EVs are loaded with common virulence factors like GP63 and EF1a and have profound immunomodulatory effects on mammalian macrophages (14, 15). *Leishmania* EVs have been further shown to be constitutively secreted within the midgut of its sandfly vector and are therefore present during initial infection (16). This co-inoculation causes heightened infection severity.

EVs are important vehicles for cell-cell communication and are constitutively secreted by both bacteria and eukaryotes (17). They carry many biologically active macromolecules, ranging from nucleic acids to proteins, from their cell of origin and deliver these to a recipient cell to induce the desired physiological change (18). EVs have been of great interest due to their importance in intercellular communication, especially in the context of immune and tumor cell research and have recently been established to play an important role in pathogen-host interaction (19, 20). Understanding the mechanisms of EV biogenesis and

release/uptake have already been proven to be key to understanding human disease progression and diagnosis, but the study of EVs in pathogens and parasites is still a developing field of research (21).

There are many distinct classes of EVs, including ectosomes, microvesicles, apoptotic bodies, exosomes, exomeres, and more (22). Most recent EV research has focused on exosomes, a class of EVs ranging from 30 to 140nm in diameter. Exosomes originate from the endocytic pathway and first form as intraluminal vesicles within multivesicular bodies (MVB). Eventually, these vesicles are released once the MVB fuses with the plasma membrane. It is this distinct biogenesis pathway with directed cargo sorting that make exosomes particularly attractive to study as the main vehicles for intercellular communication (23). However, with further research, the distinction between exosomes and other classes of EVs, especially microvesicles, has become much less defined and harder to validate, so they are often referred to as small extracellular vesicles (sEVs) instead (24, 25). Regardless, it is well established that the endosomal sorting complex required for transport (ESCRT) proteins play a key role in the formation of MVBs and resulting sEVs once MVB-membrane fusion occurs (26).

Many ESCRT analogs have been identified in *Leishmania* as well as other trypanosomes, which makes it reasonable to suspect that the disruption of ESCRT machinery in *Leishmania* would impact EV production as well (27-30). We chose to target Vps36 in particular, using CRISPR/cas9 mediated gene-editing in *Leishmania major* to insert a puromycin resistance gene in the Vps36 exon located on *L. major* chromosome 19. The resulting impact on EV production was assessed using nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and proteomic analysis in order to satisfy the gold standard set by MISEV directives (31). In vivo and In vitro infection experiments were also conducted to compare WT and Vps36<sup>(-/-)</sup> *L. major* infectivity. A pSP72αPUROαVps36 *L. major* addback, henceforth referred to as Vps36 *L. major*, was also generated to verify that observed effects were caused by Vps36 disruption.

## Methods

### *L. major* culture

*L. major* Seidman (MHOM/SN/74/Seidman) was cultured in 25°C liquid Schneider's Drosophila Medium (SDM) (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µl/ml streptomycin (complete Schneider's). 100 µg/ml puromycin and 1600 µg/ml paromomycin were used to maintain *Vps36*<sup>(-/-)</sup> *L. major* and *Vps36* *L. major*, respectively. To make solid SDM agar, un-supplemented SDM was mixed with 2.5% agar solution and heat inactivated FBS in a ratio of 5:4:1, then this mixture was heated to 56°C, poured into 100mm petri dishes to a thickness of 5mm, and cooled. Puromycin was added to the cooling SDM-agar mixture to a final concentration of 50 µg/ml. 2×10<sup>6</sup> log phase (3-4 day old liquid culture) *L. major* promastigotes were spread onto the SDM-agar plates and incubated at 25°C for 10 days. Individual colonies were then carefully picked under magnification and cultured in liquid SDM. The resulting liquid cultures were then frozen after 3-4 days.

### CRISPR/Cas9-mediated *Vps36* disruption

The CRISPR-associated protein 9 (Cas9) plasmid used in this study was generously provided by the Ouellette lab. CRISPR/Cas9-mediated gene disruption was carried out as previously described (32). Briefly, the ORF coding for the Cas9 nuclease of *Streptococcus pyogenes* was amplified from the CMV-CAS9-2A-GFP commercial vector (Sigma-Aldrich) and cloned in the *Leishmania* expression vector pSP72 αHYGα within XbaI and HindIII sites. In order to track the expression of Cas9, an HA tag (Y P Y D V P D Y A) was included at the C-terminal of Cas9 during the amplification of the ORF. This Cas9-containing pSP72 plasmid was transfected into *L. major* by electroporation and stable expression was confirmed by selection using hygromycin. Proper Cas9 expression was verified by western blot using a rabbit anti-HA polyclonal antibody (supp fig 1A). CRISPR gRNA (GAAGACGGATGGGGCAGGAC) was designed to target the *Vps36* analog gene on *L. major* chromosome 19 and ordered from IDT along with the tracerRNA. A puromycin repair cassette was then designed based on the puromycin resistance gene from the W432-1 plasmid. A

forward (TACTGGGAGTGGGAAGACGGATGGGGCAGGAATGACCGAGTA) and reverse (GACGGTGATGCACACCTCGTCCGCCTCCAGTCAGGCACCGGGCT) primer based on this puromycin resistance gene were designed to add sticky ends complimentary to the CRISPR-cut region of *L. major* *Vps36* onto the resistance gene to form a repair cassette. The gRNA, tracerRNA, and puromycin repair cassette were then transfected directly into the nuclei of *L. major* by Nucleofection, using the Amaxa human T cell nucleofector kit and the U033 program on the Amaxa 2B Nucleofector. Successful CRISPR-mediated *Vps36* disruption by the insertion of the puromycin repair cassette was verified by 100 µg/ml puromycin selection in liquid SDM culture. Surviving *L. major* parasites were then plated on solid SDM-agar plates with 50 µg/ml puromycin. Colonies were picked after 10 days of growth and assessed by PCR (Supp. Figure 1C) and Sanger sequencing (McGill Genome Centre) (Supp Figure 1B).

#### EV isolation

Purification of *Leishmania* exosomes was performed according to standard methods for mammalian cells. *L. major* stationary phase promastigotes (day 6-8) were washed twice with PBS, resuspended in RPMI 1640 medium (Life Technologies, Rockville, MD) without FBS and phenol red at a concentration of  $2-4 \times 10^8$  promastigotes/ml, and incubated for 4 hours at 37°C for the release of EVs in the culture medium. At the end of a 4-hour incubation, the sample was centrifuged at 4000 RPM to remove parasites and large debris, and the supernatant was filtered through a 0.45 µm syringe filter, followed by a 0.20 µm syringe filter. Next, EVs were pelleted by 2 rounds of 1-hour centrifugation at 100 000 g at 4°C and resuspended in exosome buffer (137 mM NaCl, 20 mM Hepes, pH 7.5). Resulting sEV samples were aliquoted and frozen at -80°C, with each aliquot being thawed no more than twice. sEV protein content was determined using Thermo Fisher MicroBCA kit according to manufacturer instructions.

#### NTA

WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* samples were initially diluted by 1:200 in 0.22 µm filtered EV buffer and NTA was performed using the NanoSight NS500 (Malvern Panalytical). Samples were loaded into the machine at a minimum initial volume of 500 µl. Focus and

camera level were then adjusted based on the first sample before standard script was run (camera level was unchanged for subsequent samples). Three 30 second videos were taken using the standard camera and analysis was performed using the NTA software to determine particle quantity and size as well as to perform statistical analysis. Comparisons performed between WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* were performed using ZetaView NTA (Particle Metrix). The samples were run at 25°C using 0.22 µm filtered EV buffer as a diluent. For video acquisition, a shutter frame rate of 45 was used and the sensitivity was set at 85, according to the system's software guidance algorithms. Before measurements were taken, accuracy of the ZetaView was verified using 100 nm standard beads. Samples were diluted in EV buffer with a dilution factor of 1:1000–1:10 000 to achieve a particle count in the range of 1000–2000.

### TEM

In vitro purified *L. major* EVs were coated directly onto carbon-coated copper grids by dropping 5 µl on to the grid and allowing to dry for 2 minutes. The grids were then dried using the edge of a Kimwipe and fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 minute, washed 3 times in miliQ water, and stained with 1% uranyl acetate for 1 minute. After drying, grids were visualized in the FEI Tecnai G2 Spirit Twin 120 kV Cryo-TEM and images were taken with the Gatan Ultrascan 4000 4k x 4k CCD Camera System Model 895. Microscopy and sample preparation were performed at the Facility for Electron Microscopy Research (FEMR) at McGill University.

### Scanning electron microscopy (SEM)

*L. major* promastigotes were fixed in 2.5% glutaraldehyde solution overnight at 4°C. Samples were then added to poly-L-lysine-coated slides and dehydrated in ethanol, followed by amyl acetate and supercritical CO<sub>2</sub>. Dehydrated samples were coated with Au–Pd and visualized using a Hitachi S-4700 cold field emission gun scanning electron microscope (Facility for Electron Microscopy Research, McGill University). TEM. For negative staining, nanoparticle preparations were coated directly on Formvar carbon grids, fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1min and stained with 1% uranyl acetate for 1min. For

ultrastructure analysis, parasite pellets were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. After washes in the same buffer, samples were included in 1% ultrapure agarose, post-fixed with osmium tetroxide and dehydrated in a graded acetone series before the embedding in epoxy resin. Ultrathin sections (70–80 nm) were cut from resin blocks using a Reichert-Jung Ultracut E Ultramicrotome. Formvar grids covered with isolated vesicles or with ultrathin sections were visualized in the FEI Tecnai 12 120 kV transmission electron microscope. Images were taken with the AMT XR-80C CCD Camera System (Facility for Electron Microscopy Research, McGill University).

#### LC-MS/MS

LC-MS/MS was performed at the Institute de Recherches Cliniques de Montréal (IRCM, University of Montreal, Canada). EVs were diluted in ultrapure water and protein content was determined using the microBCA protein assay kit (ThermoFisher Scientific) to account for the high dilution ratio (1:20). Whole parasite (WP) protein content was extracted using 0.1% NP-40 lysis buffer with protease inhibitor cocktail (Roche). Final WP protein content was determined using Bradford protein assay kit (ThermoFisher Scientific). Protein concentration was determined using an Infinite 200 Pro spectrophotometer (Tecan) and interpolation with a BSA standard curve. 5 µg *L. major* EV/WP protein was then precipitated using 15% trichloroacetic acid (TCA)/acetone and processed for LC-MS/MS analysis. After precipitation, in solution digestion was performed by the addition of trypsin at a ratio of 1:25 protease: protein. After an overnight incubation at 37°C, the reactions were quenched by the addition of formic acid to a final concentration of 0.2% and cleaned with C18 Zip Tip pipette tips (Millipore, Billerica, MA), before mass spectrometry analysis. Extracted peptides were injected onto a Zorbax Extended-C18 desalting column (Agilent) and subsequently chromatographically separated on a Biobasic 18 Integragrit capillary column (ThermoFisher Scientific) on a Nano High-Performance liquid chromatography system (1100 series unit; Agilent). Eluted peptides were electrosprayed as they exited the capillary column and were analyzed on a QTRAP 4000 linear ion trap mass spectrometer (SCIEX/ABI).

#### Proteomic bioinformatics

Individual sample tandem mass spectrometry spectra were peak listed using the Distiller version 2.1.0.0 software ([www.matrixscience.com/distiller](http://www.matrixscience.com/distiller)) with peak picking parameters set at 1 for signal noise ratio and at 0.3 for correlation threshold. The peak-listed data was then searched against the NCBI database with the Mascot software version 2.3.02 (Matrix Science). Mascot was set up to search the *L. major* database (txid5664, 28 107 proteins) with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 1.5 Da. Carbamidomethyl was specified in both search engines as a fixed modification. Oxidation of methionine residues was specified in Mascot as a variable modification. Scaffold software version 4.0.6.1 (Proteome Software Inc.) was used to validate MS/MS peptide and protein identifications. Identification of peptides was accepted if it could be established at greater than 95.0% probability. Identification of proteins was accepted if it could be established at greater than 95.0% probability and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The final spectral count was represented by the average of the biological replicas after normalization to the total spectral count. Unidentified proteins were removed from analysis. Gene ontology bar graphs comparing WT *L. major* and Vps36<sup>(-/-)</sup> *L. major* WP and EVs were generated using PANTHER searching against *L. major* reference database (33). Unidentified proteins were excluded from final gene ontology analysis. STRING diagrams were generated based on unique or shared proteins when comparing between WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* EVs using default settings (34).

#### Vps36 addback

Vps36 *L. major* was generated by nucleofection with a Vps36-containing plasmid. Briefly, *L. major* Seidmans genomic DNA was extracted using a standard DNazol protocol. The whole Vps36 exon was amplified using PCR with primers adding XbaI and HindIII restriction sites to the 3' and 5' ends of the amplified exon. After gel migration, the band was extracted using a DNA gel extraction kit and polyadenylation was performed by on both ends of the exon. After PCR cleanup, the polyadenylated Vps36 exon was ligated into pGEM and bacterial

transformation was performed with *Escherichia coli*. After bacterial expansion on solid agar plates, 10 white colonies containing the *Vps36*-pGEM vector were picked and expanded in liquid LB culture. A standard miniprep protocol was used to extract the *Vps36*-pGEM vector. The cloned *Vps36* exon was then excised by XbaI and HindIII digestion. After gel migration, the band was extracted using a DNA gel extraction kit and re-ligated in a pSP72 plasmid containing an aphomomycin element. Plasmid cloning was again performed as described with *E. coli* transformation and miniprep plasmid extraction. XbaI and HindIII digestion and PCR were used to confirm successful *Vps36*-pSP72paro cloning. *Vps36*-pSP72paro was then directly transfected into the nuclei of *Vps36*<sup>(-/-)</sup> *L. major* promastigotes in the logarithmic phase via Nucleofection, using the Amaxa human T cell nucleofector kit and the U033 program on the Amaxa 2B Nucleofector. *Vps36* *L. major* was then selected for using a combination of puromycin (100 µg/ml) and paromomycin (1600 µg/ml) in liquid SDM culture. Successful *Vps36* addback was verified using PCR along with positive and negative Nucleofection controls (Supp. Figure 1C).

#### Mice footpad infections

All experiments with mice were carried out in pathogen-free housing and in accordance with the regulations of the Canadian Council of Animal Care, approved by the McGill University Animal Care Committee. 4 groups of 5 male Balb/c mice (6 to 8 weeks old) were injected in the right hind footpad with 5×10<sup>6</sup> stationary phase WT *L. major* or *Vps36*<sup>(-/-)</sup> *L. major* promastigotes either alone or co-inoculated with 10µg of In vitro purified EVs from WT *L. major* or *Vps36*<sup>(-/-)</sup> *L. major*. This infection was also repeated with 3 groups of 5 male Balb/c mice, comparing WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* parasites alone. Disease progression was monitored by measuring footpad swelling weekly with a metric caliper up to 10 weeks post-infection. Mice were sacrificed at the end of each experiment by isoflurane and CO<sub>2</sub> asphyxiation, followed by cervical dislocation. Infected footpads were imaged and collected to perform parasitemia. Footpads were washed in 70% ethanol and PBS and crushed in pyrex tissue homogenizers to release *L. major* parasites. The footpad parasite burden was then determined by limiting dilution assay from the resulting

supernatant, assuming an initial dilution factor of 1:500. Statistical analysis was performed using Graphpad Prism 8.

#### RNA extraction and sRNA sequencing

RNA was extracted from either  $2 \times 10^6$  parasites or  $2 \times 10^8$  particles, comparing between WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, using microRNeasy columns (QIAGEN). RNA integrity was assessed on a Bioanalyzer RNA pico chip, followed by poly A mRNA enrichment and library preparation using the TruSeq RNA sample preparation kit (Illumina). The RNA seq libraries were sequenced on a NovaSeq 6000 (Illumina) in a paired-end 100-bp configuration. The quality of sequence reads obtained for each sequence read was confirmed using the FastQC tool (Babraham Bioinformatics). Sequence reads were trimmed using Trimmomatic (35) and rRNA reads were removed. Reads were mapped to the *L. major* reference assembly (ASM272v2) using STAR (36), and gene expression was quantified by counting the number of uniquely mapped reads with featureCounts using default parameters (37). Normalization and differential expression analysis was conducted using the edgeR Bioconductor package (38). We retained genes that had an expression level of a minimum of five counts per million reads in at least 2 of the 12 samples, and pairwise differential gene expression analysis was performed following PCA by comparing WT *L. major* or *Vps36*<sup>(-/-)</sup> *L. major* EVs or WP. Genes with changes in expression  $\geq |2|$  and adjusted p-values ( $< 10^{-5}$ ) were considered significant.

#### Mice intraperitoneal (IP) infections

4 groups of 3 mice each were infected with 108 WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* stationary promastigotes by injection into the IP cavity. PBS was used as a negative control. 6 hours post-infection, the mice were sacrificed isoflurane and CO<sub>2</sub> asphyxiation, followed by cervical dislocation cervical dislocation. and 5ml of ice-cold endotoxin-free PBS was used to obtain lavages of the cavities. The number of live cells present in the lavages was counted using a hemocytometer with 1:2 dilution in trypan blue to determine cell viability. Cells were prepared for microscopy using the Cytospin 4 cytocentrifuge (Thermo Scientific). The cells were fixed and stained using the Differential Quik (diff-quick) Stain Kit (Ral Diagnostics), then counted and differentiated by cell type. 200  $\mu$ l of each lavage was

plated in 4-well chamber slides and supplemented with Dulbecco's modified eagle medium (Wisent, St-Bruno, QC, Canada) with 10% FBS and 1% penicillin-streptomycin-glutamine up to 1000  $\mu$ l. Cells were incubated at 37°C with 5% CO<sub>2</sub>. 24-, 48-, and 72-hours post-plating, cells were fixed and stained using the diff-quick stain kit and counted.

#### Macrophage infections

C57BL6 bone marrow derived macrophages (BMDMs) were adhered to 4-well chamber slides (Nunc Lab-Tek) with DMEM (Sigma-Aldrich) at 37°C 5% CO<sub>2</sub> at a concentration of 3×10<sup>5</sup> cells per well. BMDMs were allowed to rest and adhere overnight and then infected with stationary promastigotes from WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, or Vps36 *L. major* cultures for 3 hours at a ratio of 1:5 BMDMs:parasites. Then, cells were washed gently with warmed PBS, DMEM cell media was replaced, and the infection was allowed to continue. Cell infection rate and amastigote burden was assessed at 3-, 24-, 48-, and 72-hours post-infection by diffquick staining and counting.

#### PBS starvation assay

WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* parasites were cultured to stationary phase in liquid SDM culture. 5×10<sup>6</sup> promastigotes were washed and resuspended in PBS in triplicate and incubated at 37°C for 0, 2, and 4 hours. PBS-starved parasites were then transferred to a 96-well plate, pipetting 200  $\mu$ l of PBS with suspended promastigotes per well along with 200  $\mu$ l of stationary promastigotes in SDM in triplicate as a control. 100  $\mu$ g MTT was added per well and plate was then incubated for a further 1 hour at 37°C. After incubation, the plate was spun down at 2000 rpm for 5 minutes. Supernatant was carefully removed by pipetting and the pellet was resuspended using 200  $\mu$ L DMSO. Plate was read at 492nm frequency using a Tecan Infinite 200 Pro spectrophotometer.

#### Amastigote differentiation

WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* parasites were cultured to log phase in liquid SDM culture. 5×10<sup>6</sup> promastigotes from each culture were then resuspended in phenol-free RPMI media adjusted to a pH of 5.5 and supplemented with 10% heat-

inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µl/ml streptomycin. Parasites were then incubated at 33°C in triplicate for 15 days. Parasite growth was determined every 2-3 days by haemocytometer counting and spectrophotometry at a frequency of 600 nm using a Tecan Infinite 200 Pro spectrophotometer. Final amastigote quantity was determined using a Histopaque1119 (Sigma-Aldrich) discontinuous density gradient. On day 15, parasite cultures were centrifuged at 2000 rpm for 5 minutes and resuspended in 50% Histopaque1119 and layered on top of a discontinuous gradient ranging from 70% to 100% Histopaque1119 in 10% intervals. The interphase between 50% and 70% as well as between 70% and 80% were collected, diluted in 10 ml PBS, and centrifuged again at 2000 rpm for 5 minutes. Pellet was resuspended in 1 ml PBS and counted by haemocytometer.

#### Initial sandfly Infections

*Phlebotomus duboscqi* flies were collected into infection pots 2-5 days before infection. Mouse blood was drawn by cardiac exsanguination and heparinized. The blood was spun down to separate the serum, which was denatured in a 56°C water bath for 1 hour. The phase containing cells was washed with saline and recombined with the serum. WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* were cultured in M199C media, paromomycin (1600 µg/mL), and puromycin (100 µg/mL) leading up to the infection. The last passage was done without antibiotics 24 hours before processing for infection. All three strains were then spun down and washed with saline. Stock concentration of parasites per strain was determined, after which the parasites were added to the processed blood for a final concentration 5x10<sup>6</sup> parasites/ml of blood. This blood was placed in artificial feeders with chick skins, from which flies fed until engorgement.

#### Gut dissections and parasite counting

Live PDMA flies were caught using a filtered aspirator, then killed with CO<sub>2</sub>. The dead flies were ejected into falcon tubes filled with soapy water for 2 minutes. After the flies were poured out over a net and rinsed with water, they were placed into petri dishes with saline. A glass slide was placed under a stage microscope and saline was dropped on the slide in

separate beads. Individual flies were placed in each bead and dissected using 27G needles. The isolated guts went into separate microcentrifuge tubes containing 35 µl of M199C media. An individual fly gut was placed into a microcentrifuge tube with 35 µl of M199C media. The gut is macerated using a plastic pestle and pellet mixer, then counted under a hemocytometer.

## Results

### Vps36 disruption significantly impairs but does not completely abrogate small extracellular vesicle secretion

The *Vps36* gene homolog in *L. major* was targeted for KO using CRISPR/Cas9 and the generated *Vps36*<sup>(-/-)</sup> *L. major* was verified using PCR and sanger sequencing (Supp. Figure 1B, C). WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* were cultured in liquid-SDM media and we observed that the parasites did not exhibit any significant differences in physical characteristics, including growth and morphology. We isolated EVs from liquid cultures of WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* after a 4-hour 37C TS using a combination of filtration and differential ultracentrifugation. The resulting EV samples were analyzed immediately by MicroBCA, NanoSight, and TEM. It was immediately apparent that although the *Vps36* KO by CRISPR/Cas9 was successful, *Vps36*<sup>(-/-)</sup> *L. major* parasites were still able to produce and secrete EVs (Figure 1A). Although the morphology of *L. major* EVs did not appear to be significantly altered by *Vps36* disruption (Supp. Figure 2A, B), we found that *Vps36*<sup>(-/-)</sup> *L. major* parasites secreted significantly less sEVs (EVs 140nm in diameter or less) compared to WT *L. major*. This reduction in sEV secretion was consistently observed in 3 separate experiments and was also observed when isolating EVs from the liquid WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* cultures themselves (Figure 1B). It was also promising to see that 37C TS significantly increased the proportion of sEVs compared to the whole EV population.

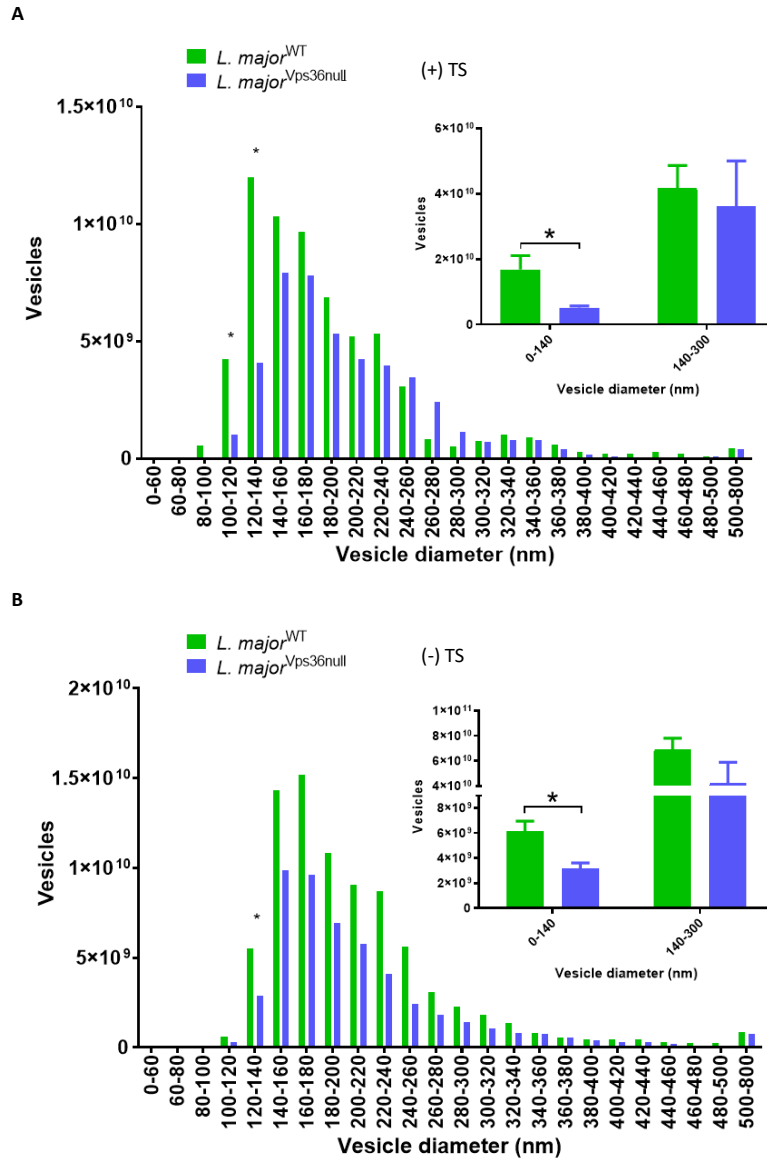


Figure 1. WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* promastigote EV release was assessed using NanoSight. To create a discontinuous bar graph, vesicles have been grouped based on diameter, typically with a range of 20nm. After 37°C TS, *Vps36*<sup>(-/-)</sup> *L. major* parasites release significantly less vesicles ranging from 100-140 nm in diameter, as well as an increased amount of vesicles ranging from 260-280 nm, though this increase was not significant (A). Though *Vps36*<sup>(-/-)</sup> *L. major* parasites seem to release less vesicles of all sizes in standard culture conditions, there was only a significant decrease in vesicles ranging from 100-120 nm in diameter (B). In both cases, *Vps36*<sup>(-/-)</sup> *L. major* released significantly less sEVs (vesicles smaller than 140 nm in diameter), as shown in the right-most graphs. Significance was calculated using multiple T-tests,  $P=0.05$ ,  $n=3$ .

### Vps36 disruption significantly alters the protein content of *L. major* EVs but not of the WP

Proteins were precipitated from lysed WT *L. major* and Vps36<sup>(-/-)</sup> *L. major* WP and EVs and compared between each other. We found that TS-induced Vps36<sup>(-/-)</sup> *L. major* EVs contained significantly fewer unique proteins when compared to WT *L. major* EVs, with there being 147 more unique proteins found in WT *L. major* EVs (Figure 2A). Despite this significant difference, the majority of proteins are shared between WT *L. major* and Vps36<sup>(-/-)</sup> *L. major* EVs (277 unique proteins) and important virulence factors were not significantly altered. Amongst the proteins that differed by more than 2-fold, Vps4 was found to be underrepresented in Vps36<sup>(-/-)</sup> *L. major* EVs (Figure 2B). Comparatively, Vps36 disruption caused little to no change in protein content in *L. major* WP, with over 85% of proteins being shared (Figure 2C). Notably, several nucleoside/nucleobase transporters were found to be differentially represented (Figure 2D). Additionally, many carbon metabolism-related proteins were found to be uniquely missing from Vps36<sup>(-/-)</sup> *L. major* EVs. When assessing differing EV protein content by gene ontology, we found that Vps36<sup>(-/-)</sup> *L. major* EVs contained almost no proteins tagged as translator regulator activity and membrane traffic protein (Figure 3). Proteins tagged as signaling and response to stimulus were also greatly underrepresented in Vps36<sup>(-/-)</sup> *L. major* EVs. Otherwise, variations within GO categories corresponded to the observed overall reduction in unique protein content in Vps36<sup>(-/-)</sup> *L. major* EVs.

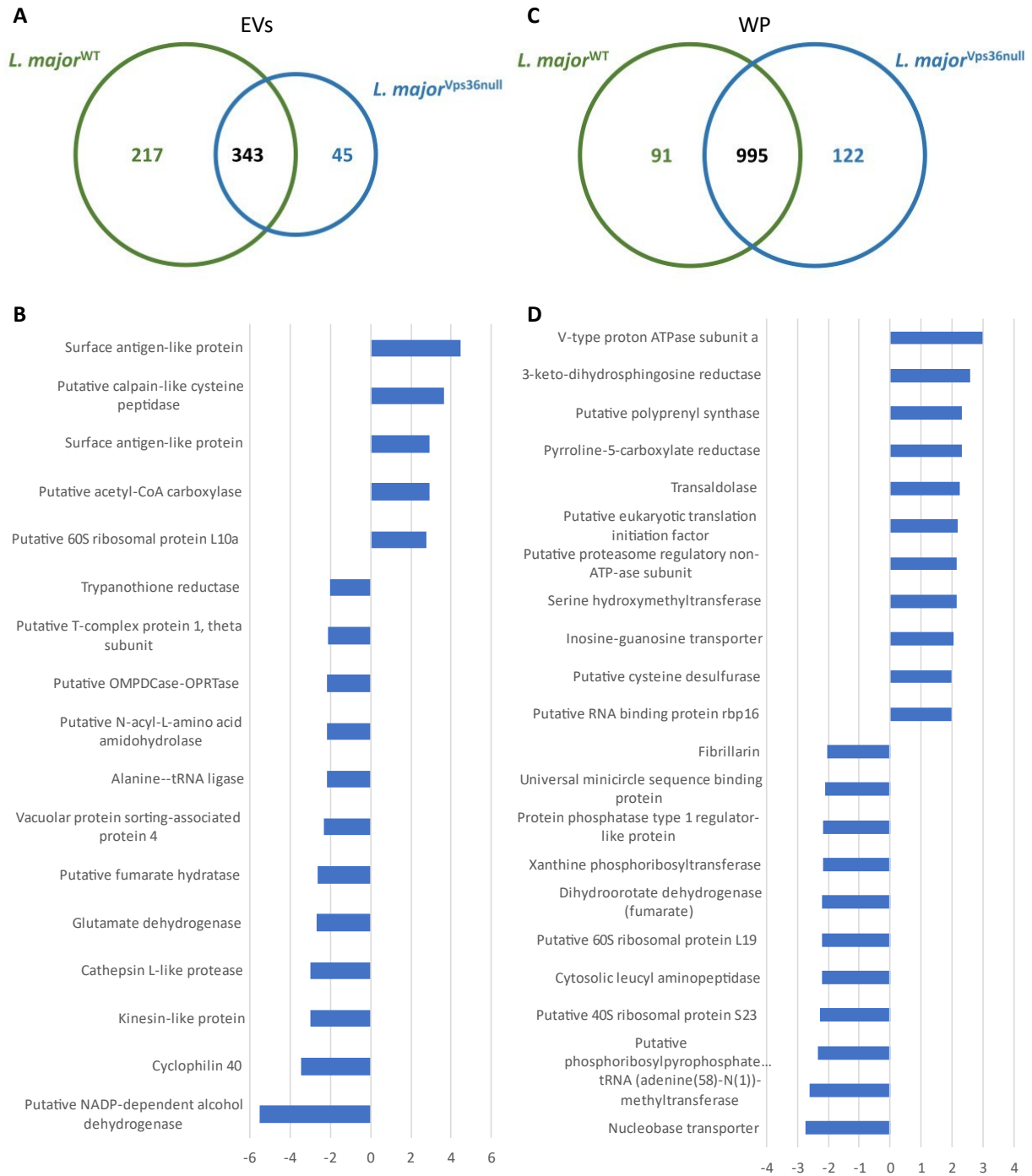


**Figure 2.** Proteomic analysis was performed on protein content extract from both EVs released after TS and *L. major* WP. Venn diagrams display proteins unique to WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, or shared. There is significant variation in EV protein content (A), while WP protein content remains largely unchanged (C). Very few shared proteins differed by >|2| fold change in either EVs (B) or WP (D). Fold changed was calculated using spectral count after normalization.



**Figure 3.** Gene ontology was determined using PANTHER for general categories of biological process, molecular function, and protein class. Graphs were generated based on shared or unique proteins between WT *L. major* and *Vps36*<sup>-/-</sup> *L. major* to assess any broad changes to EV protein cargo caused by *Vps36* disruption.

### EV RNA content remains unchanged

Nucleic acids including RNA have been reported to be common EV cargoes in previous studies. We also noted a high number of ribosomal/ribosome-associated proteins appearing in our EV proteomic data, and so decided to proceed with RNA sequencing for WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* EVs and WP. Through this analysis, we found that RNA content remained largely unaffected by *Vps36* disruption, and although *Vps36* may have a role in sorting EV protein cargo, these mechanisms seem separate from RNA packaging into EVs (Figure 4B) or RNA transcription in the WP (Figure 4A). However, when comparing the RNA content of the EVs compared to the WP, we noticed that EV RNA enrichment does vary between WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* (Figure 4C-D). Specifically, we saw more RNA enrichment in *Vps36*<sup>(-/-)</sup> *L. major* EVs compared to WT *L. major*, contrasting with the reduction of unique protein content in *Vps36*<sup>(-/-)</sup> *L. major* EVs. There were 245 RNA sequences uniquely enriched in *Vps36*<sup>(-/-)</sup> *L. major* EVs and only 57 uniquely enriched in WT *L. major* EVs (Figure 5).

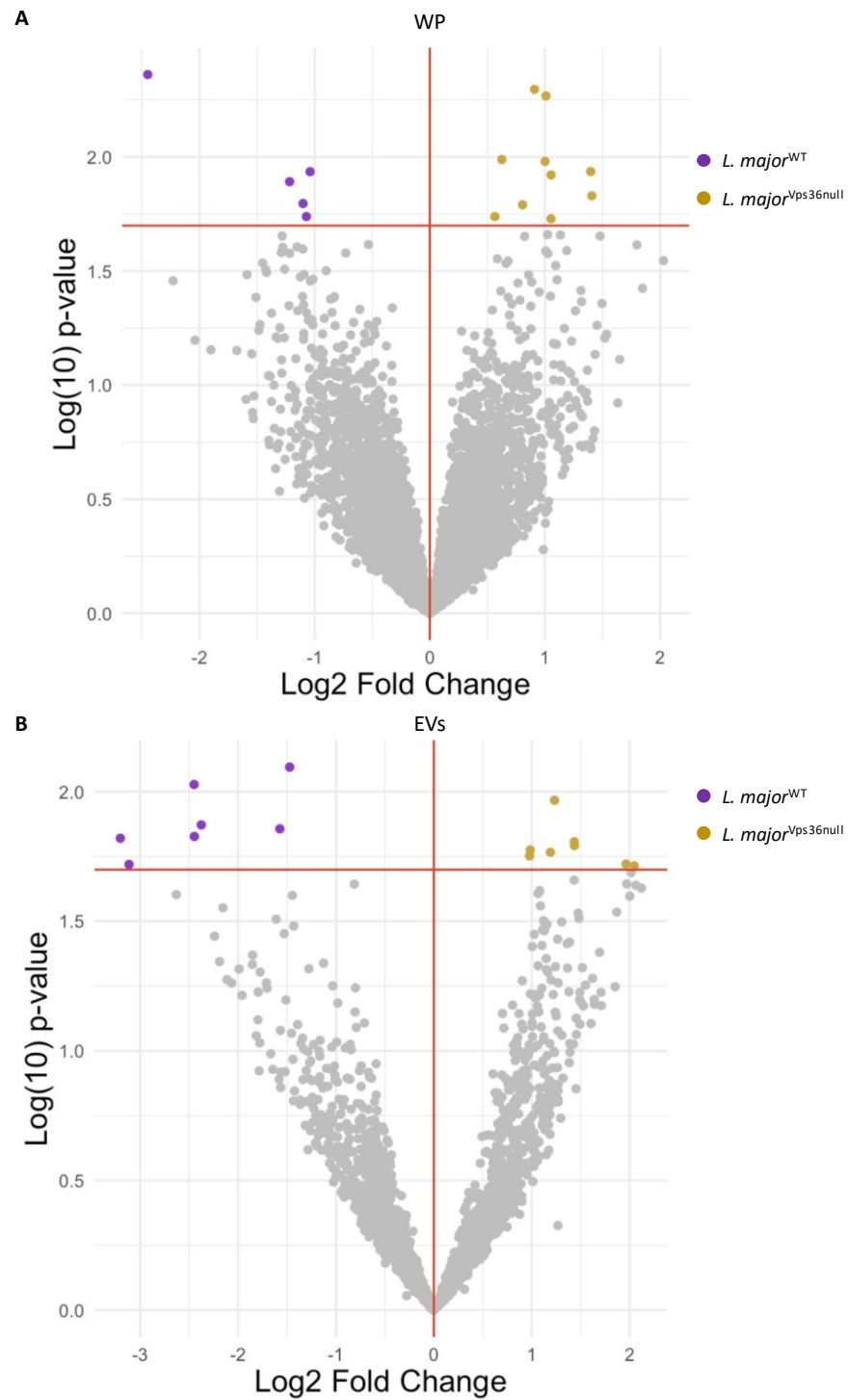


Figure 4. Volcano plots generated based on pairwise differential gene expression analysis after sRNA seq performed on both WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* EVs (B) and WP (A). No sRNA sequences were significantly enriched in either WT *L. major* or *Vps36*<sup>(-/-)</sup> *L. major*, with the cutoff line only representing a P-value of 0.02, n=3.

Vps36<sup>(-/-)</sup> *L. major* parasites exhibit a severe loss of infectivity in vivo and fail to cause significant footpad lesion swelling

WT *L. major* and Vps36<sup>(-/-)</sup> *L. major* cultures were allowed to reach stationary phase (6-8 days) and used to subcutaneously infect Balb/c mice footpads. These infections were additionally supplemented with purified WT *L. major* EVs. Over the course of the 8-week infection, we observed that co-inoculation with WT *L. major* parasite and EVs significantly augmented infection compared to parasite alone, causing significantly increased footpad swelling at all timepoints measured except at week 8 (Figure 5A). In contrast, Vps36<sup>(-/-)</sup> *L. major* infection failed to cause significant footpad swelling and lesion size remaining consistently low throughout the infection. Surprisingly, co-inoculation with Vps36<sup>(-/-)</sup> *L. major* and WT *L. major* EVs did not have any effect on lesion size, though it did result in slightly higher parasite burden at week 8 compared to Vps36<sup>(-/-)</sup> *L. major* alone (Figure 5B). Unsurprisingly, WT *L. major* parasite burden was several orders of magnitude higher. Infection severity was also visibly clear by experiment endpoint based on not only lesion size but footpad injury as well, with several WT *L. major*-infected mice experiencing hemorrhaging at their footpad lesion, whereas Vps36<sup>(-/-)</sup> *L. major*-infected mice had small, benign lesions that never broke the skin.

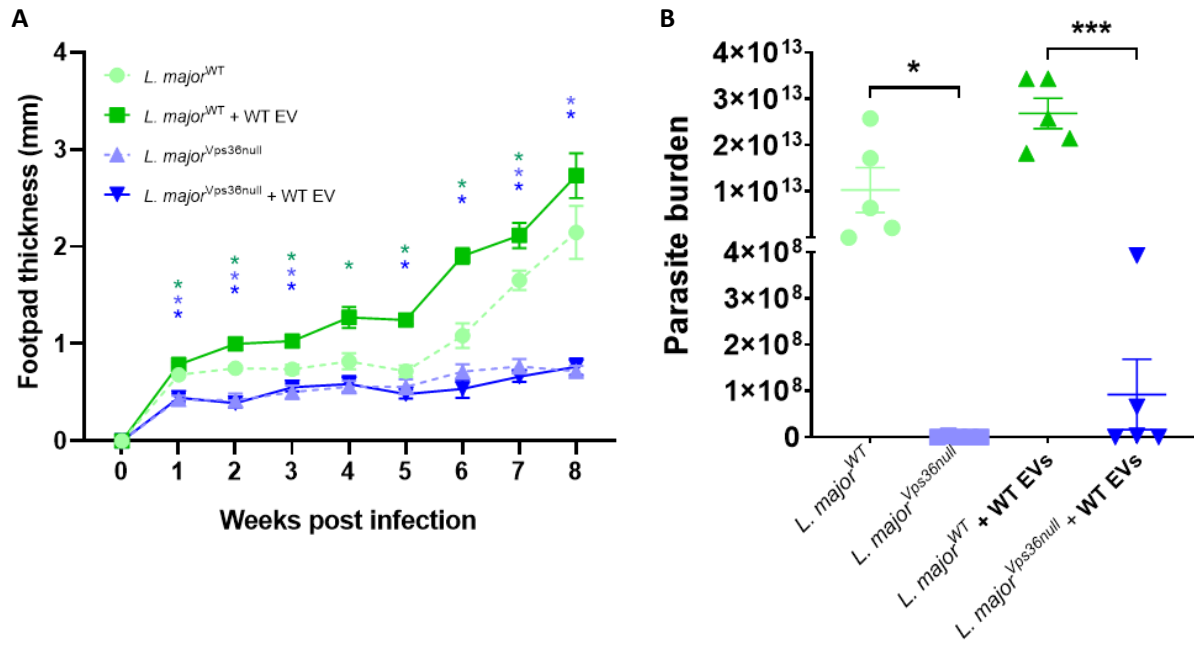


Figure 5. WTL. major and *Vps36*<sup>(-/-)</sup> *L. major* infectivity was assessed using subcutaneous Balb/c mice footpad infections. Infections were performed with either stationary promastigotes alone or a co-inoculation with WT *L. major* EVs. *Vps36*<sup>(-/-)</sup> *L. major* parasites were unable to cause significant footpad swelling at any timepoint of infection (A). Furthermore, there was no augmented infection upon co-inoculation with WT *L. major* EVs as seen with WT *L. major* parasites. Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=10$ . *Vps36*<sup>(-/-)</sup> *L. major* infection also resulted in significantly reduced parasite burden in the footpad at infection endpoint (B). Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=5$ .

Vps36 addback is sufficient in fully restoring sEV secretion and infectivity but only partially restores EV protein cargo

*Vps36 L. major* was generated via Nucleofection of a *Vps36*-pSP72paro vector in *Vps36*<sup>(-/-)</sup> *L. major* parasites (Supp. Figure 1C). We then isolated EVs from *Vps36 L. major* liquid cultures and compared EV morphology and quantity with WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* EVs as done previously, this time using ZetaView in place of the NanoSight for NTA. We found that, again, there was no significant modifications to EV morphology by *Vps36* gene disruption, but we did see a complete restoration of sEV release in *Vps36 L. major* (Figure 6A). This effect

can further be observed using SEM performed on the parasite surface, making it clear that there is much less EVs being secreted by Vps36<sup>(-/-)</sup> *L. major* compared to Vps36 *L. major* and WT *L. major* (Figure 6B). After performing proteomic analysis, we found that the overall protein content of Vps36 *L. major* EVs significantly differed from both WT *L. major* and Vps36<sup>(-/-)</sup> *L. major* EVs (Figure 6C) while the WP protein content was mostly unchanged as expected (Figure 6D). STRING diagrams were used to Next, we used parasites alone to infect Balb/c footpads and found that Vps36 addback successfully restored Vps36<sup>(-/-)</sup> *L. major* infectivity, causing significant parasite burden and lesion size by week 7 (Figure 8A, B). Although Vps36 *L. major* lesions were slightly smaller compared to WT *L. major* lesions, this difference was negligible and both parasites caused significantly greater infection compared to Vps36<sup>(-/-)</sup> *L. major*.

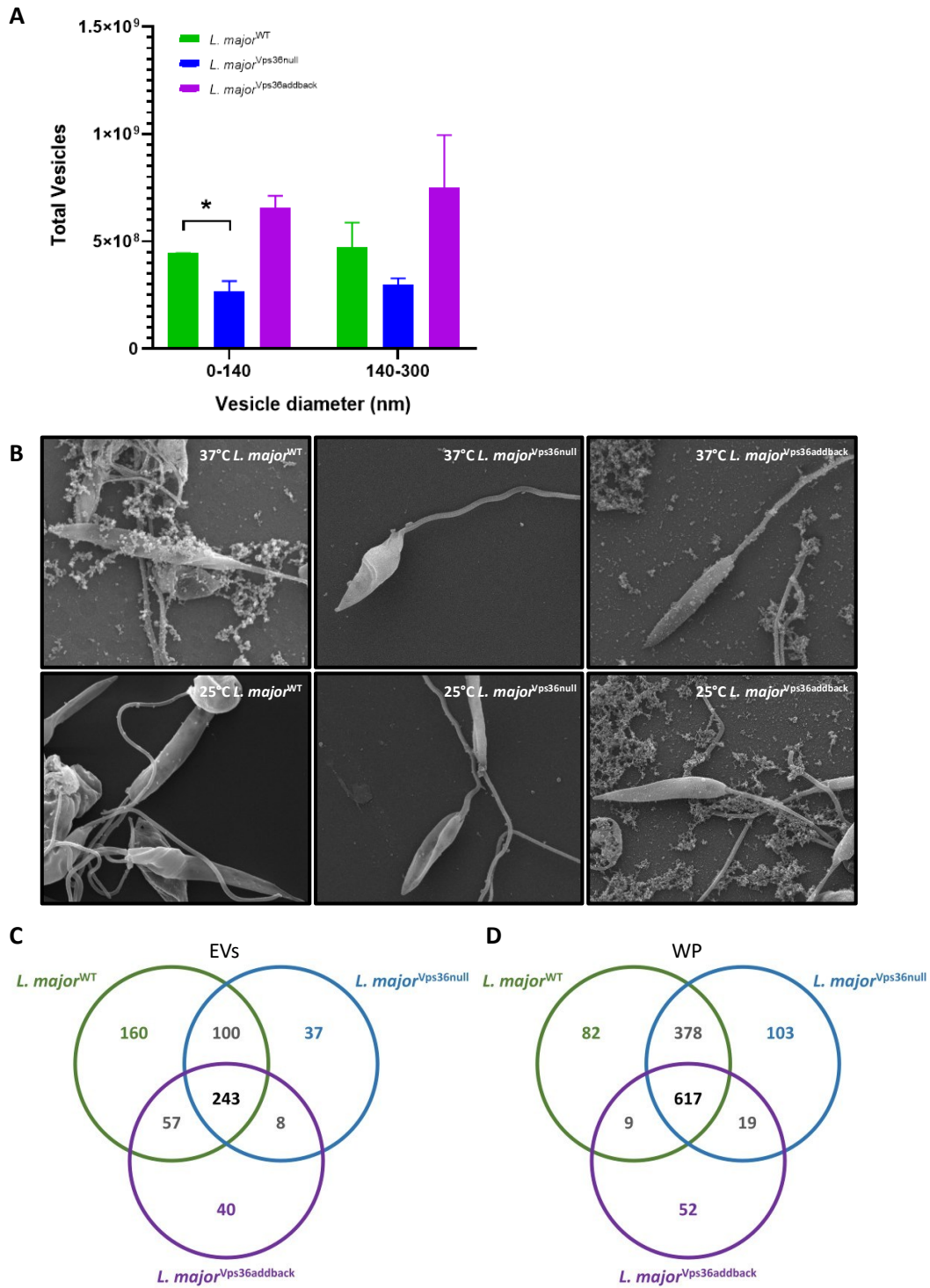


Figure 6. *Vps36* addback fully restored EV release but still resulted in unique *L. major* EV protein profile. When analyzed by ZetaView, *Vps36* *L. major* promastigotes exhibited similar levels of EV

*release after TS compared to WT L. major, whereas Vps36<sup>(-/-)</sup> L. major promastigotes released significantly less sEVs as expected (A). Significance was calculated by T-test, n=2, P=0.05. This restored EV release is evident from SEM, showing significantly more particles on the surface of WT L. major, and Vps36 L. major promastigotes after TS compared to Vps36<sup>(-/-)</sup> L. major promastigotes, which were largely unaffected by TS (B). Micrographs were taken with a magnification of 9000×. When assessing EV and WP protein content based on unique or shared proteins, we found that Vps36 L. major EV protein content was itself unique compared to both WT L. major and Vps36<sup>(-/-)</sup> L. major EVs (C). Surprisingly, Vps36 L. major WP protein content was also more unique, sharing few proteins with WT L. major or Vps36<sup>(-/-)</sup> L. major, while WT L. major and Vps36<sup>(-/-)</sup> L. major shared 378 proteins absent from Vps36 L. major.*

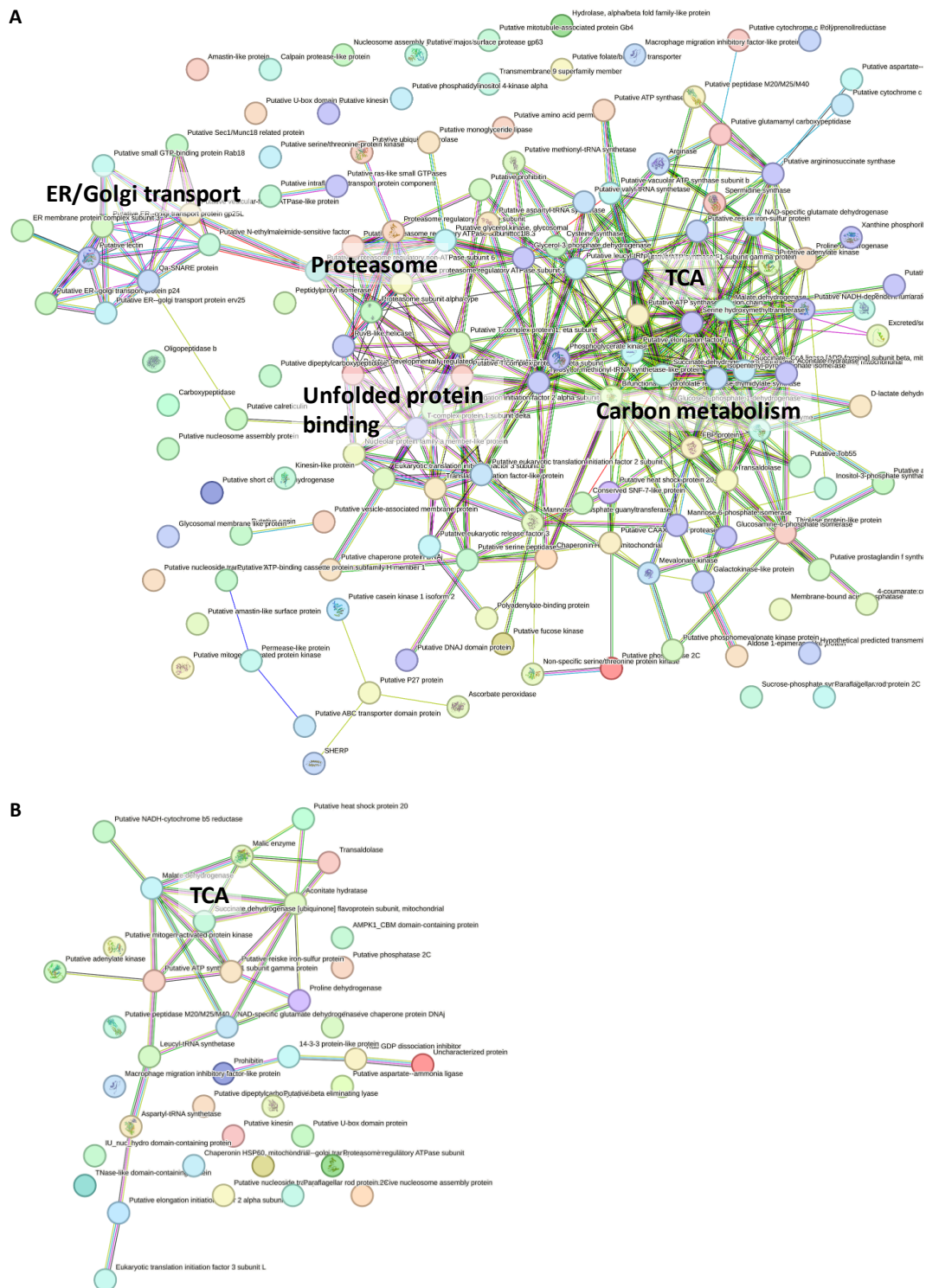


Figure 7. STRING diagrams generated with WT L. major EV proteins missing from *Vps36*<sup>(-/-)</sup> L. major EVs (A) as well as the “restored” proteins in *Vps36* L. major EVs (B). Where possible, labels have been added to clusters of proteins with shared functions.

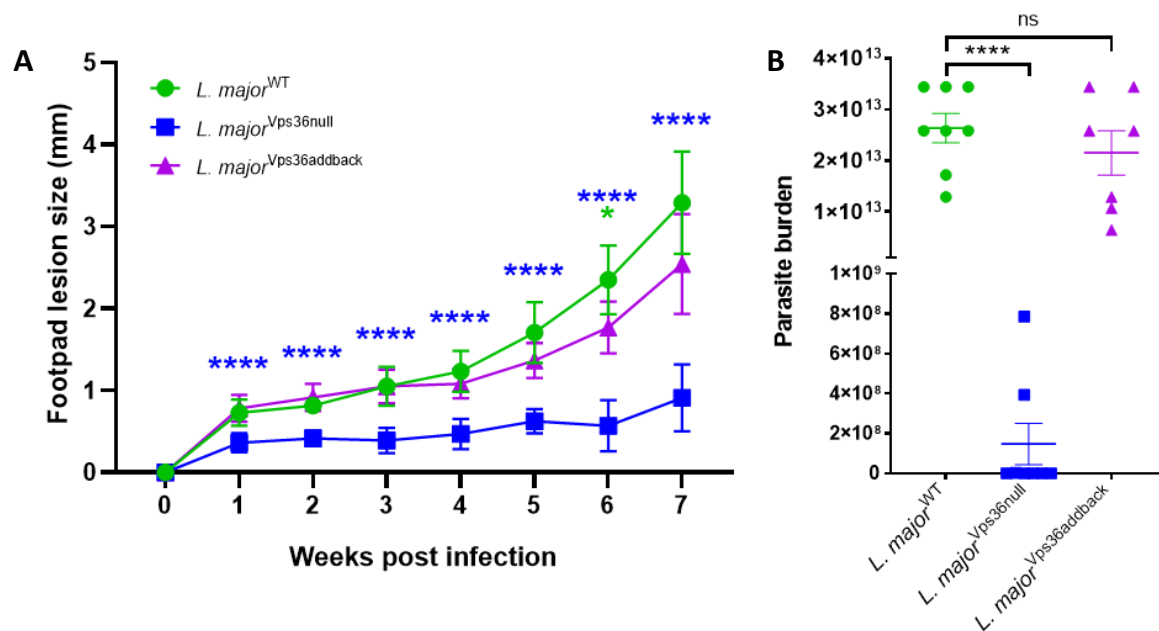


Figure 8. WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* infectivity was assessed using subcutaneous Balb/c mice footpad infections. Infections were performed with stationary promastigotes alone. *Vps36* *L. major* exhibited similar levels of virulence compared to WT *L. major*, whereas *Vps36*<sup>(-/-)</sup> *L. major* caused significantly reduced footpad swelling (A) and footpad parasite burden (B) as expected. Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=10$ .

*Vps36*<sup>(-/-)</sup> *L. major* parasites struggle to maintain BMDM infection in vitro, but *Vps36*<sup>(-/-)</sup> *L. major* EVs enhance *L. major* infectivity similarly to WT *L. major* and *Vps36* *L. major* EVs

To further validate the results from our in vivo infection experiments, we infected Balb/c mice IP with WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* stationary parasites and performed an IP lavage after 6 hours of infection to assess cell recruitment and infection. We observed no significant differences in cell recruitment between WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* IP infections (Figure 9A), but did find *Vps36*<sup>(-/-)</sup> *L. major* parasites infected fewer cells after 6 hours, especially neutrophils, which we found a significant reduction in infection rate (Figure 9B). After collecting the IP lavage cells, we further cultured these cells in DMEM and allowed the infection to persist for up to 72 hours. We found that

even by 24 hours post-lavage, Vps36<sup>(-/-)</sup> *L. major* infection had already mostly been cleared by the IP cells (Figure 9C). It should be noted that after culturing the IP cells, neutrophils were not maintained well in the culture media and mostly macrophages/monocytes remained. This reduced Vps36<sup>(-/-)</sup> *L. major* infection can also be observed in the dwindling number of Vps36<sup>(-/-)</sup> *L. major* amastigotes found in infected IP cells (Figure 9D). We also performed in vitro macrophage infections using C57BL6 BMDMs with stationary *L. major*, comparing WT *L. major*, Vps36<sup>(-/-)</sup> *L. major* and Vps36 *L. major* in vitro infectivity and establishing a timeline of infection. Like the IP infections, we found that although there is a noticeable but not significant reduction in Vps36<sup>(-/-)</sup> *L. major* macrophage infection rate during the initial infection period (3 hours), it was really 24-hours post-infection and beyond that demonstrated the remarkable loss of infectivity of Vps36<sup>(-/-)</sup> *L. major* parasites compared to WT *L. major* and Vps36 *L. major* parasites, with almost complete clearance achieved by 48-hours post-infection (Figure 10A). This was accompanied by the same reduction in amastigotes per infected cell, suggesting a loss of fitness in Vps36<sup>(-/-)</sup> *L. major* parasites during infection (Figure 10B). Despite these promising results, repeating the in vivo mice footpad infections comparing the infectivity of WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* EVs when co-inoculating with WT *L. major* promastigotes (Figure 10C). This combined with previous footpad infections comparing the parasites alone suggests a defect in the Vps36<sup>(-/-)</sup> *L. major* parasites themselves that is the cause of the observed reduction in virulence.

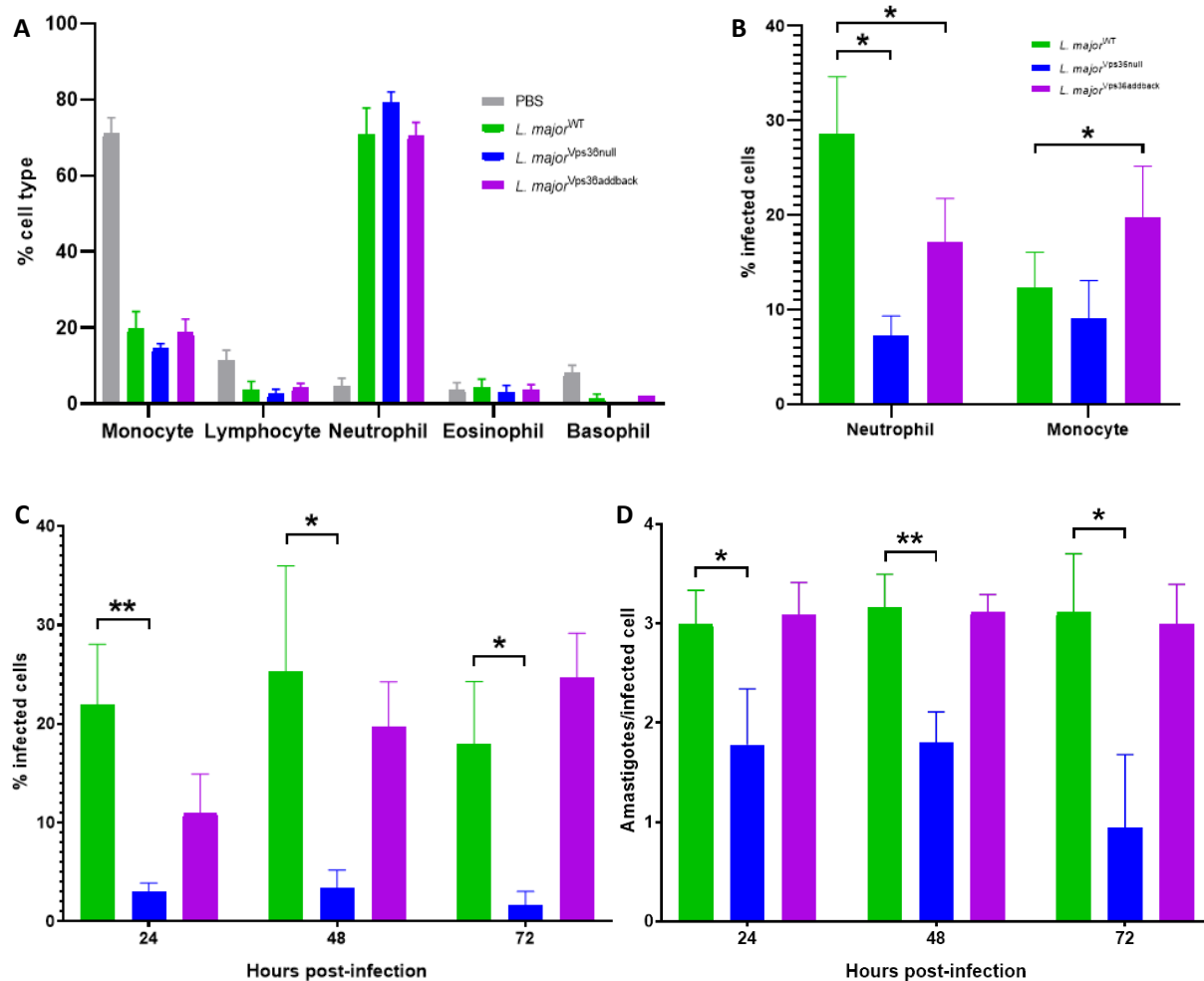


Figure 9. WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* infectivity was also assessed using Balb/c IP infections. After a 6-hour infection with stationary promastigotes alone, IP cells were collected by lavage, stained by diff-quick, and counted/differentiated (A). While there was no significant difference in cell recruitment between WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* infection, significantly more neutrophils were infected by WT *L. major* compared to *Vps36*<sup>(-/-)</sup> *L. major* and *Vps36* *L. major*, while significantly more monocytes were infected by *Vps36* *L. major* (B). After plating IP cells, the infection caused by *Vps36*<sup>(-/-)</sup> *L. major* continued to remain low when considering both percent infection (C) and amastigotes per infected cell (D). Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=6$ .

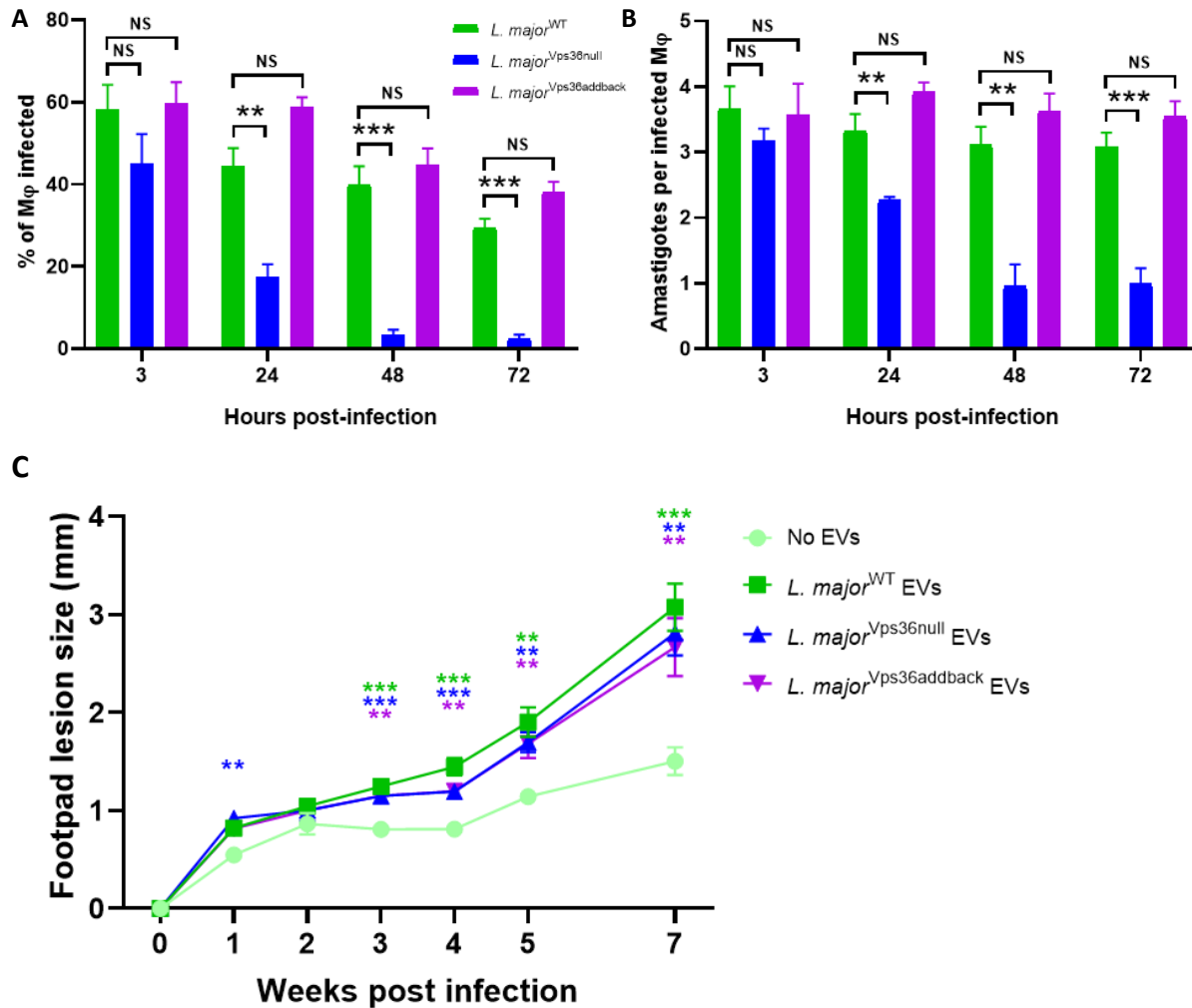


Figure 10. WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* infectivity was also assessed using BMDMs. While initial infection (3-hours) was similar between the different parasites, *Vps36*<sup>(-/-)</sup> *L. major* infection dropped significantly 24-, 48-, and 72- hours post-infection compared to WT *L. major* and *Vps36* *L. major* with regards to both percent infection (A) and amastigotes per infected cell (B). Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=6$ .

*Vps36* disruption does not significantly impact *L. major* starvation resistance, but severely limits amastigote transformation

From our proteomic data, it made sense to investigate potential metabolic deficiencies of *Vps36*<sup>(-/-)</sup> *L. major*. To assess parasite resistance to starvation, a necessary metabolic

adaptation *Leishmania* parasites undergo when transforming from a promastigote to amastigote form, an MTT assay was used to determine parasite viability after PBS starvation (39, 40). Disappointingly, only Vps36 *L. major* promastigotes demonstrated any significant variation, possessing seemingly higher resistance to starvation. However, this increased resistance was not significant during the whole course of the starvation, with WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* all exhibiting relatively similar levels of survival over the course of 5 hours of PBS starvation when assessing by raw OD or by percent survival (Figure 11A, B). Instead, it seems to be the transformative capabilities of Vps36<sup>(-/-)</sup> *L. major* parasites that have been impaired by Vps36 disruption. Under transformation conditions, Vps36<sup>(-/-)</sup> *L. major* promastigotes failed to transform into amastigotes or indeed grow at all (Figure 12A). Furthermore, unlike with previous experiments, we found that Vps36 *L. major* also did not return to WT *L. major* levels of growth/transformation. We confirmed this by purifying amastigotes at day 15 of the differentiation cultures using a ficoll density gradient, showing that the Vps36<sup>(-/-)</sup> *L. major* culture resulted in significantly fewer axenic amastigotes and Vps36 *L. major* showed only a partial return to WT levels (Figure 12B).

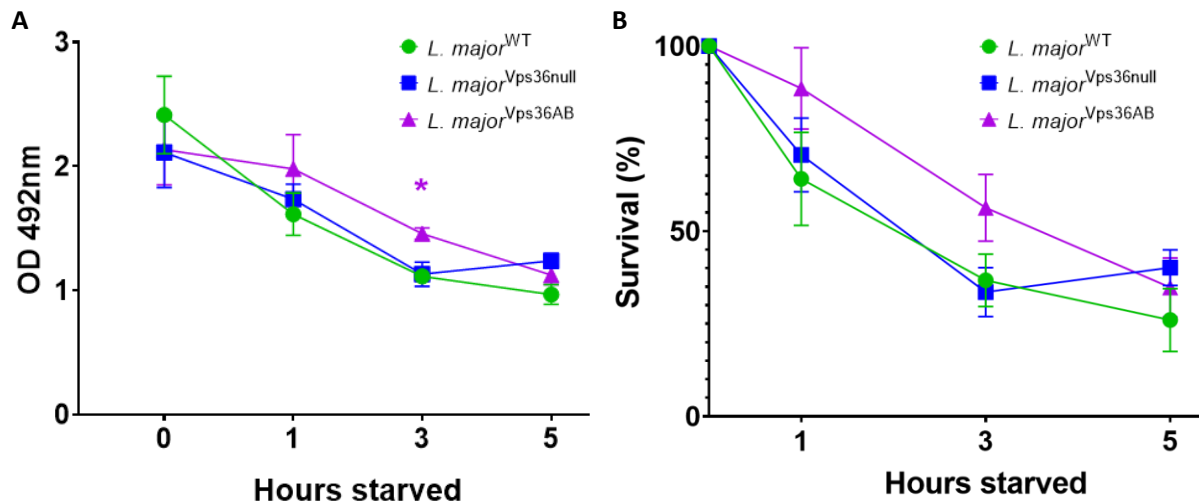


Figure 11. WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* PBS starvation was assessed using an MTT assay. *L. major* starvation resistance was largely unaffected by Vps36 manipulation, though Vps36 *L. major* demonstrated potentially higher resistance. Overall differences were nonsignificant when measuring by adjusted OD (A) or with computed percent survival (B). Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=6$ .

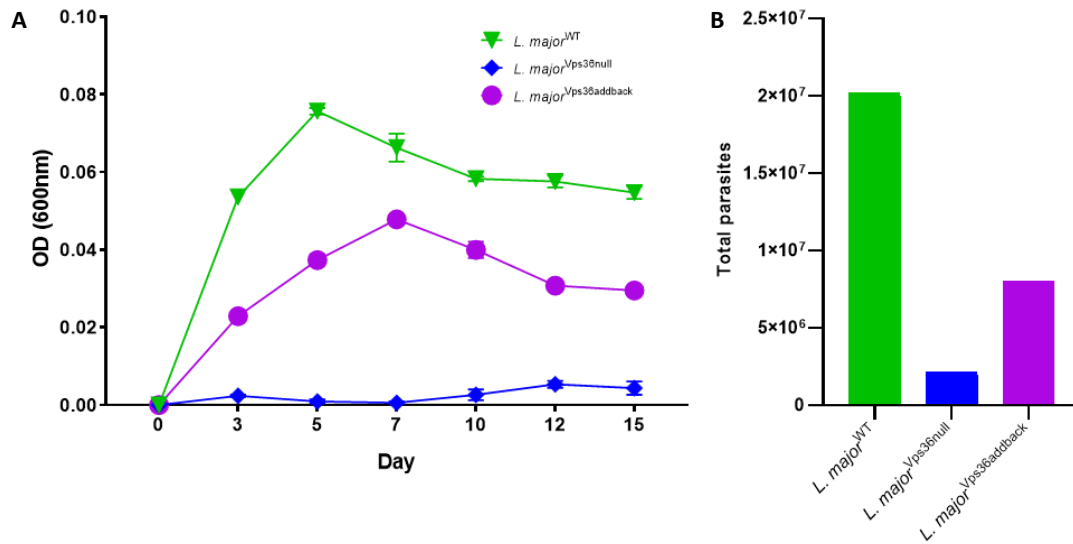


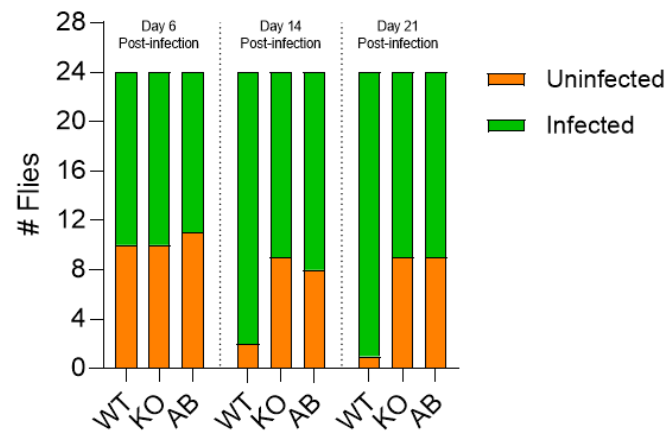
Figure 12. WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* amastigote transformation was assessed over 15 days. *Vps36*<sup>(-/-)</sup> *L. major* log phase promastigotes failed to grow in transformation conditions, while even *Vps36* *L. major* demonstrated significantly lower growth compared to WT *L. major* (A). Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=3$ . This resulted in fewer collected amastigotes as well (B).

### *Vps36* disruption negatively impacts *L. major* survivability in sandflies and metacyclogenesis

Concurrently with our amastigote transformation experiments, WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* parasites were also used to infect sandflies to understand the effects of *Vps36* disruption in true in vivo experiments. Unlike with our in vivo and in vitro mice experiments, we found that both *Vps36*<sup>(-/-)</sup> *L. major* and *Vps36* *L. major* parasites infected sandflies at a lower level compared to WT *L. major* (Figure 13A). This was true when look at the number of flies infected as well as the overall parasite burden in infected flies (Figure 13B). Another indicator of impeded parasite function within the sandfly midgut is the significantly reduced metacyclogenesis of *Vps36*<sup>(-/-)</sup> *L. major* and *Vps36* *L. major* parasites (Figure 13C). While *Vps36* *L. major* metastatic promastigotes could be identified in sandfly midguts at 14 days post-infection and were present at a significantly higher quantity compared to *Vps36*<sup>(-/-)</sup> *L. major*, these mostly disappeared by 21 days post-infection and *Vps36*<sup>(-/-)</sup> *L. major* and *Vps36* *L. major* metacyclogenesis was comparable.

A

### Infection Frequency of *L. major* SD in *P. duboscqi* -MA



B

### % Metacyclic Stage - *L. major* SD in *P. duboscqi* -MA (Infected Flies Only)

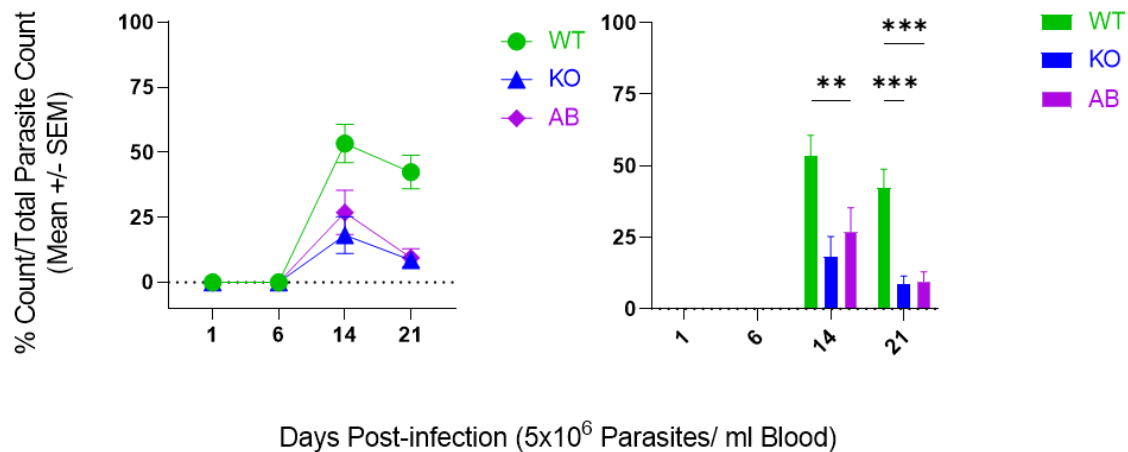


Figure 13. WT *L. major*, *Vps36*<sup>-/-</sup> *L. major*, and *Vps36* *L. major* *in vivo* viability was tested by infecting *Phlebotomus duboscqi* sandflies. While midgut infection rate was comparable between parasites at 6 days post-infection, both *Vps36*<sup>-/-</sup> *L. major* and *Vps36* *L. major* demonstrated reduced infection compared to WT *L. major* 14- and 21-days post-infection (A). Furthermore, *Vps36*<sup>-/-</sup> *L. major* and *Vps36* *L. major* demonstrated significantly reduced metacyclogenesis compared to WT *L. major* (B). Significance was calculated by multiple T-test,  $P=0.05$ ,  $n=3$ .

## Discussion

When deciding the methodology for inhibiting EV production/release in *Leishmania*, we decided to target Vps36 due to it appearing in some previous *Leishmania* EV proteomic data and many studies performed with other eukaryotes showing the efficacy of targeting Vps36 to prevent ESCRTII formation and completely abrogate EV production (41-43). Additionally, a Vps36 analog in *Trypanosoma brucei* has been previously targeted and demonstrated EV abrogation, as well as inhibiting *T. brucei* social motility (30). However, our initial results made it immediately clear that Vps36 disruption in *L. major* does not entirely prevent EV production. It is unclear if this is due to a failure to completely disrupt the function of the Vps36 protein itself or if there are more robust compensatory EV production mechanisms in *L. major*. Despite this, Vps36 disruption did significantly reduce sEV secretion by Vps36<sup>(-/-)</sup> *L. major*. Vps36 disruption also greatly altered the protein contents of Vps36<sup>(-/-)</sup> *L. major* EVs whereas WP protein content remained unchanged. This is consistent with reports on the role of ESCRTII in protein sorting to the MVB (44). Upon closer inspection, we found that the ESCRT protein Vps4, while still present, was greatly reduced in Vps36<sup>(-/-)</sup> *L. major* EVs compared to WT *L. major* EVs. Vps4 is part of the ESCRT III complex which is responsible for the abscission of the MVB to form intraluminal vesicles, which eventually become exosomes (22). The significant reduction of vesicular Vps4 in Vps36<sup>(-/-)</sup> *L. major* further lends support for the induction of EV dysfunction in Vps36<sup>(-/-)</sup> *L. major* as a result of Vps36 disruption.

To fully assess the impact of Vps36 disruption on *L. major* infectivity, a Balb/c mice footpad infection model was used. Balb/c mice are typically susceptible to cutaneous leishmaniasis due to them possessing a Th2-biased immune response, which will make any loss in parasite infectivity immediately apparent (45, 46). Despite this susceptibility, we found that Vps36<sup>(-/-)</sup> *L. major* parasites failed to induce significant footpad inflammation, especially compared to the lesions caused by WT *L. major* infection. However, we found that the co-injection of Vps36<sup>(-/-)</sup> *L. major* parasites with WT *L. major* EVs did not restore parasite infectivity. We expected that the addition of WT EVs would sufficiently restore infectivity due to the important role of *Leishmania* EVs during early infection, yet the lack of augmentation

observed flew in the face of these expectations (16). Indeed, further experiments failed to demonstrate a difference in infection when WT *L. major* parasites were co-injected with EVs from WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* parasites (Figure 10C). This suggests that a mechanism other than EV dysfunction is the major cause of the significant loss of *L. major* infectivity. Furthermore, Vps36 addback was sufficient in restoring both EV release and *L. major* infectivity both in vivo and in vitro, demonstrating that both these properties are related to Vps36 function.

Based on the proteomic analysis results, especially noting the absence of a cluster of tricarboxylic acid cycle (TCA)-related proteins from Vps36<sup>(-/-)</sup> *L. major* EVs, parasite metabolism dysfunction seemed like a likely culprit for the loss of infectivity. This was further reinforced by the return of some of these proteins in Vps36 *L. major* EVs like malate and succinate dehydrogenase. A necessary metabolic quiescence occurs upon amastigote transformation, and it has been previously noted that Vps4 disruption can lead to increased susceptibility to starvation (29). However, our metabolomic analysis and other experiments testing WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* metabolic differences yielded no significant results. Because these experiments were all performed using the promastigote forms of the parasites and Vps36<sup>(-/-)</sup> *L. major* parasites consistently demonstrated severely abrogated infectivity and viability during infection, it made sense to next assess the amastigote forms of WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major*. Surprisingly, while attempting to generate axenic amastigotes, we discovered that Vps36<sup>(-/-)</sup> *L. major* parasites were almost completely unable to transform into an axenic amastigote form compared to WT *L. major*, aligning with how Vps4 disruption also impacts *L. mexicana* transformation and metacyclogenesis (29). We repeated the amastigote transformation process using proper experimental parameters by culturing our parasites in axenic amastigote transformation conditions and discovered significant impairment of amastigote transformation in Vps36<sup>(-/-)</sup> *L. major*, with almost no Vps36<sup>(-/-)</sup> *L. major* being collected at experiment endpoint. Surprisingly, Vps36 addback was insufficient to fully restore amastigote transformation. This result was reinforced in subsequent sandfly infections using *Phlebotomus duboscqi*, where Vps36<sup>(-/-)</sup> *L. major* development was significantly impaired in the sandfly midgut resulting in

negligible metacyclogenesis. In this case, Vps36 addback was ineffective at restoring metacyclogenesis, although it is unclear if this is due to loss of the Vps36 addback element from lack of antibiotic pressure within the sandfly.

In conclusion, *Vps36* disruption greatly impacts regular EV machinery of *L. major*, significantly reducing sEV release and altering EV protein cargo. However, observed reductions in *L. major* infectivity are likely caused by impaired amastigote transformation and metacyclogenesis rather than being a product of the observed EV disfunction. In addition, *Vps36* disruption did not cause any variations in EV sRNA content nor did it have any metabolic consequences on the parasites themselves. Through each infection model, *Vps36*<sup>(-/-)</sup> *L. major* infection remained low at all timepoints of each experiment, suggesting its use as an attenuated strain. In the future, we would like to further investigate *Vps36*<sup>(-/-)</sup> *L. major* metabolism and expand our metabolomic analysis to confirm if there is no metabolic impact. It is also of utmost importance to conduct a truly in vivo infection experiment with infected *P. duboscqi* sandflies and Balb/c mice.

## Conflict of interest

MO is supported by the Canadian Institutes of Health Research (CIHR, grant number PJT-159765) and The Natural Sciences and Engineering Research Council of Canada (NSERC). GD was supported by a MSc studentship from Fonds de Recherche du Québec en Santé (FRQS) and the Canada Graduate Scholarships Doctoral Award (CGS-D) provided by the CIHR. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author contributions

The project was conceptualized by MO, GD, and CFP. All experiments and analysis, including the EV purifications, NTA analysis, microBCA, TEM imaging, proteomic analysis, *in vitro* macrophage infections, and *in vivo* mice infections, were performed by GD. Mice footpad measurements were performed with support from MO. Vps36 addback and PCR confirmation was performed by AC. RNA sequencing and analysis was performed by DL. Sandfly experiments and dissections were performed by GG in the lab of NP. All figures were generated by GD except figure 4, which was generated by LM, and figure 13, which was generated by GG.

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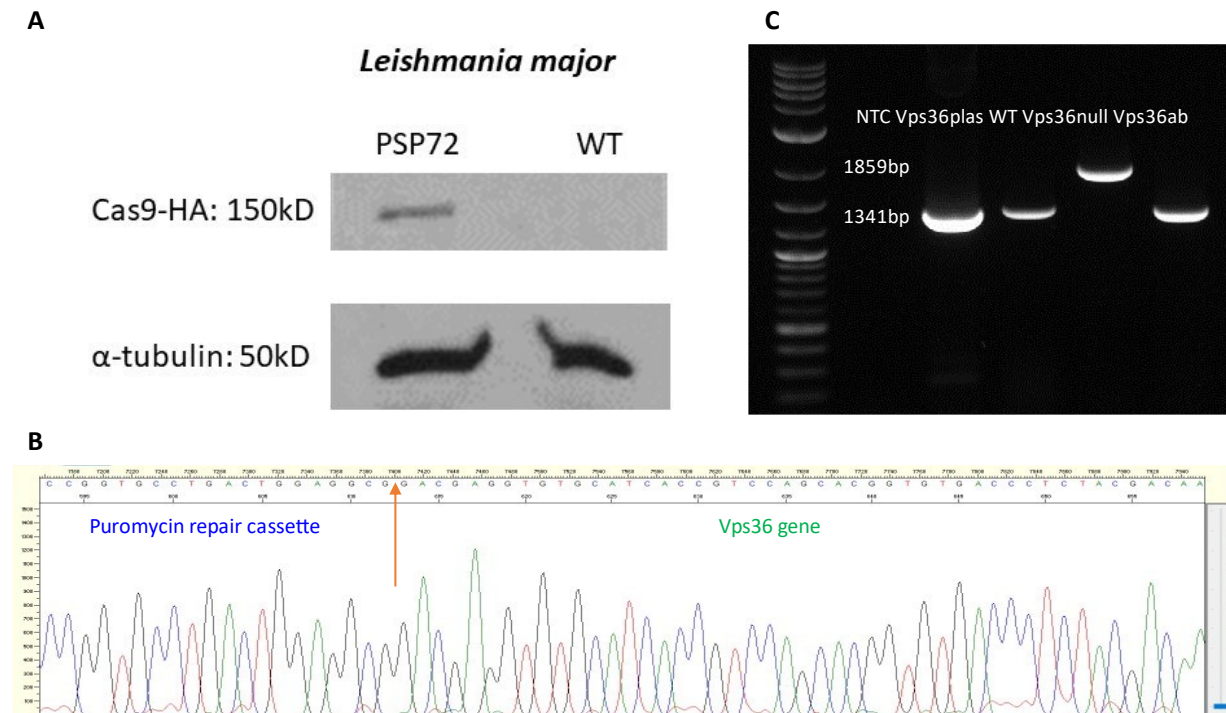
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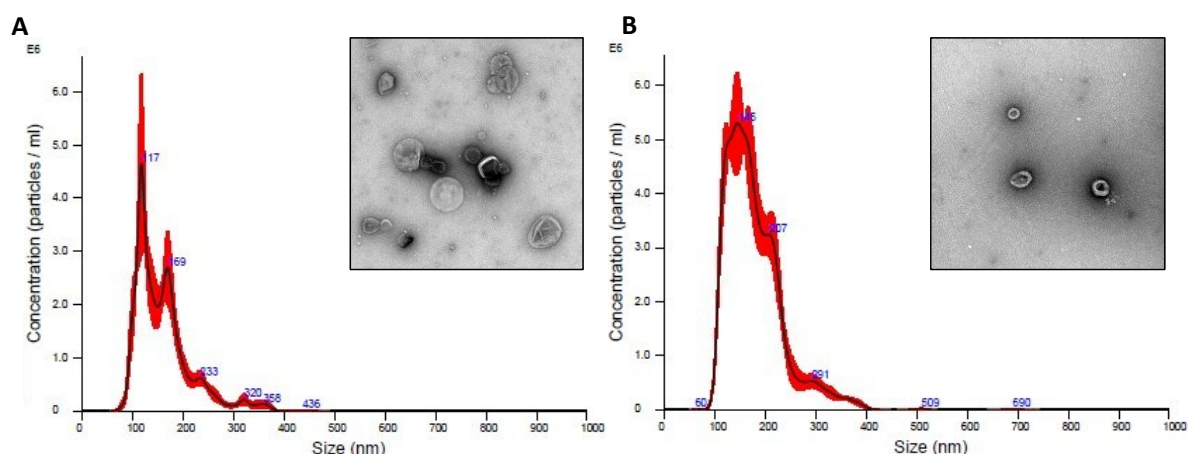
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## Supplemental figures and tables



**Supplemental Figure 1. Verification steps performed to confirm successful *Vps36* disruption and addback.** After the transfection of the Cas9-pSP72 vector into *L. major*, Cas9 expression was verified by western blot (A). α-tubulin was used as a loading control with rabbit anti-HA polyclonal antibody and rabbit anti-α-tubulin antibody being used. *Vps36*<sup>(-/-)</sup> *L. major* genomic DNA was extracted and Sanger sequencing was used with primers against the whole *Vps36* exon to verify *Vps36* disruption (B). The red arrow indicates the site of the CRISPR/Cas9-mediated nick, with the preceding codons belonging to the 3' terminus of the puromycin resistance gene and the proceeding codons belonging to the *Vps36* exon. To confirm successful *Vps36* addback, WT *L. major*, *Vps36*<sup>(-/-)</sup> *L. major*, and *Vps36* *L. major* DNA was extracted and PCR was performed using primers targeting *Vps36* (C). 1341bp indicates the size of the *Vps36* fragment, while 1859bp indicates the addition of the puromycin resistance gene.



*Supplemental Figure 2. Examples of typical particle distribution graphs generated by the NTA software provided by Malvern Panalytical as well as the typical appearance of L. major EVs resulting from TEM. EVs from WT L. major are displayed on the left (A) and Vps36<sup>(-/-)</sup> L. major EVs on the right (B).*

| Simplified protein name                       | Uniprot accession | Molecular weight | Vps36<br><i>L. major</i><br>EVs<br>average<br>spectral<br>count | Vps36<br><i>L. major</i><br>EVs SEM<br>spectral<br>count | Vps36 <sup>(-/-)</sup><br><i>L. major</i><br>EVs<br>average<br>spectral<br>count | Vps36 <sup>(-/-)</sup><br><i>L. major</i><br>EVs SEM<br>spectral<br>count | WT<br><i>L. major</i><br>EVs<br>average<br>spectral<br>count | WT<br><i>L. major</i><br>EVs SEM<br>spectral<br>count |
|-----------------------------------------------|-------------------|------------------|-----------------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------|-------------------------------------------------------|
| Leishmanolysin                                | GP63_LEIMA        | 64 kDa           | 434                                                             | 72                                                       | 343.3333                                                                         | 102.4212                                                                  | 415.6667                                                     | 134.0713                                              |
| Putative heat-shock protein hsp70             | Q4Q7Y4_LEIMA      | 72 kDa           | 96.5                                                            | 3.5                                                      | 121                                                                              | 28.47806                                                                  | 139.3333                                                     | 24.12698                                              |
| Elongation factor 1-alpha                     | Q4QE19_LEIMA      | 49 kDa           | 117                                                             | 9                                                        | 110                                                                              | 15.6205                                                                   | 121                                                          | 20.51828                                              |
| Plasma membrane ATPase                        | Q4QDN8_LEIMA      | 107 kDa          | 89.5                                                            | 0.5                                                      | 91.66667                                                                         | 12.78454                                                                  | 86.33333                                                     | 24.91541                                              |
| Glyceraldehyde-3-phosphate dehydrogenase      | Q4Q6Z5_LEIMA      | 39 kDa           | 35.5                                                            | 2.5                                                      | 86.33333                                                                         | 30.8671                                                                   | 93                                                           | 15.04438                                              |
| Tubulin alpha chain                           | Q4QGC5_LEIMA      | 50 kDa           | 79                                                              | 19                                                       | 80.66667                                                                         | 17.74198                                                                  | 58.33333                                                     | 11.55182                                              |
| Putative glucose-regulated protein 78         | A4I3J9_LEIIN      | 72 kDa           | 53.5                                                            | 0.5                                                      | 76.66667                                                                         | 25.36621                                                                  | 75.33333                                                     | 20.03608                                              |
| Elongation factor 2                           | Q4Q259_LEIMA      | 94 kDa           | 65                                                              | 4                                                        | 67.33333                                                                         | 18.66667                                                                  | 69                                                           | 18.17507                                              |
| Putative surface antigen protein              | Q4QGL8_LEIMA      | 65 kDa           | 47                                                              | 6                                                        | 59.33333                                                                         | 14.31006                                                                  | 79                                                           | 13.79613                                              |
| Peroxidoxin                                   | Q9TZS4_LEIMA      | 22 kDa           | 65                                                              | 15                                                       | 54.33333                                                                         | 8.212457                                                                  | 65.66667                                                     | 4.977728                                              |
| Tubulin beta chain                            | E9B3R4_LEIMU      | 55 kDa           | 72.5                                                            | 8.5                                                      | 56.66667                                                                         | 19.67514                                                                  | 51.66667                                                     | 8.089774                                              |
| Uncharacterized protein                       | Q4Q8Z6_LEIMA      | 90 kDa           | 76.5                                                            | 6.5                                                      | 35.33333                                                                         | 17.37175                                                                  | 56.33333                                                     | 12.71919                                              |
| Phosphopyruvate hydratase                     | Q4QFL8_LEIMA      | 46 kDa           | 70.5                                                            | 4.5                                                      | 48                                                                               | 10.58301                                                                  | 55.33333                                                     | 11.56623                                              |
| 40S ribosomal protein S4                      | Q4QG31_LEIMA      | 31 kDa           | 19.5                                                            | 7.5                                                      | 72                                                                               | 14.84363                                                                  | 63.33333                                                     | 9.333333                                              |
| ATP synthase subunit beta                     | Q4Q9X6_LEIMA      | 56 kDa           | 79                                                              | 2                                                        | 46.33333                                                                         | 0                                                                         | 42.66667                                                     | 13.86042                                              |
| Putative long chain fatty Acyl CoA synthetase | E9ACH0_LEIMA      | 79 kDa           | 51                                                              | 4                                                        | 45.66667                                                                         | 10.26861                                                                  | 53.33333                                                     | 11.86498                                              |
| Uncharacterized protein                       | E9ADF0_LEIMA      | 38 kDa           | 38                                                              | 4                                                        | 53.33333                                                                         | 9.597453                                                                  | 56.33333                                                     | 12.19745                                              |
| Putative 60S ribosomal protein L10            | O97213_LEIMA      | 25 kDa           | 0                                                               | 0                                                        | 56                                                                               | 6.506407                                                                  | 50.33333                                                     | 4.977728                                              |

|                                                    |              |         |      |      |          |          |          |          |
|----------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Metallo-peptidase, Clan ME, Family M16             | E9AEW1_LEIMA | 55 kDa  | 71.5 | 18.5 | 32.33333 | 18.36966 | 41.66667 | 11.1704  |
| Putative ribosomal protein L3                      | Q4Q2Q6_LEIMA | 47 kDa  | 34.5 | 9.5  | 50.66667 | 11.05039 | 50.33333 | 11.85093 |
| Probable sodium stibogluconate resistance protein  | Q9BHE5_LEIMA | 69 kDa  | 34.5 | 5.5  | 42       | 14.1892  | 54       | 10.59874 |
| 40S ribosomal protein S3a                          | RS3A_LEIMA   | 30 kDa  | 46   | 19   | 49.33333 | 5.696002 | 41.33333 | 5.044249 |
| Heat shock protein 83-1                            | Q4Q4I6_LEIMA | 81 kDa  | 38.5 | 3.5  | 36       | 7.371115 | 50.66667 | 8.685876 |
| Putative 40S ribosomal protein S3                  | Q4Q4A0_LEIMA | 24 kDa  | 0    | 0    | 60.33333 | 10.68228 | 55       | 9.814955 |
| 40S ribosomal protein S7                           | E9AC32_LEIMA | 24 kDa  | 12.5 | 3.5  | 56       | 11.23981 | 40.33333 | 10.86789 |
| Putative 60S ribosomal protein L13a                | A4HWP9_LEIIN | 25 kDa  | 34.5 | 14.5 | 43.33333 | 11.14052 | 45.33333 | 8.950481 |
| H(+)-exporting diphosphatase                       | Q4Q6E1_LEIMA | 84 kDa  | 38.5 | 4.5  | 32.66667 | 11.1704  | 50.33333 | 9.83757  |
| Putative surface antigen protein 2                 | Q4QGJ0_LEIMA | 67 kDa  | 0    | 0    | 30.33333 | 16.33333 | 43.66667 | 7.310571 |
| ATP-dependent 6-phosphofructokinase                | E9AEB3_LEIMA | 54 kDa  | 11.5 | 5.5  | 39.66667 | 20.21825 | 60       | 15.27525 |
| 40S ribosomal protein S6                           | A4HZI4_LEIIN | 28 kDa  | 26   | 8    | 48.66667 | 11.09554 | 39.66667 | 7.688375 |
| Pyruvate, phosphate dikinase                       | Q4QGX9_LEIMA | 101 kDa | 15   | 3    | 39       | 22.50185 | 55.33333 | 22.69606 |
| Protein disulfide-isomerase                        | Q4Q059_LEIMA | 52 kDa  | 28   | 5    | 37.66667 | 18.22392 | 40.66667 | 9.905105 |
| Uncharacterized protein                            | Q4QB61_LEIMA | 16 kDa  | 53.5 | 10.5 | 31       | 8.504901 | 28       | 6.506407 |
| Putative cytoskeleton-associated protein CAP5.5    | Q4Q6L9_LEIMA | 80 kDa  | 41   | 8    | 30.33333 | 11.83685 | 37.33333 | 11.62373 |
| Putative ATPase alpha subunit                      | E8NHQ7_LEIMU | 63 kDa  | 49.5 | 3.5  | 29       | 16.09348 | 37.33333 | 11.05039 |
| Putative 40S ribosomal protein S9                  | Q4Q1V1_LEIMA | 22 kDa  | 17   | 2    | 42.33333 | 6.936217 | 40.66667 | 5.364492 |
| Putative heat shock protein                        | Q4QDQ2_LEIMA | 92 kDa  | 35   | 8    | 26       | 13.20353 | 39       | 10.14889 |
| 60S ribosomal protein L18a                         | Q4Q5J4_LEIMA | 21 kDa  | 14   | 5    | 43.66667 | 10.26861 | 36.33333 | 4.371626 |
| Putative ribosomal protein L1a                     | E9ADW5_LEIMA | 41 kDa  | 25.5 | 12.5 | 36       | 4.041452 | 32       | 6.658328 |
| Uncharacterized protein                            | Q4QIK4_LEIMA | 245 kDa | 15.5 | 11.5 | 51.33333 | 0        | 20.33333 | 0        |
| ADP/ATP translocase                                | Q4QDK0_LEIMA | 35 kDa  | 37.5 | 5.5  | 27.66667 | 13.24554 | 35       | 6.027714 |
| Glucose transporter, Imgt2                         | Q4Q0D1_LEIMA | 61 kDa  | 35   | 0    | 31.66667 | 4.255715 | 25.66667 | 8.819171 |
| 40S ribosomal protein S2                           | Q4Q5P0_LEIMA | 29 kDa  | 10   | 2    | 39.66667 | 8.743251 | 34       | 9.848858 |
| Putative 60S ribosomal protein L7                  | Q4Q9H4_LEIMA | 29 kDa  | 15   | 3    | 36.33333 | 5.364492 | 34       | 3.785939 |
| Putative Transitional endoplasmic reticulum ATPase | Q4Q1T9_LEIMA | 87 kDa  | 14.5 | 1.5  | 33       | 7.211103 | 33.66667 | 16.75642 |
| Putative 60S ribosomal protein L7a                 | A4HT77_LEIIN | 37 kDa  | 16.5 | 8.5  | 37.66667 | 7.055337 | 28.33333 | 3.382964 |
| Phosphotransferase                                 | Q4VT70_LEIMA | 52 kDa  | 14   | 4    | 36.66667 | 0        | 31       | 0        |
| Peptidyl-prolyl cis-trans isomerase                | O02614_LEIMA | 19 kDa  | 28   | 1    | 29.33333 | 4.409586 | 29       | 4.041452 |
| Heat shock protein 70-related protein              | Q4Q970_LEIMA | 71 kDa  | 34   | 6    | 20.33333 | 6.385748 | 29       | 7.234178 |
| Nucleoside diphosphate kinase                      | Q9GP00_LEIIN | 17 kDa  | 28.5 | 6.5  | 28.33333 | 8.11035  | 30       | 5.131601 |
| Putative 40S ribosomal protein L14                 | Q4QBJ1_LEIMA | 20 kDa  | 24.5 | 4.5  | 30.66667 | 5.547772 | 27.33333 | 2.333333 |
| Nodulin-like domain-containing protein             | E9AE09_LEIMA | 74 kDa  | 31   | 1    | 30.66667 | 9.279607 | 21       | 7.211103 |
| 60S ribosomal protein L11 (L5, L16)                | A4HS42_LEIIN | 22 kDa  | 22.5 | 10.5 | 29       | 5.131601 | 29.33333 | 5.487359 |
| Putative surface antigen protein                   | Q4QGI0_LEIMA | 54 kDa  | 0    | 0    | 0        | 0        | 25.33333 | 12.78454 |
| ATP pyrophosphate-lyase                            | Q4QEI3_LEIMA | 151 kDa | 13   | 5    | 35       | 0        | 24.66667 | 11.68094 |
| Clathrin heavy chain                               | Q4Q1R2_LEIMA | 190 kDa | 20.5 | 5.5  | 30       | 10.2632  | 19.66667 | 7.333333 |
| Metallo-peptidase, Clan ME, Family M16             | Q4QG67_LEIMA | 58 kDa  | 44   | 21   | 17.33333 | 0        | 22.66667 | 9.207485 |

|                                                       |              |         |      |     |          |          |          |          |
|-------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| PDZ domain-containing protein                         | Q4Q7P2_LEIMA | 80 kDa  | 22   | 4   | 24.33333 | 9.404491 | 29.33333 | 11.83685 |
| 40S ribosomal protein S8                              | RS8_LEIMA    | 25 kDa  | 22.5 | 1.5 | 29.66667 | 6.119187 | 24.66667 | 4.977728 |
| 60S ribosomal protein L18                             | RL18_LEIMA   | 22 kDa  | 0    | 0   | 34.66667 | 4.630815 | 30       | 1.527525 |
| Putative 60S ribosomal protein L21                    | Q4Q2H9_LEIMA | 18 kDa  | 19.5 | 8.5 | 28       | 6.244998 | 26.66667 | 3.179797 |
| Putative folate/biopterin transporter                 | Q4QHI0_LEIMA | 75 kDa  | 15   | 3   | 32.66667 | 9.171211 | 23.66667 | 6.17342  |
| DUF1935 domain-containing protein                     | Q4QCR9_LEIMA | 20 kDa  | 21.5 | 1.5 | 28       | 9.165151 | 23.66667 | 4.666667 |
| Uncharacterized protein L7836.08                      | Q9U0V4_LEIMA | 63 kDa  | 15   | 3   | 24.66667 | 9.171211 | 31.66667 | 6.887993 |
| Metallo-peptidase, Clan ME, Family M16                | Q4QCI1_LEIMA | 51 kDa  | 46   | 24  | 14       | 0        | 17       | 6.928203 |
| Putative 40S ribosomal protein S16                    | Q4Q9A5_LEIMA | 17 kDa  | 0    | 0   | 31       | 4.932883 | 29.33333 | 5.783117 |
| Putative calpain-like cysteine peptidase              | E9AD27_LEIMA | 700 kDa | 7    | 1   | 41.33333 | 0        | 11.33333 | 3.333333 |
| Putative small myristoylated protein 4                | Q5SDH3_LEIMA | 17 kDa  | 21.5 | 2.5 | 25.66667 | 4.977728 | 24.33333 | 4.371626 |
| Putative glycosomal phosphoenolpyruvate carboxykinase | E9ADF9_LEIMA | 58 kDa  | 6    | 2   | 24       | 0        | 35       | 10.53565 |
| Putative 60S ribosomal protein L9                     | Q4Q6V5_LEIMA | 22 kDa  | 7.5  | 1.5 | 30       | 5.131601 | 26       | 3.05505  |
| Putative polyubiquitin                                | A4HPM0_LEIBR | 111 kDa | 13   | 2   | 32       | 9.609024 | 20.33333 | 6.69162  |
| Putative calpain-like cysteine peptidase              | Q5SDH5_LEIMA | 15 kDa  | 23   | 2   | 26       | 4.582576 | 22       | 2.081666 |
| Glycosomal membrane protein                           | E9AFJ4_LEIMA | 25 kDa  | 7.5  | 0.5 | 21       | 0        | 36       | 16.70329 |
| GMP reductase                                         | GMPR_LEIMA   | 52 kDa  | 0    | 0   | 21.66667 | 0        | 37       | 12.09683 |
| Histone H4                                            | A4HW98_LEIIN | 11 kDa  | 13.5 | 1.5 | 21       | 3.605551 | 26.66667 | 2.027588 |
| Uncharacterized protein                               | Q4Q9C8_LEIMA | 41 kDa  | 10.5 | 3.5 | 29       | 16.62328 | 25.33333 | 6.565905 |
| Phosphoglycerate kinase                               | E9AGU0_LEIIN | 45 kDa  | 14   | 1   | 20.66667 | 7.535103 | 29       | 4.041452 |
| Kinesin-like protein                                  | Q4QDJ5_LEIMA | 93 kDa  | 21.5 | 8.5 | 15.66667 | 8.293237 | 28.33333 | 8.293237 |
| Histone H2A                                           | E9AE33_LEIMA | 14 kDa  | 25   | 13  | 22.66667 | 3.382964 | 19.66667 | 1.20185  |
| 40S ribosomal protein S19-like protein                | E9AEE8_LEIMA | 18 kDa  | 15   | 3   | 26.33333 | 4.484541 | 24       | 4.041452 |
| Putative ribonucleoprotein p18, mitochondrial         | Q4QFF2_LEIMA | 21 kDa  | 14.5 | 2.5 | 20.33333 | 0        | 26.66667 | 8.875685 |
| Inosine-5'-monophosphate dehydrogenase                | Q4QD53_LEIMA | 56 kDa  | 0    | 0   | 20.33333 | 0        | 36.66667 | 14.42606 |
| Uncharacterized protein                               | Q4Q9N7_LEIMA | 33 kDa  | 16   | 1   | 19.66667 | 7.753136 | 26.33333 | 8.373238 |
| Cytochrome c oxidase subunit IV                       | Q4QGM6_LEIMA | 39 kDa  | 27.5 | 4.5 | 14.66667 | 0        | 20       | 7.937254 |
| Putative rab1 small GTP-binding protein               | Q4QH98_LEIMA | 25 kDa  | 13   | 2   | 22       | 4.50925  | 26       | 4        |
| Ribosomal protein L15                                 | Q9N9V8_LEIIN | 24 kDa  | 0    | 0   | 27       | 7.549834 | 22.66667 | 7.055337 |
| Uncharacterized protein                               | Q4QIY5_LEIMA | 49 kDa  | 23.5 | 4.5 | 13.66667 | 7.310571 | 26.33333 | 9.905105 |
| Putative glycosomal membrane protein                  | Q4Q838_LEIMA | 24 kDa  | 8.5  | 0.5 | 18.66667 | 9.820613 | 31.33333 | 7.423686 |
| Putative 60S ribosomal protein L2                     | A4HL27_LEIBR | 28 kDa  | 16   | 10  | 24.66667 | 7.512952 | 19.66667 | 3.282953 |
| ATP-dependent RNA helicase eIF4A                      | Q4Q5P5_LEIMA | 67 kDa  | 18.5 | 0.5 | 17.33333 | 6.359595 | 24       | 3.605551 |
| GTP-binding protein homologue                         | Q25324_LEIMA | 22 kDa  | 19   | 1   | 17.66667 | 1.333333 | 20       | 4.041452 |
| Putative 60S ribosomal protein L6                     | Q4QF84_LEIMA | 21 kDa  | 0    | 0   | 28       | 7.549834 | 26       | 3.21455  |
| Putative RNA binding protein                          | Q4Q5K7_LEIMA | 25 kDa  | 13   | 4   | 23       | 3.605551 | 21       | 3.605551 |
| 40S ribosomal protein SA                              | RSSA_LEIMA   | 27 kDa  | 10   | 2   | 25       | 9.165151 | 20.66667 | 4.255715 |
| Putative cystathione gamma lyase                      | E9AFE7_LEIMA | 45 kDa  | 15   | 5   | 21.66667 | 3.282953 | 20.66667 | 4.807402 |

|                                                              |              |         |      |     |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Putative 40S ribosomal protein S11                           | E9AUU6_LEIMU | 16 kDa  | 12.5 | 3.5 | 21.33333 | 6.839428 | 20.66667 | 2.185813 |
| Histone H2B                                                  | Q4QDL6_LEIMA | 12 kDa  | 17   | 3   | 19.66667 | 4.333333 | 17       | 1        |
| Actin                                                        | A4HSC2_LEIIN | 42 kDa  | 18.5 | 4.5 | 20.66667 | 3.179797 | 18.33333 | 2.333333 |
| Putative serine/threonine-protein kinase                     | Q4Q9K2_LEIMA | 44 kDa  | 7    | 2   | 20       | 5.567764 | 24.33333 | 5.206833 |
| Putative voltage-dependent anion-selective channel           | E9ACB9_LEIMA | 30 kDa  | 14.5 | 2.5 | 17.66667 | 7.666667 | 24.33333 | 3.282953 |
| Uncharacterized protein                                      | Q4QB63_LEIMA | 26 kDa  | 12   | 1   | 17       | 5.033223 | 24.33333 | 5.364492 |
| Putative calcium motive P-type ATPase                        | E9AF31_LEIMA | 122 kDa | 12   | 4   | 20       | 8.621678 | 15.33333 | 0        |
| Uncharacterized protein                                      | Q9N852_LEIMA | 54 kDa  | 12.5 | 7.5 | 23       | 11.59023 | 17       | 4.041452 |
| Uncharacterized protein                                      | Q4QGX6_LEIMA | 28 kDa  | 39.5 | 9.5 | 9.333333 | 0        | 11.66667 | 3.527668 |
| Putative 60S ribosomal protein L13                           | E9AEA8_LEIMA | 25 kDa  | 22   | 13  | 15.33333 | 2.333333 | 19       | 2.516611 |
| 40S ribosomal protein S24                                    | Q4Q1D2_LEIMA | 16 kDa  | 0    | 0   | 28.66667 | 7.218803 | 19.66667 | 5.174725 |
| Putative GTP-binding protein                                 | E9AE82_LEIMA | 77 kDa  | 8    | 3   | 17       | 8.504901 | 21       | 7.937254 |
| Histone H4                                                   | Q4QJ78_LEIMA | 11 kDa  | 19.5 | 1.5 | 16.33333 | 2.603417 | 19       | 2.081666 |
| Putative calpain-like cysteine peptidase                     | Q4QCS9_LEIMA | 108 kDa | 11.5 | 0.5 | 19.66667 | 0        | 17       | 4.582576 |
| Putative adenylate kinase                                    | Q4QC71_LEIMA | 30 kDa  | 16.5 | 3.5 | 17.33333 | 8.950481 | 18       | 6.110101 |
| Putative heat shock 70-related protein 1, mitochondrial      | A4I5T0_LEIIN | 72 kDa  | 17.5 | 0.5 | 13       | 5.033223 | 19.33333 | 8.452482 |
| Adenosylhomocysteinase                                       | Q4Q124_LEIMA | 48 kDa  | 17   | 1   | 17.33333 | 7.356025 | 16       | 6.082763 |
| Serine/threonine-protein phosphatase                         | Q4QGM7_LEIMA | 109 kDa | 7.5  | 2.5 | 13.66667 | 4.096069 | 24.66667 | 5.783117 |
| Arginyl-tRNA synthetase                                      | E9ADB2_LEIMA | 78 kDa  | 20   | 1   | 9.666667 | 0        | 18.66667 | 9.735388 |
| Uncharacterized protein                                      | E9AJG4_LEIMU | 51 kDa  | 16   | 0   | 15.66667 | 6.333333 | 18.66667 | 4.409586 |
| Uncharacterized protein                                      | Q4QB64_LEIMA | 81 kDa  | 16   | 3   | 8.666667 | 0        | 23       | 8.717798 |
| D-lactate dehydrogenase-like protein                         | E9ADN0_LEIMA | 53 kDa  | 4    | 0   | 19.33333 | 10.86789 | 22.33333 | 9.492687 |
| Phosphoglycerate mutase (2,3-diphosphoglycerate-independent) | Q4Q090_LEIMA | 61 kDa  | 14.5 | 2.5 | 14       | 6.082763 | 20.66667 | 4.096069 |
| Putative ADP-ribosylation factor                             | A4I784_LEIIN | 20 kDa  | 19   | 2   | 11       | 1.154701 | 17.33333 | 2.962731 |
| Uncharacterized protein                                      | Q4QH89_LEIMA | 37 kDa  | 12.5 | 0.5 | 15.33333 | 3.480102 | 17.66667 | 5.547772 |
| 9,11-endoperoxide prostaglandin H2 reductase                 | PGFS_LEIMA   | 32 kDa  | 9    | 2   | 16       | 0        | 20.66667 | 2.728451 |
| Uncharacterized protein                                      | Q4Q350_LEIMA | 17 kDa  | 17.5 | 6.5 | 11       | 2.081666 | 20       | 2.309401 |
| Putative long-chain-fatty-acid-CoA ligase                    | A4HRH5_LEIIN | 78 kDa  | 7    | 3   | 19       | 7.767453 | 16.66667 | 4.176655 |
| Putative small GTP-binding protein Rab11                     | Q4QHC3_LEIMA | 23 kDa  | 10   | 3   | 17.66667 | 4.176655 | 19       | 4        |
| Surface antigen-like protein                                 | Q9XZX9_LEIMA | 74 kDa  | 6.5  | 1.5 | 29.66667 | 16.75642 | 6.666667 | 0        |
| Acetyl-coenzyme A synthetase                                 | Q9U0W1_LEIMA | 77 kDa  | 11.5 | 1.5 | 13.66667 | 6.765928 | 18.33333 | 6.887993 |
| Superoxide dismutase                                         | Q4Q597_LEIMA | 21 kDa  | 11.5 | 0.5 | 17.66667 | 5.783117 | 15       | 2        |
| Putative trypanothione synthetase                            | Q711P7_LEIMA | 74 kDa  | 13   | 0   | 10.66667 | 0        | 18.33333 | 5.456902 |
| PHB domain-containing protein                                | Q4QD86_LEIMA | 31 kDa  | 10   | 3   | 15.33333 | 7.535103 | 18       | 6.110101 |
| 60S ribosomal protein L27                                    | Q4Q504_LEIMA | 15 kDa  | 8    | 2   | 18.33333 | 2.603417 | 18.66667 | 2.027588 |
| Uncharacterized protein                                      | Q4Q9S9_LEIMA | 17 kDa  | 13.5 | 6.5 | 13       | 0        | 18.33333 | 2.905933 |
| Putative ADP ribosylation factor 3                           | E9ADU4_LEIMA | 20 kDa  | 11   | 3   | 13.66667 | 4.702245 | 18       | 4.932883 |
| Putative lipophosphoglycan biosynthetic protein              | E9ADS8_LEIMA | 87 kDa  | 7    | 2   | 15.33333 | 0        | 17.33333 | 6.064468 |

|                                                                                |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative adenylate kinase                                                      | Q4Q3J7_LEIMA | 24 kDa  | 18.5 | 10.5 | 0        | 0        | 16       | 3.511885 |
| Putative dipeptylcarboxypeptidase                                              | E9ACE8_LEIMA | 77 kDa  | 12   | 1    | 0        | 0        | 17       | 0        |
| Putative aldose 1-epimerase                                                    | E9AES2_LEIMA | 41 kDa  | 16.5 | 1.5  | 13.66667 | 6.765928 | 13.33333 | 4.910307 |
| Uncharacterized protein                                                        | Q4QI59_LEIMA | 42 kDa  | 14.5 | 4.5  | 0        | 0        | 18.66667 | 5.456902 |
| Rab GDP dissociation inhibitor                                                 | E9AE78_LEIMA | 50 kDa  | 17.5 | 6.5  | 0        | 0        | 11       | 0        |
| Stress-inducible protein STI1 homolog                                          | Q4Q271_LEIMA | 29 kDa  | 15.5 | 0.5  | 11       | 5.686241 | 14.33333 | 4.910307 |
| Dimethylargininase                                                             | Q4QI38_LEIMA | 32 kDa  | 13.5 | 1.5  | 5        | 0        | 22.33333 | 3.282953 |
| Histone H2A                                                                    | Q4QCA4_LEIMA | 14 kDa  | 0    | 0    | 17.33333 | 3.480102 | 13.66667 | 0.881917 |
| Putative 60S ribosomal protein L23                                             | E9AFK3_LEIMA | 15 kDa  | 0    | 0    | 19       | 2.886751 | 18.66667 | 2.403701 |
| Putative 40S ribosomal protein S13                                             | E9ARZ1_LEIMU | 17 kDa  | 0    | 0    | 18.33333 | 2.333333 | 16       | 2.309401 |
| Putative ribosomal protein l35a                                                | Q4QHL3_LEIMA | 16 kDa  | 0    | 0    | 19.33333 | 6.359595 | 16       | 3        |
| Putative heat shock protein DNAJ                                               | E9ACW0_LEIMA | 44 kDa  | 9.5  | 3.5  | 14       | 4.041452 | 15.66667 | 4.702245 |
| Glucose-6-phosphate isomerase                                                  | Q4QGN9_LEIMA | 67 kDa  | 8.5  | 0.5  | 11.33333 | 0        | 17       | 8.888194 |
| Uncharacterized protein                                                        | Q4QB65_LEIMA | 38 kDa  | 0    | 0    | 8.333333 | 1.452966 | 25.66667 | 7.264832 |
| Putative amino acid transporter aATP11                                         | Q4Q6N0_LEIMA | 55 kDa  | 12.5 | 3.5  | 13       | 6.506407 | 12.33333 | 4.484541 |
| Uncharacterized protein                                                        | Q4QC37_LEIMA | 46 kDa  | 19   | 3    | 11       | 0        | 11.33333 | 5.925463 |
| Cysteine peptidase C (CPC)                                                     | E9ADT5_LEIMA | 37 kDa  | 6    | 0    | 12       | 0        | 15.33333 | 6.17342  |
| Uncharacterized protein                                                        | E9ACC0_LEIMA | 32 kDa  | 10   | 1    | 8        | 2.645751 | 20       | 3.05505  |
| Myosin XXI                                                                     | Q4Q4N5_LEIMA | 119 kDa | 0    | 0    | 15.66667 | 0        | 13       | 4.725816 |
| Putative small myristoylated protein-3                                         | Q4QFP8_LEIMA | 13 kDa  | 21.5 | 0.5  | 9.666667 | 3.333333 | 8.666667 | 1.855921 |
| 40S ribosomal protein S5                                                       | E9ANP0_LEIMU | 21 kDa  | 0    | 0    | 20       | 5.686241 | 10.66667 | 5.174725 |
| Putative surface protein amastin                                               | Q4Q7L7_LEIMA | 21 kDa  | 19.5 | 5.5  | 9.666667 | 1.20185  | 11.66667 | 1.20185  |
| Putative 40S ribosomal protein S15A                                            | Q4QGW3_LEIMA | 15 kDa  | 0    | 0    | 18       | 5.507571 | 15.33333 | 3.844188 |
| Phospholipid-transporting ATPase                                               | Q4QG01_LEIMA | 124 kDa | 0    | 0    | 15.33333 | 8.353309 | 11.33333 | 5.206833 |
| Putative cytochrome c1, heme protein, mitochondrial                            | Q4QIT8_LEIMA | 30 kDa  | 14   | 9    | 10.66667 | 0        | 12.66667 | 1.763834 |
| Elongation factor 1-beta                                                       | Q4Q3C1_LEIMA | 26 kDa  | 13   | 0    | 9.666667 | 0        | 11       | 2.516611 |
| Amastin-like protein                                                           | Q4Q3F7_LEIMA | 24 kDa  | 15   | 4    | 10.33333 | 2.848001 | 13.66667 | 1.452966 |
| Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex | A4I3X3_LEIIN | 42 kDa  | 10   | 5    | 16       | 7.371115 | 10.66667 | 1.763834 |
| Pyruvate kinase                                                                | E9AEI0_LEIMA | 54 kDa  | 12   | 2    | 11.66667 | 4.484541 | 11.33333 | 4.841946 |
| Putative nima-related protein kinase                                           | Q4Q1S4_LEIMA | 56 kDa  | 9.5  | 2.5  | 10.66667 | 5.811865 | 12.66667 | 3.711843 |
| Uncharacterized protein                                                        | Q4Q0P9_LEIMA | 30 kDa  | 0    | 0    | 11       | 0        | 16.66667 | 4.630815 |
| Uridine 5'-monophosphate synthase                                              | Q4QEW5_LEIMA | 50 kDa  | 0    | 0    | 9.666667 | 0        | 20.66667 | 7.310571 |
| Inosine-guanosine transporter                                                  | Q4Q1M9_LEIMA | 54 kDa  | 9.5  | 1.5  | 14.66667 | 6.333333 | 9.666667 | 5.174725 |
| Putative long-chain-fatty-acid-CoA ligase                                      | E9AC40_LEIMA | 77 kDa  | 0    | 0    | 14       | 7.094599 | 11.33333 | 0        |
| Putative lanosterol 14-alpha-demethylase                                       | Q4QGX0_LEIMA | 54 kDa  | 0    | 0    | 15.66667 | 7.623064 | 13       | 6.506407 |
| Putative 60S ribosomal protein L28                                             | Q4QGW7_LEIMA | 16 kDa  | 6.5  | 2.5  | 13.33333 | 2.333333 | 13.66667 | 3.179797 |
| Putative mitochondrial phosphate transporter                                   | E9AFS0_LEIMA | 35 kDa  | 9    | 2    | 11       | 6.110101 | 14       | 2.886751 |
| Kinesin-like protein                                                           | Q9NF78_LEIMA | 75 kDa  | 0    | 0    | 6.666667 | 3.666667 | 20       | 8        |

|                                                    |              |         |      |     |          |          |          |          |
|----------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Putative 40S ribosomal protein S18                 | A4ICP1_LEIIN | 19 kDa  | 0    | 0   | 16       | 5.033223 | 15       | 2        |
| Putative thimet oligopeptidase                     | Q4Q937_LEIMA | 77 kDa  | 13.5 | 2.5 | 11       | 0        | 10.33333 | 5.783117 |
| FGE-sulfatase domain-containing protein            | E9ADV2_LEIMA | 87 kDa  | 13.5 | 0.5 | 7.333333 | 0        | 13       | 4.041452 |
| 40S ribosomal protein S26                          | A4I3C8_LEIIN | 13 kDa  | 0    | 0   | 11.66667 | 3.527668 | 0        | 0        |
| Putative reiske iron-sulfur protein                | E9AEX6_LEIMA | 34 kDa  | 27   | 5   | 0        | 0        | 6.333333 | 0        |
| META domain containing protein                     | Q4QEA3_LEIMA | 48 kDa  | 8    | 2   | 13.66667 | 5.238745 | 11       | 2.081666 |
| Fructose-bisphosphate aldolase                     | Q4Q1V0_LEIMA | 41 kDa  | 7    | 1   | 13.66667 | 0        | 11       | 2        |
| Putative 60S ribosomal protein L27A/L29            | A4IBR5_LEIIN | 16 kDa  | 7    | 2   | 11       | 3.21455  | 14.33333 | 3.844188 |
| Dipeptidyl peptidase 3                             | Q4QJA6_LEIMA | 76 kDa  | 21   | 1   | 7.333333 | 0        | 7.666667 | 0        |
| Putative 60S ribosomal protein L17                 | A4HD62_LEIBR | 19 kDa  | 9.5  | 3.5 | 12       | 1.527525 | 10.33333 | 3.179797 |
| Uncharacterized protein                            | Q4Q5Q8_LEIMA | 140 kDa | 10   | 6   | 7        | 0        | 15.66667 | 3.282953 |
| Transketolase                                      | Q4QAC4_LEIMA | 72 kDa  | 8.5  | 1.5 | 11.66667 | 0        | 11.66667 | 4.255715 |
| DUF4139 domain-containing protein                  | E9AE72_LEIMA | 69 kDa  | 8    | 3   | 10       | 5.131601 | 13.33333 | 3.480102 |
| Alkyl dihydroxyacetonephosphate synthase           | Q7YWB6_LEIMA | 70 kDa  | 3.5  | 1.5 | 11.66667 | 0        | 15.66667 | 8.569973 |
| Metallo-peptidase, Clan MA(E), Family M32          | Q4Q3T3_LEIMA | 57 kDa  | 8.5  | 0.5 | 12.66667 | 6.765928 | 10       | 4.041452 |
| Uncharacterized protein                            | Q4Q353_LEIMA | 38 kDa  | 12   | 3   | 7.333333 | 0        | 14.33333 | 4.484541 |
| Vesicle-fusing ATPase                              | E9AEB2_LEIMA | 50 kDa  | 3.5  | 0.5 | 6.333333 | 0        | 14.66667 | 4.977728 |
| C2 domain protein                                  | Q9BHF8_LEIMA | 30 kDa  | 8    | 2   | 13.66667 | 2.185813 | 10.33333 | 1.666667 |
| Uncharacterized protein                            | Q4Q3G2_LEIMA | 248 kDa | 0    | 0   | 28       | 0        | 0        | 0        |
| Peptidyl-prolyl cis-trans isomerase                | Q4QJ67_LEIMA | 20 kDa  | 6.5  | 0.5 | 9.333333 | 4.484541 | 13.33333 | 2.603417 |
| Glutathione peroxidase                             | Q4Q9B2_LEIMA | 19 kDa  | 3    | 0   | 10.66667 | 0        | 15       | 2.645751 |
| Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase | Q4QJH5_LEIMA | 25 kDa  | 0    | 0   | 12       | 6.244998 | 13.33333 | 3.382964 |
| Adenosine kinase                                   | Q4Q7L5_LEIMA | 37 kDa  | 11   | 0   | 10.33333 | 3.527668 | 10.33333 | 4.484541 |
| Sacchrp_dh_NADP domain-containing protein          | Q4Q8N2_LEIMA | 42 kDa  | 7.5  | 3.5 | 11       | 0        | 10       | 5.291503 |
| Putative aminopeptidase P                          | E9AF59_LEIMA | 54 kDa  | 7.5  | 1.5 | 7.666667 | 0        | 14       | 6.082763 |
| 14-3-3 protein II                                  | Q4QH45_LEIMA | 29 kDa  | 7.5  | 3.5 | 7.666667 | 0        | 13.66667 | 3.711843 |
| Uncharacterized protein                            | E9AE81_LEIMA | 155 kDa | 0    | 0   | 9.666667 | 0        | 11.33333 | 5.840472 |
| Putative NADH-dependent fumarate reductase         | E9AEU1_LEIMA | 123 kDa | 0    | 0   | 0        | 0        | 13.33333 | 7.423686 |
| HASPA1                                             | P90552_LEIMA | 8 kDa   | 5.5  | 1.5 | 10.33333 | 0.881917 | 13.33333 | 1.20185  |
| Uncharacterized protein                            | Q4QBS4_LEIMA | 50 kDa  | 8.5  | 4.5 | 10       | 0        | 11.33333 | 5.840472 |
| Putative 60S ribosomal protein L5                  | E9AF09_LEIMA | 34 kDa  | 3.5  | 0.5 | 14       | 2.516611 | 10.33333 | 2.185813 |
| Uncharacterized protein                            | Q4Q0W8_LEIMA | 17 kDa  | 9.5  | 3.5 | 9        | 5.033223 | 10.33333 | 1.763834 |
| Peptidase_M28 domain-containing protein            | Q4Q990_LEIMA | 60 kDa  | 5.5  | 1.5 | 9.333333 | 0        | 14       | 6.506407 |
| Kinesin motor domain-containing protein            | Q4Q0P7_LEIMA | 71 kDa  | 9    | 3   | 7.333333 | 0        | 11.33333 | 3.179797 |
| ATP-dependent RNA helicase eIF4A                   | E9AFD4_LEIMA | 100 kDa | 10   | 4   | 8        | 0        | 11.33333 | 3.756476 |
| Cytochrome c                                       | Q4QEN5_LEIMA | 12 kDa  | 16   | 9   | 6        | 0        | 8        | 1.154701 |
| Malate dehydrogenase                               | Q4Q7X6_LEIMA | 34 kDa  | 13   | 0   | 0        | 0        | 6.666667 | 3.382964 |
| Uncharacterized protein                            | Q4Q079_LEIMA | 29 kDa  | 13   | 1   | 0        | 0        | 4.666667 | 2.333333 |

|                                                              |              |         |      |     |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Uncharacterized protein                                      | Q4Q9F3_LEIMA | 30 kDa  | 17.5 | 1.5 | 3.333333 | 0        | 10.66667 | 2.333333 |
| Putative 60S ribosomal protein L19                           | A4HST1_LEIIN | 28 kDa  | 10.5 | 4.5 | 10.66667 | 0.881917 | 9        | 0        |
| Uncharacterized protein                                      | Q4QD99_LEIMA | 41 kDa  | 9    | 1   | 8        | 4.041452 | 10       | 3.05505  |
| Threonyl-tRNA synthetase                                     | E9AEW4_LEIMA | 89 kDa  | 0    | 0   | 8.666667 | 4.702245 | 13.66667 | 5.174725 |
| Kinetoplastid membrane protein-11                            | E9B6A1_LEIMU | 11 kDa  | 0    | 0   | 8        | 0        | 11.33333 | 2.848001 |
| Malic enzyme                                                 | E9AWR7_LEIMU | 63 kDa  | 13.5 | 3.5 | 0        | 0        | 8        | 0        |
| Transmembrane 9 superfamily member                           | Q4Q2H8_LEIMA | 71 kDa  | 9.5  | 3.5 | 7.333333 | 0        | 8.666667 | 4.255715 |
| Uncharacterized protein                                      | Q4QE89_LEIMA | 72 kDa  | 0    | 0   | 8        | 4.50925  | 12.33333 | 1.763834 |
| Chaperonin HSP60, mitochondrial                              | Q4Q1M0_LEIMA | 59 kDa  | 10   | 1   | 0        | 0        | 11.33333 | 6.009252 |
| Putative 40S ribosomal protein S23                           | Q4QC89_LEIMA | 16 kDa  | 0    | 0   | 12       | 4.50925  | 12.33333 | 3.844188 |
| Aldose 1-epimerase-like protein                              | E9AES3_LEIMA | 42 kDa  | 0    | 0   | 0        | 0        | 14.66667 | 7.446103 |
| Putative 60S Ribosomal protein L36                           | A4HZ73_LEIIN | 12 kDa  | 5    | 0   | 9.666667 | 2.185813 | 12       | 1        |
| PPM-type phosphatase domain-containing protein               | Q4QA19_LEIMA | 45 kDa  | 12.5 | 0.5 | 6        | 0        | 8        | 4.041452 |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QJ76_LEIMA | 76 kDa  | 8.5  | 2.5 | 5.666667 | 2.728451 | 9.333333 | 4.484541 |
| Putative 40S ribosomal protein S17                           | Q4Q806_LEIMA | 17 kDa  | 0    | 0   | 10       | 1.527525 | 12       | 2.081666 |
| Histone H3                                                   | A4HUJ9_LEIIN | 15 kDa  | 0    | 0   | 12.33333 | 1.452966 | 9.666667 | 1.20185  |
| Putative eukaryotic initiation factor 5a                     | A4I1B5_LEIIN | 29 kDa  | 6    | 0   | 9        | 1.527525 | 11       | 1.527525 |
| Putative ATP-binding cassette protein subfamily G, member 2  | Q4QJ70_LEIMA | 74 kDa  | 7.5  | 3.5 | 11.66667 | 0        | 7        | 0        |
| Putative beta-fructofuranosidase                             | Q9XTP3_LEIMA | 72 kDa  | 0    | 0   | 8        | 0        | 7.333333 | 0        |
| Putative 60S ribosomal protein L26                           | Q4QAC5_LEIMA | 16 kDa  | 0    | 0   | 11.33333 | 3.929942 | 9        | 3.511885 |
| LmRab7 GTP-binding protein                                   | Q9N2P5_LEIMA | 24 kDa  | 10   | 2   | 6        | 2.309401 | 10.33333 | 0.666667 |
| S-adenosylmethionine synthase                                | Q4Q6T6_LEIMA | 43 kDa  | 3.5  | 0.5 | 8.666667 | 4.910307 | 9.333333 | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QCL0_LEIMA | 47 kDa  | 3    | 0   | 4        | 1.154701 | 15.33333 | 3.711843 |
| Phosphoglycerate kinase                                      | Q4Q6V1_LEIMA | 58 kDa  | 0    | 0   | 0        | 0        | 13.33333 | 5.607535 |
| Glutamate dehydrogenase                                      | Q4Q7X1_LEIMA | 49 kDa  | 0    | 0   | 5        | 0        | 13.33333 | 0        |
| Malate dehydrogenase                                         | Q4QDF0_LEIMA | 34 kDa  | 0    | 0   | 9        | 0        | 10.66667 | 3.711843 |
| Uncharacterized protein                                      | Q4Q6X5_LEIMA | 23 kDa  | 5    | 1   | 9.333333 | 5.333333 | 9.666667 | 3.756476 |
| Cysteine protease (Fragment)                                 | A1BLM5_LEIMA | 36 kDa  | 0    | 0   | 8        | 4.041452 | 10.33333 | 2.962731 |
| Uncharacterized protein                                      | Q4Q3M9_LEIMA | 821 kDa | 0    | 0   | 12       | 0        | 6.666667 | 2.185813 |
| Seryl-tRNA synthetase                                        | Q4QH70_LEIMA | 53 kDa  | 6.5  | 1.5 | 8        | 0        | 8        | 0        |
| VPS37 C-terminal domain-containing protein                   | Q4Q8L4_LEIMA | 32 kDa  | 9.5  | 4.5 | 9        | 4.041452 | 7        | 3.05505  |
| Surface antigen-like protein                                 | E9ACQ0_LEIMA | 30 kDa  | 0    | 0   | 13.66667 | 6.385748 | 4.666667 | 1.666667 |
| Putative cytochrome c oxidase subunit V                      | Q4Q922_LEIMA | 22 kDa  | 13.5 | 4.5 | 5.333333 | 0        | 6        | 3        |
| Uncharacterized protein                                      | Q4Q3U3_LEIMA | 56 kDa  | 15   | 4   | 0        | 0        | 6        | 2        |
| Putative NADH-cytochrome b5 reductase                        | Q4QBS0_LEIMA | 32 kDa  | 14   | 8   | 0        | 0        | 8.666667 | 1.855921 |
| Uncharacterized protein                                      | Q4Q9J7_LEIMA | 109 kDa | 10.5 | 6.5 | 0        | 0        | 10       | 0        |
| Uncharacterized protein                                      | Q4QD20_LEIMA | 29 kDa  | 8    | 2   | 7        | 0        | 10.33333 | 3.527668 |
| Putative fatty acyl CoA syntetase 1                          | E9AC38_LEIMA | 77 kDa  | 5    | 1   | 7.666667 | 4.176655 | 9.666667 | 3.282953 |

|                                                                        |              |         |      |     |          |          |          |          |
|------------------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| 40S ribosomal protein S12                                              | A4HVI6_LEIIN | 16 kDa  | 5.5  | 0.5 | 6.666667 | 0        | 11.33333 | 3.844188 |
| 40S ribosomal protein S14                                              | Q4Q8H1_LEIMA | 16 kDa  | 0    | 0   | 11.66667 | 2.603417 | 8.666667 | 2.185813 |
| Paraflagellar rod protein 2C                                           | Q4QEM2_LEIMA | 69 kDa  | 7.5  | 4.5 | 0        | 0        | 9.666667 | 3.179797 |
| Amastin-like surface protein-like protein                              | Q4Q7L6_LEIMA | 24 kDa  | 9    | 1   | 5.666667 | 2.962731 | 7.666667 | 1.20185  |
| Putative endoribonuclease L-PSP (Pb5)                                  | Q4QBF5_LEIMA | 17 kDa  | 15.5 | 0.5 | 4        | 0        | 7.333333 | 2.027588 |
| Putative elongation initiation factor 2 alpha subunit                  | E9ACP3_LEIMA | 49 kDa  | 7.5  | 0.5 | 0        | 0        | 9.666667 | 0        |
| I/6 autoantigen-like protein                                           | Q4QBK1_LEIMA | 23 kDa  | 8    | 2   | 8.333333 | 3.929942 | 7.333333 | 3.333333 |
| N(1),N(8)-bis(glutathionyl)spermidine reductase                        | Q4QJG7_LEIMA | 53 kDa  | 7.5  | 2.5 | 5        | 2        | 10       | 2.886751 |
| Putative 60S ribosomal protein L34                                     | A4HY10_LEIIN | 19 kDa  | 0    | 0   | 8.666667 | 1.20185  | 10       | 0.57735  |
| T-complex protein 1 subunit gamma                                      | Q4QB32_LEIMA | 60 kDa  | 0    | 0   | 6.666667 | 0        | 11.66667 | 6.17342  |
| Prohibitin                                                             | E9AEI4_LEIMA | 32 kDa  | 7    | 3   | 0        | 0        | 8        | 4.358899 |
| Non-specific serine/threonine protein kinase                           | Q9NHE1_LEIMA | 40 kDa  | 5    | 1   | 9.333333 | 3.382964 | 6        | 2        |
| Alanine--tRNA ligase                                                   | Q4QBJ3_LEIMA | 106 kDa | 4.5  | 1.5 | 5.333333 | 0        | 11.66667 | 3.179797 |
| ATP-dependent RNA helicase eIF4A                                       | E9AEL4_LEIMA | 46 kDa  | 7.5  | 0.5 | 7        | 0        | 9        | 2.081666 |
| 14-3-3 protein-like protein                                            | E9AT99_LEIMU | 30 kDa  | 8.5  | 2.5 | 0        | 0        | 6.333333 | 3.480102 |
| Tryparedoxin                                                           | E9ADX4_LEIMA | 17 kDa  | 6    | 0   | 5        | 2.645751 | 8.666667 | 0.666667 |
| Putative sarcoplasmic reticulum glycoprotein                           | Q4QDJ3_LEIMA | 69 kDa  | 0    | 0   | 7        | 0        | 8        | 4.582576 |
| Putative calpain-like cysteine peptidase                               | Q9U0T9_LEIMA | 94 kDa  | 0    | 0   | 9.333333 | 0        | 6        | 0        |
| Uncharacterized protein                                                | Q4QIX9_LEIMA | 114 kDa | 6.5  | 0.5 | 8        | 0        | 7        | 0        |
| Putative ATP-binding cassette protein subfamily A, member 8            | E9AD74_LEIMA | 202 kDa | 0    | 0   | 8        | 0        | 7.333333 | 3.711843 |
| 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase | Q4Q6R3_LEIMA | 86 kDa  | 4.5  | 2.5 | 8.666667 | 0        | 8.333333 | 0        |
| Profilin                                                               | Q4Q5N1_LEIMA | 16 kDa  | 4.5  | 2.5 | 7        | 3.605551 | 9        | 2.081666 |
| Uncharacterized protein                                                | E9ADY2_LEIMA | 79 kDa  | 8    | 1   | 3.666667 | 2.027588 | 9        | 3.785939 |
| Phosphomannomutase                                                     | Q4Q1M7_LEIMA | 28 kDa  | 6    | 1   | 8.666667 | 0        | 7.333333 | 1.763834 |
| Flagellar glycoprotein-like protein                                    | Q4QHF2_LEIMA | 60 kDa  | 6    | 4   | 8.666667 | 2.728451 | 4.666667 | 1.452966 |
| 6-phosphogluconate dehydrogenase, decarboxylating (Fragment)           | Q9NGR0_LEIMA | 52 kDa  | 5    | 0   | 6.333333 | 0        | 7.666667 | 2.848001 |
| Putative pretranslocation protein, alpha subunit                       | Q4QGX4_LEIMA | 54 kDa  | 0    | 0   | 7.333333 | 3.929942 | 8.666667 | 3.179797 |
| Putative ATP synthase F1 subunit gamma protein                         | Q4QC06_LEIMA | 34 kDa  | 7    | 0   | 0        | 0        | 7.333333 | 3.711843 |
| Putative zinc transporter                                              | Q4Q873_LEIMA | 44 kDa  | 0    | 0   | 9.333333 | 2.333333 | 9.333333 | 1.666667 |
| Cyclophilin 40                                                         | E9AFV2_LEIMA | 38 kDa  | 9.5  | 2.5 | 2.333333 | 1.20185  | 8        | 1.732051 |
| Transmembrane 9 superfamily member                                     | E9AE21_LEIMA | 76 kDa  | 4.5  | 2.5 | 6.333333 | 0        | 8        | 1.527525 |
| Putative 60S ribosomal protein L10a                                    | Q4QDX9_LEIMA | 25 kDa  | 3.5  | 0.5 | 11       | 6        | 4        | 1.527525 |
| Putative ribosomal protein L24                                         | A4HNW7_LEIBR | 15 kDa  | 0    | 0   | 6.666667 | 3.382964 | 6.666667 | 0.881917 |
| ADP-ribosylation factor-like protein 6                                 | Q4QEM9_LEIMA | 20 kDa  | 8.5  | 0.5 | 4.333333 | 0        | 0        | 0        |
| Splicing factor pstr1-like protein                                     | Q4QIK0_LEIMA | 42 kDa  | 3.5  | 1.5 | 7.333333 | 4.055175 | 8.333333 | 1.452966 |
| Putative kinesin                                                       | E9ADV5_LEIMA | 192 kDa | 0    | 0   | 0        | 0        | 7.666667 | 0        |

|                                                                                |              |         |      |     |          |          |          |          |
|--------------------------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Putative DNAJ domain protein                                                   | Q4QAS9_LEIMA | 89 kDa  | 0    | 0   | 0        | 0        | 6        | 0        |
| Putative vacuolar ATP synthase subunit b                                       | A4I3X4_LEIIN | 56 kDa  | 0    | 0   | 0        | 0        | 4.333333 | 2.185813 |
| Uncharacterized protein                                                        | Q4QDR4_LEIMA | 34 kDa  | 10.5 | 1.5 | 0        | 0        | 5.666667 | 0        |
| Putative ef-hand protein 5                                                     | Q4QHT4_LEIMA | 21 kDa  | 5    | 1   | 6.666667 | 1.666667 | 8.333333 | 2.403701 |
| Uncharacterized protein                                                        | E9ADC5_LEIMA | 25 kDa  | 11.5 | 0.5 | 3        | 0        | 7.333333 | 2.027588 |
| SB domain-containing protein                                                   | E9AEF7_LEIMA | 45 kDa  | 4    | 0   | 9        | 0        | 5.666667 | 1.452966 |
| Uncharacterized protein                                                        | Q4QFU5_LEIMA | 34 kDa  | 6    | 3   | 3.333333 | 0        | 9        | 1.527525 |
| Putative NADP-dependent alcohol dehydrogenase                                  | Q4QBD8_LEIMA | 38 kDa  | 6    | 1   | 2        | 1        | 11       | 3.785939 |
| Putative membrane-bound acid phosphatase                                       | Q4Q7Z7_LEIMA | 60 kDa  | 3.5  | 1.5 | 5.333333 | 0        | 9.666667 | 3.844188 |
| Putative IgE-dependent histamine-releasing factor                              | Q4QAI0_LEIMA | 19 kDa  | 8.5  | 1.5 | 5.333333 | 0        | 0        | 0        |
| Putative paraflagellar rod protein 1D                                          | E9AE36_LEIMA | 69 kDa  | 5    | 2   | 7        | 0        | 6.666667 | 3.756476 |
| 60S ribosomal protein L32                                                      | Q4QC11_LEIMA | 15 kDa  | 0    | 0   | 8.666667 | 1.20185  | 9.333333 | 0.881917 |
| Dynamin-type G domain-containing protein                                       | Q4Q2X3_LEIMA | 62 kDa  | 0    | 0   | 0        | 0        | 7        | 0        |
| Diphosphomevalonate decarboxylase                                              | Q4QE40_LEIMA | 42 kDa  | 0    | 0   | 6.666667 | 0        | 6.666667 | 3.756476 |
| Putative N-acyl-L-amino acid amidohydrolase                                    | Q4Q6F0_LEIMA | 44 kDa  | 5    | 1   | 4.333333 | 0        | 9.333333 | 3.844188 |
| Acetyl-CoA carboxylase                                                         | Q4Q5W1_LEIMA | 241 kDa | 0    | 0   | 11.66667 | 0        | 4        | 0        |
| Cathepsin L-like protease                                                      | P90628_LEIMA | 48 kDa  | 8.5  | 2.5 | 2.333333 | 1.20185  | 7        | 2.886751 |
| Putative kinesin                                                               | E9AF32_LEIMA | 77 kDa  | 7    | 1   | 0        | 0        | 7.666667 | 4.333333 |
| ER membrane protein complex subunit 1                                          | Q4Q2C8_LEIMA | 87 kDa  | 0    | 0   | 8.666667 | 0        | 7.666667 | 3.282953 |
| Ribosomal protein L37                                                          | E9AEH2_LEIMA | 10 kDa  | 0    | 0   | 10       | 1.527525 | 5.666667 | 2.962731 |
| Putative fumarate hydratase                                                    | E9AE57_LEIMA | 63 kDa  | 0    | 0   | 4.666667 | 0        | 12.33333 | 4.333333 |
| DUF4139 domain-containing protein                                              | Q4Q7L0_LEIMA | 63 kDa  | 0    | 0   | 5.666667 | 0        | 7        | 0        |
| Diadenosine tetraphosphate synthetase                                          | Q4Q131_LEIMA | 70 kDa  | 0    | 0   | 6.666667 | 0        | 7.666667 | 0        |
| Uncharacterized protein                                                        | Q4QFN8_LEIMA | 32 kDa  | 0    | 0   | 0        | 0        | 8.666667 | 4.176655 |
| C2 domain protein                                                              | Q9BHE8_LEIMA | 31 kDa  | 0    | 0   | 8        | 2.516611 | 6.666667 | 1.666667 |
| Citrulline--aspartate ligase                                                   | Q4QBE8_LEIMA | 46 kDa  | 4.5  | 0.5 | 0        | 0        | 10.33333 | 3.666667 |
| Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex | Q4Q1F5_LEIMA | 49 kDa  | 0    | 0   | 9.666667 | 0        | 6.666667 | 2.185813 |
| Aspartyl-tRNA synthetase                                                       | Q4Q7R2_LEIMA | 62 kDa  | 9    | 4   | 0        | 0        | 6.333333 | 0        |
| Putative amino acid transporter                                                | Q4QIH0_LEIMA | 54 kDa  | 5.5  | 1.5 | 5        | 0        | 6.666667 | 2.728451 |
| Pyridoxal kinase                                                               | Q4Q7H9_LEIMA | 33 kDa  | 8.5  | 1.5 | 6.333333 | 3.382964 | 0        | 0        |
| Putative Sec1/Munc18 related protein                                           | Q4Q8W6_LEIMA | 80 kDa  | 0    | 0   | 0        | 0        | 9.333333 | 4.977728 |
| Serine/threonine-protein phosphatase                                           | A4HSD9_LEIIN | 72 kDa  | 8    | 2   | 5        | 0        | 4        | 0        |
| Putative folate/biopterin transporter                                          | Q4QHH7_LEIMA | 78 kDa  | 0    | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Putative mitotubule-associated protein Gb4                                     | Q4Q8Z7_LEIMA | 295 kDa | 0    | 0   | 0        | 0        | 7.666667 | 0        |
| Uncharacterized protein                                                        | Q4Q230_LEIMA | 26 kDa  | 0    | 0   | 0        | 0        | 6        | 0        |
| IU_nuc_hydro domain-containing protein                                         | Q4Q9F7_LEIMA | 38 kDa  | 7.5  | 4.5 | 0        | 0        | 6.333333 | 2.603417 |

|                                                                                                                          |              |         |     |     |          |          |          |          |
|--------------------------------------------------------------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Serine/threonine-protein phosphatase                                                                                     | A4I3E3_LEIIN | 34 kDa  | 3   | 0   | 8.333333 | 0        | 4.333333 | 0        |
| Mitogen-activated protein kinase                                                                                         | Q4QHG6_LEIMA | 44 kDa  | 5.5 | 0.5 | 4.333333 | 0        | 6.333333 | 2.848001 |
| Uncharacterized protein                                                                                                  | E9AFW1_LEIMA | 39 kDa  | 4.5 | 1.5 | 0        | 0        | 7.666667 | 0        |
| Uncharacterized protein                                                                                                  | Q4Q944_LEIMA | 15 kDa  | 0   | 0   | 9        | 4.582576 | 4.666667 | 2.185813 |
| Alba domain-containing protein                                                                                           | Q4Q2T7_LEIMA | 23 kDa  | 9.5 | 2.5 | 0        | 0        | 0        | 0        |
| Methyltransferase                                                                                                        | Q4Q1I3_LEIMA | 40 kDa  | 6.5 | 2.5 | 4.333333 | 0        | 6        | 0        |
| Putative acyl-CoA dehydrogenase                                                                                          | Q4Q812_LEIMA | 69 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 0        |
| Uncharacterized protein                                                                                                  | Q4Q3K8_LEIMA | 34 kDa  | 0   | 0   | 5.666667 | 0        | 7.666667 | 2.666667 |
| Putative Gamma-soluble NSF attachment protein (SNAP-gamma) (N-ethylmaleimide-sensitive factor attachment protein, gamma) | Q4Q3F3_LEIMA | 42 kDa  | 6.5 | 2.5 | 4.333333 | 0        | 6        | 3.21455  |
| Amastin-like protein                                                                                                     | Q4QI87_LEIMA | 22 kDa  | 0   | 0   | 0        | 0        | 8        | 3.05505  |
| V-type proton ATPase subunit a                                                                                           | Q4QAY7_LEIMA | 88 kDa  | 6.5 | 2.5 | 0        | 0        | 0        | 0        |
| Protein tyrosine phosphatase-like protein                                                                                | Q4QEZ7_LEIMA | 19 kDa  | 0   | 0   | 7.333333 | 0        | 8        | 1.527525 |
| Obg-like ATPase 1                                                                                                        | E9ACV1_LEIMA | 44 kDa  | 4.5 | 0.5 | 3.666667 | 2.027588 | 6.666667 | 1.666667 |
| Asparagine--tRNA ligase                                                                                                  | Q4Q2W6_LEIMA | 99 kDa  | 0   | 0   | 5.666667 | 0        | 8        | 4.041452 |
| Programmed cell death 6 protein-like protein                                                                             | Q4QG08_LEIMA | 26 kDa  | 0   | 0   | 6.666667 | 0        | 0        | 0        |
| Prolyl-tRNA synthetase                                                                                                   | Q4QDS0_LEIMA | 82 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 0        |
| Isoleucyl-tRNA synthetase                                                                                                | Q4Q0J9_LEIMA | 126 kDa | 4   | 1   | 5        | 0        | 7        | 4        |
| Uncharacterized protein                                                                                                  | Q4Q6R0_LEIMA | 69 kDa  | 0   | 0   | 10       | 3.464102 | 0        | 0        |
| Amino acid permease                                                                                                      | Q4Q682_LEIMA | 64 kDa  | 0   | 0   | 8        | 3.05505  | 5.333333 | 0        |
| Inositol-3-phosphate synthase                                                                                            | Q4QFJ8_LEIMA | 58 kDa  | 0   | 0   | 0        | 0        | 9.333333 | 2.333333 |
| Mannose-1-phosphate guanylyltransferase                                                                                  | Q4QBG5_LEIMA | 42 kDa  | 0   | 0   | 0        | 0        | 7        | 3.785939 |
| Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial                                                 | E9AH70_LEIIN | 67 kDa  | 6   | 3   | 0        | 0        | 3.666667 | 0        |
| SHERP                                                                                                                    | Q9XTN3_LEIMA | 6 kDa   | 0   | 0   | 0        | 0        | 8.666667 | 4.910307 |
| Uncharacterized protein                                                                                                  | Q4Q483_LEIMA | 28 kDa  | 13  | 5   | 0        | 0        | 3.666667 | 0        |
| Uncharacterized protein                                                                                                  | Q4QB79_LEIMA | 57 kDa  | 0   | 0   | 0        | 0        | 12.66667 | 0        |
| Triosephosphate isomerase                                                                                                | Q4QAP8_LEIMA | 27 kDa  | 5.5 | 1.5 | 5.333333 | 2.848001 | 5        | 2.516611 |
| Uncharacterized protein                                                                                                  | Q4Q4T2_LEIMA | 67 kDa  | 3   | 1   | 4.333333 | 2.333333 | 9        | 2.081666 |
| Putative 60S ribosomal protein L35                                                                                       | A4I2G1_LEIIN | 15 kDa  | 0   | 0   | 6.666667 | 0.333333 | 5.333333 | 0.881917 |
| Calreticulin                                                                                                             | Q4Q601_LEIMA | 45 kDa  | 0   | 0   | 0        | 0        | 7.333333 | 1.763834 |
| Uncharacterized protein                                                                                                  | Q4Q0Z1_LEIMA | 123 kDa | 0   | 0   | 0        | 0        | 3.666667 | 0        |
| Aconitate hydratase                                                                                                      | Q4QDZ1_LEIMA | 97 kDa  | 3   | 0   | 0        | 0        | 6.666667 | 0        |
| ATP pyrophosphate-lyase                                                                                                  | Q4Q1A1_LEIMA | 150 kDa | 4.5 | 1.5 | 0        | 0        | 0        | 0        |
| Putative nucleoside transporter 1                                                                                        | Q4QF58_LEIMA | 54 kDa  | 3.5 | 0.5 | 0        | 0        | 7.666667 | 2.905933 |
| 1-phosphatidylinositol 4-kinase                                                                                          | A4I4K7_LEIIN | 290 kDa | 0   | 0   | 0        | 0        | 7.666667 | 3.844188 |
| NAD-specific glutamate dehydrogenase                                                                                     | Q4QF83_LEIMA | 115 kDa | 3.5 | 0.5 | 0        | 0        | 6.333333 | 0        |
| Putative adenylate kinase                                                                                                | Q9U1D3_LEIMA | 23 kDa  | 0   | 0   | 4.666667 | 0        | 5.333333 | 2.728451 |
| Peptidylprolyl isomerase                                                                                                 | Q4QD56_LEIMA | 48 kDa  | 3.5 | 0.5 | 5        | 0        | 7        | 2.516611 |

|                                           |              |        |     |     |          |          |          |          |
|-------------------------------------------|--------------|--------|-----|-----|----------|----------|----------|----------|
| Uncharacterized protein L7845.03          | Q9GRP8_LEIMA | 63 kDa | 0   | 0   | 5.333333 | 0        | 6.666667 | 1.666667 |
| 60S ribosomal protein L37a                | A4HLC4_LEIBR | 11 kDa | 0   | 0   | 6        | 2        | 6.666667 | 0.881917 |
| ADF/Cofilin                               | E9ADQ2_LEIMA | 16 kDa | 6   | 0   | 5        | 2.516611 | 4        | 0        |
| Cysteine desulphydrase                    | Q4Q511_LEIMA | 44 kDa | 4   | 1   | 4        | 0        | 4.666667 | 2.603417 |
| CCT-theta                                 | Q4Q063_LEIMA | 58 kDa | 0   | 0   | 3.333333 | 0        | 7        | 3.511885 |
| Putative serine/threonine kinase          | Q4QIJ7_LEIMA | 34 kDa | 3.5 | 0.5 | 5.666667 | 0        | 5.333333 | 1.855921 |
| Uncharacterized protein                   | Q4QAH5_LEIMA | 67 kDa | 0   | 0   | 7.666667 | 0        | 3.333333 | 0        |
| Galactokinase-like protein                | E9AF98_LEIMA | 53 kDa | 0   | 0   | 0        | 0        | 9        | 0        |
| Uncharacterized protein                   | Q4Q950_LEIMA | 84 kDa | 6   | 1   | 0        | 0        | 4.666667 | 2.603417 |
| Putative calpain-like cysteine peptidase  | Q4QCS7_LEIMA | 78 kDa | 0   | 0   | 13       | 0        | 0        | 0        |
| Uncharacterized protein                   | Q4QEX1_LEIMA | 22 kDa | 0   | 0   | 7.333333 | 2.848001 | 4.333333 | 0        |
| Putative ATP synthase, epsilon chain      | A4I651_LEIIN | 20 kDa | 0   | 0   | 0        | 0        | 5.666667 | 2.848001 |
| Glycosomal membrane like protein          | Q4QAW6_LEIMA | 24 kDa | 0   | 0   | 0        | 0        | 7        | 0        |
| Uncharacterized protein                   | Q4QHF3_LEIMA | 82 kDa | 0   | 0   | 5.333333 | 0        | 4        | 2        |
| Ecotin-like protein 3                     | ECOT3_LEIMA  | 42 kDa | 0   | 0   | 7.333333 | 0        | 4.666667 | 0        |
| Uncharacterized protein                   | Q4Q6Z3_LEIMA | 49 kDa | 0   | 0   | 5        | 0        | 6.333333 | 0        |
| Uncharacterized protein                   | E9AFR7_LEIMA | 24 kDa | 11  | 2   | 0        | 0        | 4.333333 | 1.20185  |
| Putative mitogen activated protein kinase | Q4Q449_LEIMA | 47 kDa | 5.5 | 0.5 | 0        | 0        | 7        | 3        |
| Uncharacterized protein                   | A4HXU2_LEIIN | 77 kDa | 0   | 0   | 6        | 0        | 5        | 0        |
| Cysteine protease                         | Q4Q4N3_LEIMA | 43 kDa | 0   | 0   | 5.666667 | 0        | 6.333333 | 0        |
| Fer2_3 domain-containing protein          | Q4QB10_LEIMA | 27 kDa | 9.5 | 0.5 | 0        | 0        | 0        | 0        |
| Proteasome subunit beta                   | Q4QGU1_LEIMA | 30 kDa | 4.5 | 1.5 | 4.333333 | 0        | 5.666667 | 1.666667 |
| Uncharacterized protein                   | Q4QIR5_LEIMA | 27 kDa | 0   | 0   | 0        | 0        | 4.666667 | 0        |
| Protein kinase domain-containing protein  | E9AET0_LEIMA | 54 kDa | 6   | 2   | 2.666667 | 0        | 7        | 1.732051 |
| Uncharacterized protein                   | Q4QAD7_LEIMA | 75 kDa | 0   | 0   | 4        | 0        | 5.666667 | 0        |
| Uncharacterized protein                   | Q4QJC8_LEIMA | 39 kDa | 5   | 2   | 0        | 0        | 6        | 3.21455  |
| Putative serine/threonine-protein kinase  | E9AEB9_LEIMA | 57 kDa | 0   | 0   | 0        | 0        | 8        | 3.21455  |
| Uncharacterized protein                   | Q4QH65_LEIMA | 94 kDa | 9   | 6   | 0        | 0        | 0        | 0        |
| Prolyl endopeptidase                      | Q4QHU7_LEIMA | 83 kDa | 4   | 0   | 0        | 0        | 5.666667 | 0        |
| C-8 sterol isomerase-like protein         | E9AE76_LEIMA | 25 kDa | 0   | 0   | 5.666667 | 2.185813 | 5.666667 | 1.20185  |
| Farnesyl pyrophosphate synthase           | Q4QBL1_LEIMA | 41 kDa | 8   | 2   | 3.333333 | 0        | 0        | 0        |
| Putative heat shock protein 20            | E9AEA7_LEIMA | 17 kDa | 8   | 0   | 0        | 0        | 4.333333 | 0.666667 |
| Putative 60S ribosomal protein L23a       | E9AKT6_LEIMU | 16 kDa | 0   | 0   | 5.666667 | 3.179797 | 6.333333 | 1.333333 |
| Uncharacterized protein                   | E9AGR7_LEIIN | 31 kDa | 0   | 0   | 0        | 0        | 4.333333 | 0        |
| Fructose-bisphosphatase                   | O97193_LEIMA | 39 kDa | 0   | 0   | 0        | 0        | 6.333333 | 2.333333 |
| Uncharacterized protein                   | E9ADU3_LEIMA | 53 kDa | 7   | 1   | 0        | 0        | 2.666667 | 1.452966 |
| ADP-ribosylation factor-like protein      | Q4Q756_LEIMA | 20 kDa | 4   | 0   | 3.666667 | 0        | 5.333333 | 1.452966 |
| Uncharacterized protein                   | O97220_LEIMA | 39 kDa | 0   | 0   | 5        | 0        | 4        | 0        |
| Uncharacterized protein                   | Q4QEM4_LEIMA | 40 kDa | 7   | 0   | 0        | 0        | 3.333333 | 0        |

|                                                             |              |         |     |     |          |          |          |          |
|-------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| tRNA-binding domain-containing protein                      | Q4QBU9_LEIMA | 45 kDa  | 7   | 2   | 3        | 0        | 3.333333 | 1.763834 |
| Dolichyl-diphosphooligosaccharide--protein glycotransferase | E9AET9_LEIMA | 94 kDa  | 0   | 0   | 3.666667 | 0        | 6.333333 | 3.382964 |
| Transaldolase                                               | Q4QEU3_LEIMA | 37 kDa  | 6   | 2   | 0        | 0        | 4        | 0        |
| BRO1 domain-containing protein                              | E9ADE3_LEIMA | 102 kDa | 0   | 0   | 0        | 0        | 6        | 2.081666 |
| Uncharacterized protein                                     | Q4QFI9_LEIMA | 104 kDa | 0   | 0   | 0        | 0        | 6        | 1        |
| Uncharacterized protein                                     | E9AFK1_LEIMA | 71 kDa  | 0   | 0   | 0        | 0        | 9        | 3.464102 |
| Uncharacterized protein                                     | Q4Q3F4_LEIMA | 52 kDa  | 0   | 0   | 0        | 0        | 8.666667 | 4.910307 |
| Uncharacterized protein                                     | Q4Q3Q5_LEIMA | 17 kDa  | 9.5 | 2.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4QHJ5_LEIMA | 29 kDa  | 4.5 | 1.5 | 5.333333 | 1.763834 | 3.333333 | 1.666667 |
| Elongation of fatty acids protein                           | Q4QFR7_LEIMA | 33 kDa  | 0   | 0   | 3.333333 | 1.763834 | 6        | 1.527525 |
| Putative beta eliminating lyase                             | E9AC39_LEIMA | 39 kDa  | 4.5 | 0.5 | 0        | 0        | 4.666667 | 0        |
| Metallo-peptidase, Clan MA(E), family 32                    | Q4QGE5_LEIMA | 57 kDa  | 8.5 | 1.5 | 0        | 0        | 0        | 0        |
| Putative ribosomal protein L38                              | Q4Q8X0_LEIMA | 9 kDa   | 0   | 0   | 4.666667 | 0        | 6        | 0.57735  |
| AMPK1_CBM domain-containing protein                         | Q4Q4X1_LEIMA | 97 kDa  | 5   | 3   | 0        | 0        | 3        | 0        |
| Cystathionine beta-synthase                                 | Q4QEG9_LEIMA | 39 kDa  | 3   | 1   | 4.333333 | 2.333333 | 4.666667 | 0        |
| Ubiquitin-conjugating enzyme-like protein                   | Q4QFZ6_LEIMA | 16 kDa  | 4.5 | 1.5 | 4        | 0        | 4.333333 | 0.666667 |
| Uncharacterized protein                                     | Q4Q276_LEIMA | 17 kDa  | 4   | 0   | 4        | 0        | 4        | 0        |
| Uncharacterized protein                                     | E9ABZ0_LEIMA | 37 kDa  | 2.5 | 0.5 | 6.666667 | 0        | 0        | 0        |
| Elongation of fatty acids protein                           | E9APR0_LEIMU | 32 kDa  | 0   | 0   | 2.666667 | 0        | 0        | 0        |
| Uncharacterized protein                                     | E9AEI7_LEIMA | 24 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Putative ADP-ribosylation factor                            | A4HJL1_LEIBR | 20 kDa  | 3   | 0   | 3.666667 | 1.666667 | 6        | 2        |
| Putative 60S ribosomal subunit protein L31                  | A4IBL9_LEIIN | 21 kDa  | 0   | 0   | 6        | 1        | 4        | 0        |
| Mevalonate kinase                                           | Q4Q6K7_LEIMA | 35 kDa  | 0   | 0   | 0        | 0        | 7        | 2.516611 |
| LACK                                                        | O43943_LEIMA | 34 kDa  | 0   | 0   | 3        | 0        | 5.333333 | 1.20185  |
| Putative phosphatase 2C                                     | Q4Q7S1_LEIMA | 42 kDa  | 4   | 0   | 0        | 0        | 4.666667 | 0        |
| Cysteine synthase                                           | Q4Q159_LEIMA | 35 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 2.905933 |
| D-lactate dehydrogenase-like protein                        | E9ADI2_LEIMA | 54 kDa  | 0   | 0   | 0        | 0        | 6.666667 | 0        |
| Aspartate aminotransferase                                  | Q4FX34_LEIMA | 46 kDa  | 4   | 1   | 5.333333 | 0        | 0        | 0        |
| Leucyl-tRNA synthetase                                      | Q4QG44_LEIMA | 122 kDa | 3   | 1   | 0        | 0        | 4.666667 | 2.185813 |
| Putative ER--golgi transport protein erv25                  | Q4Q1K6_LEIMA | 30 kDa  | 5   | 1   | 0        | 0        | 3.666667 | 0        |
| Uncharacterized protein                                     | E9AFU8_LEIMA | 79 kDa  | 0   | 0   | 0        | 0        | 5        | 0        |
| GTP-binding nuclear protein                                 | Q4Q9V1_LEIMA | 24 kDa  | 0   | 0   | 2.666667 | 0        | 5        | 1.154701 |
| Uncharacterized protein                                     | Q4Q8U2_LEIMA | 161 kDa | 0   | 0   | 0        | 0        | 8.333333 | 3.179797 |
| Uncharacterized protein                                     | Q4Q416_LEIMA | 72 kDa  | 3.5 | 0.5 | 0        | 0        | 4.666667 | 1.666667 |
| Uncharacterized protein                                     | E9ACC9_LEIMA | 18 kDa  | 0   | 0   | 3        | 0        | 5.333333 | 2.728451 |
| Putative lectin                                             | Q4QG87_LEIMA | 55 kDa  | 0   | 0   | 0        | 0        | 4.333333 | 0        |
| CCT-alpha                                                   | Q4Q4U5_LEIMA | 59 kDa  | 9   | 5   | 0        | 0        | 0        | 0        |
| Elongation factor 1B gamma                                  | Q66NE0_LEIMA | 46 kDa  | 6   | 1   | 0        | 0        | 0        | 0        |
| Proline dehydrogenase                                       | Q4Q933_LEIMA | 64 kDa  | 2.5 | 0.5 | 0        | 0        | 4.333333 | 2.333333 |

|                                                                 |              |         |     |     |          |          |          |          |
|-----------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative histone H3 variant                                     | Q4QDF8_LEIMA | 16 kDa  | 0   | 0   | 6.666667 | 0.881917 | 4.333333 | 0.333333 |
| Membrane-bound acid phosphatase                                 | Q4Q1G4_LEIMA | 57 kDa  | 0   | 0   | 0        | 0        | 6.666667 | 3.527668 |
| 4-coumarate:coa ligase-like protein                             | Q4QDB5_LEIMA | 66 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 0        |
| Subtilisin-like serine peptidase (Fragment)                     | A4HVN2_LEIIN | 126 kDa | 0   | 0   | 3        | 0        | 0        | 0        |
| Leishmanolysin                                                  | Q4Q8L3_LEIMA | 61 kDa  | 0   | 0   | 0        | 0        | 6.333333 | 0        |
| Uncharacterized protein                                         | A4I307_LEIIN | 22 kDa  | 4   | 0   | 3        | 1.527525 | 4        | 2.081666 |
| Probable ubiquitin-conjugating enzyme e2-17 kDa                 | Q9GNC1_LEIMA | 17 kDa  | 0   | 0   | 5        | 0        | 4.333333 | 2.333333 |
| Putative serine/threonine-protein kinase                        | Q4QC33_LEIMA | 90 kDa  | 3.5 | 0.5 | 3.333333 | 0        | 0        | 0        |
| Uncharacterized protein                                         | E9AD42_LEIMA | 20 kDa  | 12  | 6   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                         | Q4QDG5_LEIMA | 61 kDa  | 0   | 0   | 0        | 0        | 7.666667 | 0        |
| Putative RNA helicase                                           | E9AVE1_LEIMU | 49 kDa  | 0   | 0   | 3.666667 | 0        | 3        | 0        |
| Uncharacterized protein                                         | Q4QFT6_LEIMA | 30 kDa  | 0   | 0   | 6        | 3        | 0        | 0        |
| Uncharacterized protein                                         | Q4Q428_LEIMA | 36 kDa  | 0   | 0   | 3        | 1.527525 | 5.333333 | 0        |
| Putative aspartate--ammonia ligase                              | Q4Q9B1_LEIMA | 40 kDa  | 4.5 | 1.5 | 0        | 0        | 3.666667 | 2.027588 |
| Glucose-6-phosphate 1-dehydrogenase                             | Q4Q3K1_LEIMA | 63 kDa  | 0   | 0   | 0        | 0        | 6        | 3.21455  |
| Putative Qc-SNARE protein                                       | Q4QIG6_LEIMA | 24 kDa  | 0   | 0   | 0        | 0        | 3.666667 | 1.855921 |
| Uncharacterized protein                                         | Q4QEW8_LEIMA | 37 kDa  | 4.5 | 0.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                         | Q4QDG4_LEIMA | 58 kDa  | 0   | 0   | 0        | 0        | 9.666667 | 0        |
| RING-type domain-containing protein                             | Q4QHY1_LEIMA | 51 kDa  | 0   | 0   | 0        | 0        | 6.666667 | 2.403701 |
| Putative phosphatidic acid phosphatase                          | Q4QDZ8_LEIMA | 46 kDa  | 0   | 0   | 2.666667 | 0        | 0        | 0        |
| Uncharacterized protein                                         | Q4Q5Y6_LEIMA | 51 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Nucleobase transporter                                          | Q4QH25_LEIMA | 60 kDa  | 0   | 0   | 3        | 0        | 0        | 0        |
| Proteasome subunit alpha type                                   | Q4QC13_LEIMA | 25 kDa  | 0   | 0   | 3.333333 | 1.763834 | 2.666667 | 1.452966 |
| Uncharacterized protein                                         | Q4Q5N2_LEIMA | 40 kDa  | 0   | 0   | 3.666667 | 2.027588 | 4        | 0.57735  |
| Putative chaperone protein DNAj                                 | E9AU61_LEIMU | 38 kDa  | 5   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial | A4ID83_LEIIN | 44 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 0        |
| Putative universal minicircle sequence binding protein          | A4IDD1_LEIIN | 13 kDa  | 0   | 0   | 6        | 1.154701 | 4.333333 | 0.333333 |
| Transmembrane 9 superfamily member                              | Q4QG16_LEIMA | 67 kDa  | 0   | 0   | 0        | 0        | 5        | 2.516611 |
| CCT-epsilon                                                     | Q4Q5I2_LEIMA | 59 kDa  | 3.5 | 0.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                         | Q4QGD8_LEIMA | 56 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Putative cytochrome c oxidase subunit VI                        | Q4QC12_LEIMA | 19 kDa  | 0   | 0   | 0        | 0        | 3.666667 | 0.666667 |
| CCT-beta                                                        | E9ADA4_LEIMA | 58 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0        |
| Uncharacterized protein                                         | Q4Q5F6_LEIMA | 54 kDa  | 0   | 0   | 8.666667 | 0        | 0        | 0        |
| Putative peptidase M20/M25/M40                                  | Q4Q426_LEIMA | 52 kDa  | 3.5 | 0.5 | 0        | 0        | 4.333333 | 1.855921 |
| Bifunctional dihydrofolate reductase-thymidylate synthase       | DRTS_LEIMA   | 59 kDa  | 0   | 0   | 0        | 0        | 5.666667 | 3.179797 |
| Uncharacterized protein                                         | A4HYE6_LEIIN | 31 kDa  | 5   | 1   | 0        | 0        | 4        | 0        |
| Uncharacterized protein                                         | Q4Q4E4_LEIMA | 22 kDa  | 4   | 0   | 0        | 0        | 2.666667 | 1.452966 |
| Peptidylprolyl isomerase                                        | Q4QHC5_LEIMA | 17 kDa  | 0   | 0   | 4        | 0        | 3.333333 | 0        |

|                                                     |                  |         |     |     |          |          |          |          |
|-----------------------------------------------------|------------------|---------|-----|-----|----------|----------|----------|----------|
| Hydrolase, alpha/beta fold family-like protein      | Q4QE90_LEIMA     | 38 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 2.728451 |
| CAAX prenyl protease                                | E9ACY1_LEIMA     | 49 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0        |
| Uncharacterized protein                             | Q4QI82_LEIMA     | 45 kDa  | 0   | 0   | 0        | 0        | 6.333333 | 3.480102 |
| Arginase                                            | A0A145YEM0_LEIMA | 36 kDa  | 0   | 0   | 0        | 0        | 5        | 0        |
| Putative ubiquitin-activating enzyme e1             | E9AFD0_LEIMA     | 126 kDa | 0   | 0   | 3.666667 | 0        | 3        | 0        |
| Uncharacterized protein                             | E9ADB6_LEIMA     | 34 kDa  | 0   | 0   | 4        | 0.57735  | 3.333333 | 0.333333 |
| Adenine phosphoribosyltransferase                   | Q4Q9H8_LEIMA     | 26 kDa  | 0   | 0   | 3.666667 | 2.027588 | 2.666667 | 1.452966 |
| CRAL-TRIO domain-containing protein                 | Q4Q7C6_LEIMA     | 28 kDa  | 4   | 0   | 2.333333 | 1.20185  | 4.666667 | 1.20185  |
| Putative ATP-dependent DEAD/H RNA helicase          | Q4QIQ9_LEIMA     | 64 kDa  | 4   | 2   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                             | Q4Q8T2_LEIMA     | 88 kDa  | 0   | 0   | 0        | 0        | 3.666667 | 0.881917 |
| Calpain protease-like protein                       | Q4Q3Y6_LEIMA     | 151 kDa | 0   | 0   | 0        | 0        | 5.333333 | 2.848001 |
| Uncharacterized protein                             | E9ASZ8_LEIMU     | 56 kDa  | 0   | 0   | 0        | 0        | 5        | 0.57735  |
| Putative proteasome regulatory non-ATPase subunit 6 | E9ACB2_LEIMA     | 59 kDa  | 0   | 0   | 0        | 0        | 2.333333 | 0        |
| Glycerol kinase                                     | E9AFD2_LEIMA     | 56 kDa  | 0   | 0   | 0        | 0        | 4.333333 | 0        |
| Putative proteasome regulatory ATPase subunit 1     | A4HZT4_LEIIN     | 49 kDa  | 0   | 0   | 0        | 0        | 5.666667 | 1.20185  |
| Uncharacterized protein                             | E9AXP7_LEIMU     | 104 kDa | 0   | 0   | 0        | 0        | 4.333333 | 2.185813 |
| UTP--glucose-1-phosphate uridylyltransferase        | Q4QDU3_LEIMA     | 55 kDa  | 3.5 | 0.5 | 2.666667 | 1.333333 | 4        | 1.527525 |
| Uncharacterized protein                             | Q4Q561_LEIMA     | 118 kDa | 3   | 1   | 0        | 0        | 3.666667 | 0        |
| Probable citrate synthase, mitochondrial            | CISY_LEIMA       | 52 kDa  | 6   | 2   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                             | Q4QAY9_LEIMA     | 33 kDa  | 3   | 0   | 4.333333 | 0        | 0        | 0        |
| 60S ribosomal protein L30                           | E9AEK1_LEIMA     | 11 kDa  | 0   | 0   | 4        | 0        | 4.333333 | 0.666667 |
| Glucosamine-6-phosphate isomerase                   | Q4Q4U6_LEIMA     | 32 kDa  | 0   | 0   | 0        | 0        | 5        | 1.732051 |
| Uncharacterized protein                             | Q4Q6H8_LEIMA     | 48 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.603417 |
| Pyruvate dehydrogenase E1 component subunit beta    | Q4Q9R8_LEIMA     | 38 kDa  | 0   | 0   | 3        | 0        | 0        | 0        |
| Isocitrate dehydrogenase [NADP]                     | A4HUD9_LEIIN     | 48 kDa  | 0   | 0   | 3.333333 | 1.763834 | 3        | 0        |
| Reticulon-like protein                              | Q4Q737_LEIMA     | 22 kDa  | 0   | 0   | 3.333333 | 1.666667 | 4        | 0        |
| Polyadenylate-binding protein                       | A4HN85_LEIBR     | 64 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0.666667 |
| Elongation of fatty acids protein                   | Q4QFR8_LEIMA     | 33 kDa  | 3   | 0   | 0        | 0        | 0        | 0        |
| Ankyrin/TPR repeat protein                          | Q4QAW7_LEIMA     | 43 kDa  | 0   | 0   | 0        | 0        | 3.666667 | 0        |
| Proteasome subunit alpha type                       | E9AFW0_LEIMA     | 27 kDa  | 2.5 | 0.5 | 0        | 0        | 0        | 0        |
| Macrophage migration inhibitory factor-like protein | Q4Q412_LEIMA     | 13 kDa  | 3   | 0   | 0        | 0        | 3        | 0        |
| Prohibitin                                          | Q4QEK4_LEIMA     | 30 kDa  | 3.5 | 0.5 | 0        | 0        | 0        | 0        |
| T-complex protein 1 subunit delta                   | E9AGX7_LEIIN     | 60 kDa  | 0   | 0   | 0        | 0        | 3        | 0        |
| Phosphodiesterase                                   | E9AEA6_LEIMA     | 81 kDa  | 2.5 | 0.5 | 3.333333 | 0        | 3.333333 | 0        |
| AMMECR1 domain-containing protein                   | Q4Q5M0_LEIMA     | 21 kDa  | 0   | 0   | 0        | 0        | 3.666667 | 2.027588 |
| Uncharacterized protein                             | A4IB40_LEIIN     | 36 kDa  | 0   | 0   | 0        | 0        | 3        | 0        |
| Vesicle-fusing ATPase                               | E9AD85_LEIMA     | 108 kDa | 0   | 0   | 0        | 0        | 4        | 0        |
| Uncharacterized protein                             | Q4QGT3_LEIMA     | 32 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |

|                                                                     |              |         |     |     |          |          |          |          |
|---------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Uncharacterized protein                                             | Q4QAF8_LEIMA | 156 kDa | 0   | 0   | 7.333333 | 0        | 0        | 0        |
| Uncharacterized protein                                             | Q4Q3P1_LEIMA | 69 kDa  | 0   | 0   | 8        | 0        | 0        | 0        |
| Surface antigen-like protein                                        | Q4QJB2_LEIMA | 30 kDa  | 0   | 0   | 8        | 0        | 0        | 0        |
| Methionine--tRNA ligase                                             | Q4QCD2_LEIMA | 84 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 0        |
| Translation initiation factor-like protein                          | Q4QIB4_LEIMA | 38 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.603417 |
| Putative U-box domain protein                                       | E9ADT0_LEIMA | 67 kDa  | 5   | 1   | 0        | 0        | 2.666667 | 1.452966 |
| Hypothetical predicted transmembrane protein                        | Q4QAT6_LEIMA | 152 kDa | 0   | 0   | 0        | 0        | 2.666667 | 1.452966 |
| Uncharacterized protein                                             | E9ACA6_LEIMA | 74 kDa  | 0   | 0   | 3.666667 | 0        | 3        | 0        |
| ACB domain-containing protein                                       | Q4QED0_LEIMA | 11 kDa  | 4.5 | 0.5 | 2.666667 | 0        | 2        | 0        |
| Putative tyrosyl-tRNA synthetase                                    | Q4QFJ7_LEIMA | 75 kDa  | 5.5 | 0.5 | 0        | 0        | 0        | 0        |
| Excreted/secreted protein 27                                        | Q1X7K5_LEIMA | 13 kDa  | 0   | 0   | 0        | 0        | 4        | 1        |
| Putative monoglyceride lipase                                       | Q4Q6E9_LEIMA | 35 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Protein kinase domain-containing protein                            | Q4QFA8_LEIMA | 74 kDa  | 0   | 0   | 0        | 0        | 5        | 1.527525 |
| Putative nucleosome assembly protein                                | Q4QDH7_LEIMA | 40 kDa  | 2.5 | 0.5 | 0        | 0        | 4.333333 | 0        |
| Putative glutamamyl carboxypeptidase                                | E9AE16_LEIMA | 44 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 0        |
| Putative cytochrome c oxidase VII                                   | Q4Q9Y0_LEIMA | 19 kDa  | 0   | 0   | 0        | 0        | 3.666667 | 0        |
| Uncharacterized protein                                             | E9B244_LEIMU | 25 kDa  | 0   | 0   | 2.666667 | 0        | 3        | 1.527525 |
| MRH domain-containing protein                                       | Q4QBV4_LEIMA | 128 kDa | 6   | 4   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                             | Q4Q3P0_LEIMA | 71 kDa  | 0   | 0   | 8        | 4.358899 | 0        | 0        |
| Tyrosyl or methionyl-tRNA synthetase-like protein                   | Q4QEQ4_LEIMA | 20 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0        |
| Thioredoxin-like_fold domain-containing protein                     | Q4QFW6_LEIMA | 22 kDa  | 4   | 2   | 0        | 0        | 0        | 0        |
| Putative phosphomevalonate kinase protein                           | A4HWN3_LEIIN | 49 kDa  | 0   | 0   | 0        | 0        | 4.333333 | 0.881917 |
| Uncharacterized protein                                             | E9AF39_LEIMA | 48 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.452966 |
| Putative eukaryotic release factor 3                                | A4HV24_LEIIN | 83 kDa  | 0   | 0   | 0        | 0        | 4.333333 | 2.333333 |
| Uncharacterized protein                                             | Q4QEJ8_LEIMA | 88 kDa  | 5   | 0   | 0        | 0        | 3        | 0        |
| Glycerol-3-phosphate dehydrogenase                                  | Q4Q8P6_LEIMA | 67 kDa  | 0   | 0   | 0        | 0        | 5.666667 | 2.962731 |
| Polyprenol reductase                                                | Q4Q9R2_LEIMA | 34 kDa  | 0   | 0   | 0        | 0        | 6        | 3        |
| ATP-dependent Clp protease subunit, heat shock protein 100 (HSP100) | A4I4I4_LEIIN | 97 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.603417 |
| Isopentenyl-pyrophosphate isomerase                                 | Q5QQ43_LEIMA | 40 kDa  | 0   | 0   | 0        | 0        | 5        | 0        |
| Uncharacterized protein                                             | Q4QFS9_LEIMA | 51 kDa  | 0   | 0   | 7        | 3.785939 | 0        | 0        |
| Uncharacterized protein                                             | Q4QFB6_LEIMA | 54 kDa  | 0   | 0   | 2.333333 | 0        | 0        | 0        |
| CCT-eta                                                             | A4IBS4_LEIIN | 62 kDa  | 0   | 0   | 0        | 0        | 2        | 0        |
| Putative eukaryotic translation initiation factor 2 subunit         | Q4QHR7_LEIMA | 53 kDa  | 0   | 0   | 0        | 0        | 4        | 1.154701 |
| ATP pyrophosphate-lyase                                             | Q4Q8R1_LEIMA | 71 kDa  | 2.5 | 0.5 | 0        | 0        | 0        | 0        |
| Sucrose-phosphate synthase-like protein                             | Q4QES5_LEIMA | 52 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Putative proteasome regulatory ATPase subunit tcc118.3              | E9AGB0_LEIIN | 46 kDa  | 0   | 0   | 0        | 0        | 2        | 0        |
| Protein kinase domain-containing protein                            | Q4QIJ9_LEIMA | 141 kDa | 5.5 | 3.5 | 0        | 0        | 0        | 0        |

|                                                                                  |              |         |   |   |          |   |          |          |
|----------------------------------------------------------------------------------|--------------|---------|---|---|----------|---|----------|----------|
| Protein kinase domain-containing protein                                         | E9ACD1_LEIMA | 154 kDa | 3 | 1 | 0        | 0 | 0        | 0        |
| Putative ABC transporter domain protein                                          | E9AE26_LEIMA | 50 kDa  | 0 | 0 | 0        | 0 | 6.666667 | 1.855921 |
| Amastin-like protein                                                             | Q4QI85_LEIMA | 22 kDa  | 0 | 0 | 2        | 0 | 0        | 0        |
| RING-type E3 ubiquitin transferase                                               | Q4QDS7_LEIMA | 39 kDa  | 0 | 0 | 2.666667 | 0 | 0        | 0        |
| Spermidine synthase                                                              | Q95Z84_LEIMA | 33 kDa  | 0 | 0 | 0        | 0 | 3.666667 | 0        |
| Proteasome regulatory ATPase subunit                                             | Q4Q0X8_LEIMA | 46 kDa  | 4 | 1 | 0        | 0 | 2.666667 | 1.333333 |
| Uncharacterized protein                                                          | Q4QCS0_LEIMA | 87 kDa  | 5 | 1 | 0        | 0 | 2        | 0        |
| Putative epsin                                                                   | Q4QA28_LEIMA | 66 kDa  | 0 | 0 | 0        | 0 | 4        | 2        |
| Conserved SNF-7-like protein                                                     | E9AEN3_LEIMA | 26 kDa  | 0 | 0 | 0        | 0 | 4.666667 | 1.666667 |
| Putative 40S ribosomal protein S27-1                                             | Q4Q140_LEIMA | 10 kDa  | 0 | 0 | 4        | 0 | 3.333333 | 0.666667 |
| Uncharacterized protein                                                          | Q4QC98_LEIMA | 200 kDa | 0 | 0 | 0        | 0 | 2        | 0        |
| Uncharacterized protein                                                          | Q4QHJ4_LEIMA | 54 kDa  | 0 | 0 | 4.333333 | 0 | 0        | 0        |
| Putative enoyl-CoA hydratase/Enoyl-CoA isomerase/3-hydroxyacyl-CoA dehydrogenase | Q4Q3S6_LEIMA | 102 kDa | 0 | 0 | 0        | 0 | 3.666667 | 0        |
| Proteasome subunit alpha type                                                    | Q4QFV4_LEIMA | 32 kDa  | 0 | 0 | 0        | 0 | 2.333333 | 0        |
| Nucleosome assembly protein-like protein                                         | Q4Q687_LEIMA | 45 kDa  | 0 | 0 | 0        | 0 | 4        | 0        |
| TNase-like domain-containing protein                                             | Q4QIW8_LEIMA | 25 kDa  | 3 | 1 | 0        | 0 | 2.666667 | 1.452966 |
| Uncharacterized protein                                                          | Q4QGN3_LEIMA | 55 kDa  | 4 | 2 | 0        | 0 | 0        | 0        |
| Putative small GTP-binding protein                                               | E9AKA7_LEIMU | 22 kDa  | 0 | 0 | 0        | 0 | 3        | 0        |
| Qa-SNARE protein                                                                 | E9AZW7_LEIMU | 39 kDa  | 0 | 0 | 0        | 0 | 2.666667 | 1.333333 |
| Putative ubiquitin-conjugating enzyme e2                                         | Q9NED4_LEIMA | 17 kDa  | 4 | 1 | 0        | 0 | 0        | 0        |
| Uncharacterized protein                                                          | Q4QD05_LEIMA | 36 kDa  | 0 | 0 | 5        | 0 | 0        | 0        |
| Protein antigen LmSTI1 (Fragment)                                                | P90553_LEIMA | 62 kDa  | 4 | 1 | 0        | 0 | 0        | 0        |
| ER membrane protein complex subunit 3                                            | Q4Q242_LEIMA | 30 kDa  | 0 | 0 | 0        | 0 | 3.333333 | 0        |
| Electron transfer flavoprotein-ubiquinone oxidoreductase                         | Q4QIN2_LEIMA | 63 kDa  | 0 | 0 | 0        | 0 | 1.666667 | 0.881917 |
| RuvB-like helicase                                                               | A4IAB6_LEIIN | 50 kDa  | 0 | 0 | 0        | 0 | 3        | 0        |
| Putative 60S ribosomal protein L44                                               | A4H7F7_LEIBR | 12 kDa  | 0 | 0 | 3.666667 | 0 | 0        | 0        |
| Calcium-transporting ATPase                                                      | Q4QIM8_LEIMA | 122 kDa | 5 | 2 | 0        | 0 | 0        | 0        |
| Glucose-6-phosphate 1-epimerase                                                  | Q4Q2K6_LEIMA | 33 kDa  | 0 | 0 | 0        | 0 | 3.333333 | 0.881917 |
| Uncharacterized protein                                                          | Q4Q7J9_LEIMA | 29 kDa  | 0 | 0 | 0        | 0 | 2.666667 | 1.452966 |
| Amastin-like protein                                                             | Q4Q3A8_LEIMA | 23 kDa  | 0 | 0 | 4.666667 | 0 | 0        | 0        |
| Putative ras-like small GTPases                                                  | Q4Q6H7_LEIMA | 64 kDa  | 0 | 0 | 0        | 0 | 4        | 2.081666 |
| Putative proteasome alpha 7 subunit                                              | E9ACZ6_LEIMA | 26 kDa  | 0 | 0 | 2.666667 | 0 | 0        | 0        |
| Uncharacterized protein                                                          | E9AEY5_LEIMA | 49 kDa  | 3 | 0 | 2.666667 | 0 | 0        | 0        |
| Carboxypeptidase                                                                 | Q4QDZ7_LEIMA | 52 kDa  | 0 | 0 | 0        | 0 | 4.333333 | 1.20185  |
| Ascorbate peroxidase                                                             | Q4Q3K2_LEIMA | 34 kDa  | 0 | 0 | 0        | 0 | 3        | 1.527525 |
| Putative ATP synthase                                                            | Q4Q1A9_LEIMA | 25 kDa  | 0 | 0 | 0        | 0 | 2.333333 | 1.20185  |
| Mannose-6-phosphate isomerase                                                    | Q4Q5C3_LEIMA | 46 kDa  | 0 | 0 | 0        | 0 | 2.333333 | 1.20185  |

|                                                                                                                                    |              |         |     |     |          |          |          |          |
|------------------------------------------------------------------------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative ER--golgi transport protein p24                                                                                           | Q4Q574_LEIMA | 18 kDa  | 0   | 0   | 0        | 0        | 2.333333 | 0        |
| Putative amastin-like surface protein                                                                                              | Q4Q327_LEIMA | 21 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.452966 |
| Surface antigen-like protein                                                                                                       | Q9XZY1_LEIMA | 28 kDa  | 0   | 0   | 4.333333 | 0        | 0        | 0        |
| Vesicle-fusing ATPase                                                                                                              | Q4QCW5_LEIMA | 81 kDa  | 0   | 0   | 0        | 0        | 2.333333 | 0        |
| Glutamine amidotransferase                                                                                                         | Q4QBY3_LEIMA | 71 kDa  | 2   | 0   | 0        | 0        | 0        | 0        |
| Putative small GTP-binding protein Rab18                                                                                           | Q4Q404_LEIMA | 24 kDa  | 0   | 0   | 0        | 0        | 3        | 1        |
| Kinesin-like protein                                                                                                               | E9AWQ4_LEIMU | 63 kDa  | 0   | 0   | 0        | 0        | 4.333333 | 2.185813 |
| CS domain-containing protein                                                                                                       | Q4QFT9_LEIMA | 37 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0        |
| Putative ER--golgi transport protein gp25L                                                                                         | E9AF06_LEIMA | 25 kDa  | 0   | 0   | 0        | 0        | 3        | 0        |
| Eukaryotic translation initiation factor 3 subunit L                                                                               | Q4Q253_LEIMA | 73 kDa  | 2.5 | 0.5 | 0        | 0        | 2.333333 | 1.20185  |
| Valyl-tRNA synthetase                                                                                                              | Q4Q6X7_LEIMA | 110 kDa | 0   | 0   | 0        | 0        | 3        | 0        |
| Putative short chain dehydrogenase                                                                                                 | E9AEU6_LEIMA | 28 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0        |
| Kinesin-like protein                                                                                                               | Q4Q7S4_LEIMA | 68 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0        |
| Surface antigen-like protein                                                                                                       | Q9XZY0_LEIMA | 18 kDa  | 0   | 0   | 3.333333 | 1.666667 | 0        | 0        |
| Proteasome regulatory non-ATP-ase subunit 11, putative (19s proteasome regulatory subunit, metallo-peptidase, clan mp, family m67) | E9B4N4_LEIMU | 35 kDa  | 0   | 0   | 0        | 0        | 2.333333 | 1.20185  |
| Putative amino acid permease                                                                                                       | Q4QBX3_LEIMA | 54 kDa  | 0   | 0   | 0        | 0        | 2.333333 | 0        |
| Uncharacterized protein                                                                                                            | A4IAR5_LEIIN | 14 kDa  | 4   | 0   | 0        | 0        | 1.333333 | 0.666667 |
| Ubiquitin carboxyl-terminal hydrolase                                                                                              | E9AE92_LEIMA | 82 kDa  | 0   | 0   | 0        | 0        | 2        | 0        |
| Protein kinase domain-containing protein                                                                                           | Q4Q2P2_LEIMA | 99 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Uncharacterized protein                                                                                                            | Q4Q602_LEIMA | 104 kDa | 0   | 0   | 0        | 0        | 2.666667 | 0        |
| Calmodulin                                                                                                                         | E9AMU3_LEIMU | 17 kDa  | 7.5 | 0.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                                                                                            | E9ADB1_LEIMA | 60 kDa  | 0   | 0   | 0        | 0        | 2        | 0        |
| Uncharacterized protein                                                                                                            | Q4QI34_LEIMA | 19 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 0.333333 |
| Putative intraflagellar transport protein component                                                                                | Q4QDI9_LEIMA | 73 kDa  | 0   | 0   | 0        | 0        | 2        | 0        |
| 6-phosphogluconolactonase                                                                                                          | Q4Q8S1_LEIMA | 28 kDa  | 4   | 1   | 0        | 0        | 0        | 0        |
| Peptidylprolyl isomerase                                                                                                           | Q4QDB9_LEIMA | 23 kDa  | 0   | 0   | 0        | 0        | 3        | 0        |
| Uncharacterized protein                                                                                                            | E9AFA3_LEIMA | 44 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Uncharacterized protein                                                                                                            | Q4Q3P4_LEIMA | 186 kDa | 0   | 0   | 5.333333 | 0        | 0        | 0        |
| 60S acidic ribosomal protein P2                                                                                                    | Q4QF62_LEIMA | 11 kDa  | 4   | 0   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                                                                                            | Q4QGQ4_LEIMA | 44 kDa  | 4   | 2   | 0        | 0        | 0        | 0        |
| Putative P27 protein                                                                                                               | Q4Q8G9_LEIMA | 28 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Putative Tob55                                                                                                                     | E9AE43_LEIMA | 53 kDa  | 0   | 0   | 0        | 0        | 3        | 1.527525 |
| Protein kinase domain-containing protein                                                                                           | Q4Q1Y6_LEIMA | 88 kDa  | 0   | 0   | 0        | 0        | 2        | 0        |
| Uncharacterized protein                                                                                                            | Q4QD70_LEIMA | 26 kDa  | 0   | 0   | 0        | 0        | 2.333333 | 0.333333 |
| Putative small GTP-binding protein Rab28                                                                                           | Q4Q7C3_LEIMA | 26 kDa  | 0   | 0   | 1.666667 | 0.881917 | 1.666667 | 0.881917 |
| Putative casein kinase 1 isoform 2                                                                                                 | Q4Q6U1_LEIMA | 42 kDa  | 0   | 0   | 0        | 0        | 1.666667 | 0.881917 |
| Uncharacterized protein                                                                                                            | Q4Q4V1_LEIMA | 24 kDa  | 0   | 0   | 3.333333 | 0        | 0        | 0        |

|                                                               |              |         |     |     |   |   |          |          |
|---------------------------------------------------------------|--------------|---------|-----|-----|---|---|----------|----------|
| Putative 1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase | Q4Q281_LEIMA | 33 kDa  | 0   | 0   | 0 | 0 | 2.333333 | 1.20185  |
| Uncharacterized protein                                       | Q4Q3P2_LEIMA | 145 kDa | 0   | 0   | 5 | 0 | 0        | 0        |
| Xanthine phosphoribosyltransferase                            | Q4QCC2_LEIMA | 27 kDa  | 0   | 0   | 0 | 0 | 3.666667 | 0        |
| Putative prostaglandin f synthase                             | A4I7L0_LEIIN | 32 kDa  | 0   | 0   | 0 | 0 | 2        | 1        |
| Thiolase protein-like protein                                 | Q4Q698_LEIMA | 47 kDa  | 0   | 0   | 0 | 0 | 2        | 1        |
| START domain-containing protein                               | Q4QGZ1_LEIMA | 37 kDa  | 0   | 0   | 0 | 0 | 2.333333 | 1.20185  |
| VHS domain-containing protein                                 | Q4Q0P8_LEIMA | 56 kDa  | 0   | 0   | 0 | 0 | 1.666667 | 0.881917 |
| Proteasome subunit alpha type                                 | Q4QH56_LEIMA | 28 kDa  | 0   | 0   | 3 | 0 | 0        | 0        |
| Putative ubiquitin hydrolase                                  | E9B0P9_LEIMU | 111 kDa | 0   | 0   | 0 | 0 | 1.666667 | 0.881917 |
| Uncharacterized protein                                       | Q4QCD8_LEIMA | 78 kDa  | 0   | 0   | 0 | 0 | 1.333333 | 0.666667 |
| Putative elongation factor Tu                                 | Q4QDW8_LEIMA | 52 kDa  | 0   | 0   | 0 | 0 | 2.666667 | 1.452966 |
| Putative 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase      | Q4Q9N8_LEIMA | 30 kDa  | 2.5 | 0.5 | 0 | 0 | 0        | 0        |
| Uncharacterized protein                                       | Q4Q1W3_LEIMA | 50 kDa  | 0   | 0   | 0 | 0 | 2.666667 | 1.452966 |
| Uncharacterized protein                                       | A4I1M3_LEIIN | 17 kDa  | 0   | 0   | 4 | 0 | 0        | 0        |
| Uncharacterized protein                                       | E9AF04_LEIMA | 82 kDa  | 0   | 0   | 0 | 0 | 3        | 0        |
| Permease-like protein                                         | Q4QBC9_LEIMA | 122 kDa | 0   | 0   | 0 | 0 | 3        | 0        |
| Serine hydroxymethyltransferase                               | Q4QFK2_LEIMA | 51 kDa  | 0   | 0   | 0 | 0 | 2.666667 | 1.452966 |
| Nucleolar protein family a member-like protein                | Q4Q2E3_LEIMA | 17 kDa  | 0   | 0   | 0 | 0 | 2.666667 | 1.452966 |
| Uncharacterized protein                                       | E9AFI1_LEIMA | 133 kDa | 0   | 0   | 0 | 0 | 3.333333 | 0        |
| Putative developmentally regulated GTP-binding protein 1      | A4IDJ9_LEIIN | 41 kDa  | 0   | 0   | 0 | 0 | 3.333333 | 0        |
| Uncharacterized protein                                       | Q4Q130_LEIMA | 26 kDa  | 5.5 | 1.5 | 0 | 0 | 0        | 0        |
| ANAPC4_WD40 domain-containing protein                         | Q4QI50_LEIMA | 139 kDa | 0   | 0   | 0 | 0 | 1.333333 | 0.666667 |
| Cilia- and flagella-associated protein 36                     | Q4Q1T6_LEIMA | 48 kDa  | 0   | 0   | 2 | 0 | 0        | 0        |
| Uncharacterized protein                                       | E9AFJ3_LEIMA | 27 kDa  | 0   | 0   | 0 | 0 | 2.333333 | 0        |
| Uncharacterized protein                                       | Q4Q387_LEIMA | 75 kDa  | 0   | 0   | 0 | 0 | 3        | 0        |
| Putative fucose kinase                                        | Q4QEX2_LEIMA | 127 kDa | 0   | 0   | 0 | 0 | 3        | 0        |
| Uncharacterized protein                                       | Q4Q7P7_LEIMA | 71 kDa  | 0   | 0   | 0 | 0 | 2        | 0        |
| Uncharacterized protein                                       | Q4Q101_LEIMA | 22 kDa  | 0   | 0   | 3 | 0 | 0        | 0        |
| Kinesin-like protein                                          | Q4QFZ3_LEIMA | 81 kDa  | 0   | 0   | 0 | 0 | 2.333333 | 1.20185  |
| Uncharacterized protein                                       | Q4Q4S4_LEIMA | 67 kDa  | 0   | 0   | 0 | 0 | 2.333333 | 0        |
| Uncharacterized protein                                       | Q4QDY7_LEIMA | 41 kDa  | 0   | 0   | 0 | 0 | 1.333333 | 0.666667 |
| Putative ATP-binding cassette protein subfamily H member 1    | Q4QH76_LEIMA | 33 kDa  | 0   | 0   | 0 | 0 | 1.333333 | 0.666667 |

*Supplemental Table 1. L. major EVs protein content comparing WT L. major, Vps36<sup>(-/-)</sup> L. major, and Vps36 L. major EVs. Protein quantity is represented by total spectral count averaged between each replicate with accompanying SEM.*

| Simplified protein name                                 | Uniprot accession | Molecular weight | Vps36<br><i>L.major</i><br>WP<br>average<br>spectral<br>count | Vps36<br><i>L.major</i><br>WP SEM<br>spectral<br>count | Vps36 <sup>(-/-)</sup><br><i>L.major</i><br>WP<br>average<br>spectral<br>count | Vps36 <sup>(-/-)</sup><br><i>L.major</i><br>WP SEM<br>spectral<br>count | WT<br><i>L.major</i><br>WP<br>average<br>spectral<br>count | WT<br><i>L.major</i><br>WP SEM<br>spectral<br>count |
|---------------------------------------------------------|-------------------|------------------|---------------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------------------------------|-------------------------------------------------------------------------|------------------------------------------------------------|-----------------------------------------------------|
| Putative heat-shock protein hsp70                       | Q4Q7Y4_LEIMA      | 72 kDa           | 309                                                           | 89                                                     | 835.3333                                                                       | 1.333333                                                                | 988                                                        | 165.606                                             |
| Putative heat shock 70-related protein 1, mitochondrial | Q4Q744_LEIMA      | 72 kDa           | 240.5                                                         | 81.5                                                   | 626                                                                            | 20.52641                                                                | 786.6667                                                   | 73.99399                                            |
| Putative calpain-like cysteine peptidase                | E9AD27_LEIMA      | 700 kDa          | 266.5                                                         | 127.5                                                  | 593.3333                                                                       | 43.33333                                                                | 453.3333                                                   | 13.96822                                            |
| Elongation factor 2                                     | E9ASD6_LEIMU      | 94 kDa           | 225                                                           | 31                                                     | 529.3333                                                                       | 34.26044                                                                | 528                                                        | 35.38361                                            |
| Heat shock protein 83-1                                 | Q4Q4I6_LEIMA      | 81 kDa           | 232                                                           | 97                                                     | 432.6667                                                                       | 26.89073                                                                | 528.6667                                                   | 145.8416                                            |
| Elongation factor 1-alpha                               | Q4QEI9_LEIMA      | 49 kDa           | 234.5                                                         | 50.5                                                   | 362.6667                                                                       | 52.23451                                                                | 457.3333                                                   | 78.56491                                            |
| Putative glucose-regulated protein 78                   | Q4Q8E6_LEIMA      | 72 kDa           | 122                                                           | 33                                                     | 372.6667                                                                       | 33.15284                                                                | 380.6667                                                   | 22.78401                                            |
| Leishmanolysin                                          | GP63_LEIMA        | 64 kDa           | 200.5                                                         | 70.5                                                   | 317.3333                                                                       | 29.53717                                                                | 368                                                        | 72.59017                                            |
| NAD-specific glutamate dehydrogenase                    | Q4QF83_LEIMA      | 115 kDa          | 150                                                           | 26                                                     | 315.3333                                                                       | 13.33333                                                                | 287.3333                                                   | 17.37175                                            |
| Aconitate hydratase                                     | Q4QDZ1_LEIMA      | 97 kDa           | 128.5                                                         | 21.5                                                   | 331.3333                                                                       | 14.43761                                                                | 303.3333                                                   | 25.9829                                             |
| Glyceraldehyde-3-phosphate dehydrogenase                | Q4Q6Z5_LEIMA      | 39 kDa           | 248.5                                                         | 59.5                                                   | 232                                                                            | 47.0319                                                                 | 300.6667                                                   | 31.52424                                            |
| ATP-dependent RNA helicase eIF4A                        | Q4Q5P5_LEIMA      | 67 kDa           | 111                                                           | 24                                                     | 290.6667                                                                       | 19.91091                                                                | 308.6667                                                   | 11.21507                                            |
| Phosphopyruvate hydratase                               | Q4QFL8_LEIMA      | 46 kDa           | 103.5                                                         | 32.5                                                   | 289.3333                                                                       | 23.21877                                                                | 276                                                        | 14                                                  |
| Putative ribosomal protein L3                           | Q4Q2Q6_LEIMA      | 47 kDa           | 71.5                                                          | 14.5                                                   | 250.6667                                                                       | 12.97861                                                                | 274                                                        | 13.01281                                            |
| Putative glycosomal phosphoenolpyruvate carboxykinase   | E9ADF9_LEIMA      | 58 kDa           | 78                                                            | 8                                                      | 261.3333                                                                       | 6.666667                                                                | 296                                                        | 21.93931                                            |
| Peroxidoxin                                             | Q9TZS4_LEIMA      | 22 kDa           | 102.5                                                         | 13.5                                                   | 232                                                                            | 19.21805                                                                | 305.3333                                                   | 98.3757                                             |
| Heat shock protein 70-related protein                   | Q4Q970_LEIMA      | 71 kDa           | 119.5                                                         | 17.5                                                   | 248.6667                                                                       | 19.67514                                                                | 246.6667                                                   | 20.40697                                            |
| Pyruvate, phosphate dikinase                            | Q4QGX9_LEIMA      | 101 kDa          | 92.5                                                          | 29.5                                                   | 214                                                                            | 38.10512                                                                | 208                                                        | 33.60556                                            |
| Phosphotransferase                                      | Q4VT70_LEIMA      | 52 kDa           | 107                                                           | 14                                                     | 211.3333                                                                       | 6.359595                                                                | 185.3333                                                   | 22.78401                                            |
| ATP synthase subunit beta                               | Q4Q9X6_LEIMA      | 56 kDa           | 60                                                            | 5                                                      | 214                                                                            | 5.291503                                                                | 211.3333                                                   | 14.43761                                            |
| Putative aminopeptidase                                 | Q4QH17_LEIMA      | 57 kDa           | 76                                                            | 22                                                     | 199.3333                                                                       | 1.333333                                                                | 199.3333                                                   | 3.527668                                            |
| H(+)-exporting diphosphatase                            | Q4Q6E1_LEIMA      | 84 kDa           | 37.5                                                          | 5.5                                                    | 204.6667                                                                       | 16.42491                                                                | 223.3333                                                   | 17.52459                                            |
| HMG_CoA_synt_N domain-containing protein                | Q4QAB9_LEIMA      | 55 kDa           | 92.5                                                          | 22.5                                                   | 219.3333                                                                       | 6.359595                                                                | 174                                                        | 20.78461                                            |
| Multifunctional fusion protein                          | E9ACG7_LEIMA      | 62 kDa           | 65.5                                                          | 19.5                                                   | 208                                                                            | 8.326664                                                                | 182.6667                                                   | 22.4004                                             |
| Putative NADH-dependent fumarate reductase              | E9AEU1_LEIMA      | 123 kDa          | 68.5                                                          | 19.5                                                   | 199.3333                                                                       | 10.72898                                                                | 172.6667                                                   | 17.52459                                            |
| 40S ribosomal protein S4                                | Q4QG31_LEIMA      | 31 kDa           | 28.5                                                          | 14.5                                                   | 178                                                                            | 15.01111                                                                | 202                                                        | 11.01514                                            |
| Putative heat shock protein                             | Q4QDQ2_LEIMA      | 92 kDa           | 117                                                           | 15                                                     | 173.3333                                                                       | 25.41216                                                                | 183.3333                                                   | 7.512952                                            |
| Uncharacterized protein                                 | Q4QB61_LEIMA      | 16 kDa           | 39.5                                                          | 8.5                                                    | 94.66667                                                                       | 11.85093                                                                | 231.3333                                                   | 0                                                   |
| Plasma membrane ATPase                                  | Q4QDN8_LEIMA      | 107 kDa          | 16.5                                                          | 1.5                                                    | 210.6667                                                                       | 11.68094                                                                | 209.3333                                                   | 34.0653                                             |
| Protein disulfide-isomerase                             | Q4Q059_LEIMA      | 52 kDa           | 57.5                                                          | 15.5                                                   | 186.6667                                                                       | 15.33333                                                                | 173.3333                                                   | 16.42491                                            |
| Peptidyl-prolyl cis-trans isomerase                     | O02614_LEIMA      | 19 kDa           | 71                                                            | 26                                                     | 194                                                                            | 17.0098                                                                 | 177.3333                                                   | 16.42491                                            |
| Cysteine peptidase C (CPC)                              | E9ADT5_LEIMA      | 37 kDa           | 35.5                                                          | 9.5                                                    | 192.6667                                                                       | 12.12894                                                                | 181.3333                                                   | 8.819171                                            |
| Histone H4                                              | A4HW98_LEIIN      | 11 kDa           | 0                                                             | 0                                                      | 156                                                                            | 11.01514                                                                | 226                                                        | 73.02055                                            |
| Phosphoglycerate kinase, glycosomal                     | PGKC_LEIMA        | 52 kDa           | 67.5                                                          | 26.5                                                   | 150                                                                            | 5.291503                                                                | 143.3333                                                   | 10.66667                                            |
| Clathrin heavy chain                                    | Q4Q1R2_LEIMA      | 190 kDa          | 88                                                            | 40                                                     | 155.3333                                                                       | 20.66667                                                                | 152.6667                                                   | 11.09554                                            |

|                                                               |              |         |      |      |          |          |          |          |
|---------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Pyruvate kinase                                               | E9AEI0_LEIMA | 54 kDa  | 72   | 12   | 177.3333 | 14.62114 | 146      | 13.31666 |
| Tubulin alpha chain                                           | Q4QGC5_LEIMA | 50 kDa  | 64.5 | 15.5 | 158      | 20.81666 | 160.6667 | 14.25171 |
| Putative 2-oxoglutarate dehydrogenase E1 component            | Q4Q171_LEIMA | 111 kDa | 48   | 18   | 174      | 6.429101 | 156      | 13.01281 |
| Putative Transitional endoplasmic reticulum ATPase            | Q4Q1T9_LEIMA | 87 kDa  | 53.5 | 18.5 | 172.6667 | 24.66667 | 145.3333 | 44.51716 |
| Metallo-peptidase, Clan ME, Family M16                        | E9AEW1_LEIMA | 55 kDa  | 22   | 5    | 180.6667 | 14.25171 | 161.3333 | 6.359595 |
| Histone H2A                                                   | E9AE33_LEIMA | 14 kDa  | 0    | 0    | 143.3333 | 7.512952 | 209.3333 | 38.6839  |
| Putative heat shock protein DNAJ                              | E9ACW0_LEIMA | 44 kDa  | 63   | 5    | 170.6667 | 5.696002 | 131.3333 | 15.07021 |
| Histone H2B                                                   | Q4QDL6_LEIMA | 12 kDa  | 0    | 0    | 142.6667 | 23.33333 | 209.3333 | 64.33592 |
| Succinyl-CoA:3-ketoacid-coenzyme A transferase                | Q4Q3V3_LEIMA | 53 kDa  | 71   | 21   | 167.3333 | 7.512952 | 130.6667 | 4.807402 |
| Putative lipophosphoglycan biosynthetic protein               | E9ADS8_LEIMA | 87 kDa  | 56   | 25   | 138      | 19.69772 | 138      | 12.49    |
| Putative GTP-binding protein                                  | E9AE82_LEIMA | 77 kDa  | 59   | 15   | 153.3333 | 20.53723 | 145.3333 | 5.696002 |
| Uncharacterized protein                                       | Q4Q6V8_LEIMA | 41 kDa  | 28   | 11   | 166      | 5.291503 | 149.3333 | 3.333333 |
| Transketolase                                                 | Q4QAC4_LEIMA | 72 kDa  | 70   | 19   | 141.3333 | 19.36779 | 124.6667 | 13.53186 |
| FGE-sulfatase domain-containing protein                       | E9ADV2_LEIMA | 87 kDa  | 36.5 | 11.5 | 162      | 6.429101 | 128      | 17.0098  |
| Uncharacterized protein                                       | Q4QEJ8_LEIMA | 88 kDa  | 48.5 | 12.5 | 142.6667 | 3.527668 | 142.6667 | 14.43761 |
| 60S ribosomal protein L18                                     | RL18_LEIMA   | 22 kDa  | 0    | 0    | 147.3333 | 12.34684 | 131.3333 | 16.70662 |
| Tubulin beta chain                                            | A4HLC9_LEIBR | 50 kDa  | 55   | 4    | 143.3333 | 24.69368 | 133.3333 | 22.69606 |
| ATP-dependent 6-phosphofructokinase                           | E9AEB3_LEIMA | 54 kDa  | 18   | 9    | 158.6667 | 7.423686 | 142.6667 | 7.859884 |
| Putative 60S ribosomal protein L13a                           | Q4Q3B9_LEIMA | 25 kDa  | 21   | 9    | 120      | 15.27525 | 154.6667 | 7.512952 |
| Putative aldose 1-epimerase                                   | E9AES2_LEIMA | 41 kDa  | 58   | 12   | 146.6667 | 0.666667 | 128      | 7.023769 |
| Dihydrolipoyl dehydrogenase                                   | Q4Q4U1_LEIMA | 51 kDa  | 42.5 | 13.5 | 142      | 8.082904 | 126.6667 | 12.23837 |
| Uncharacterized protein                                       | Q4Q8Z6_LEIMA | 90 kDa  | 78.5 | 25.5 | 118.6667 | 18.41497 | 120      | 11.01514 |
| Putative IgE-dependent histamine-releasing factor             | Q4QAI0_LEIMA | 19 kDa  | 59.5 | 37.5 | 115.3333 | 3.527668 | 126      | 16.04161 |
| Putative eukaryotic translation initiation factor 3 subunit 8 | Q4Q055_LEIMA | 82 kDa  | 59   | 19   | 123.3333 | 6.565905 | 110.6667 | 19.36779 |
| Putative 60S ribosomal protein L21                            | Q4QEX4_LEIMA | 18 kDa  | 15.5 | 5.5  | 123.3333 | 30.8617  | 150      | 32.02083 |
| Chaperonin HSP60, mitochondrial                               | Q4Q1M0_LEIMA | 59 kDa  | 48   | 21   | 128      | 41.1015  | 126      | 14.04754 |
| Activated protein kinase C                                    | J9UU49_LEITR | 34 kDa  | 35.5 | 4.5  | 129.3333 | 7.512952 | 134.6667 | 5.696002 |
| Putative 60S ribosomal protein L13                            | E9AEA8_LEIMA | 25 kDa  | 15   | 9    | 137.3333 | 34.49316 | 142.6667 | 43.34872 |
| Cyclophilin 40                                                | E9AFV2_LEIMA | 38 kDa  | 43.5 | 13.5 | 115.3333 | 2.905933 | 118      | 9.237604 |
| ATPase alpha subunit                                          | Q4QJF1_LEIMA | 63 kDa  | 0    | 0    | 128      | 13.31666 | 146      | 5.291503 |
| PPM-type phosphatase domain-containing protein                | Q4QA19_LEIMA | 45 kDa  | 36   | 8    | 122      | 2        | 118.6667 | 12.12894 |
| Acetyl-coenzyme A synthetase                                  | Q9U0W1_LEIMA | 77 kDa  | 54.5 | 12.5 | 108.6667 | 8.192137 | 122      | 11.71893 |
| Peptidylprolyl isomerase                                      | Q4QD56_LEIMA | 48 kDa  | 34.5 | 10.5 | 121.3333 | 4.807402 | 123.3333 | 10.47749 |
| Uncharacterized protein                                       | E9ACL4_LEIMA | 247 kDa | 49.5 | 18.5 | 114.6667 | 9.683893 | 110.6667 | 11.62373 |
| Fructose-bisphosphate aldolase                                | Q4Q1V0_LEIMA | 41 kDa  | 50.5 | 8.5  | 118      | 16.16581 | 105.3333 | 13.92041 |
| Uncharacterized protein                                       | Q4QHS3_LEIMA | 67 kDa  | 38   | 14   | 110      | 5.291503 | 108.6667 | 7.333333 |
| Putative 60S ribosomal protein L6                             | Q4QF84_LEIMA | 21 kDa  | 33   | 14   | 108.6667 | 6.359595 | 132      | 29.95552 |
| Putative 40S ribosomal protein S3                             | Q4Q4A0_LEIMA | 24 kDa  | 0    | 0    | 109.3333 | 11.68094 | 146.6667 | 8.11035  |

|                                                              |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative 60S ribosomal protein L5                            | E9AF09_LEIMA | 34 kDa  | 30   | 12   | 98.66667 | 5.925463 | 102.6667 | 32.33849 |
| ATP-dependent RNA helicase eIF4A                             | E9AFD4_LEIMA | 100 kDa | 98.5 | 29.5 | 87.33333 | 14.66667 | 106      | 6.110101 |
| 40S ribosomal protein S3a                                    | RS3A_LEIMA   | 30 kDa  | 18.5 | 4.5  | 98       | 23.09401 | 145.3333 | 42.11624 |
| ADP/ATP translocase                                          | Q4QDK0_LEIMA | 35 kDa  | 17   | 4    | 111.3333 | 13.77599 | 139.3333 | 19.64123 |
| Splicing factor pstr1-like protein                           | Q4QIK0_LEIMA | 42 kDa  | 36.5 | 14.5 | 113.3333 | 4.666667 | 119.3333 | 17.37175 |
| Putative 60S ribosomal protein L10                           | O97213_LEIMA | 25 kDa  | 0    | 0    | 93.33333 | 9.820613 | 114      | 7.211103 |
| Putative ribonucleoprotein p18, mitochondrial                | Q4QFF2_LEIMA | 21 kDa  | 18   | 7    | 124.6667 | 4.055175 | 119.3333 | 8.192137 |
| Putative heat shock protein                                  | Q4Q3U8_LEIMA | 72 kDa  | 53   | 21   | 119.3333 | 15.07021 | 100      | 12.2202  |
| Putative cystathione gamma lyase                             | E9AFE7_LEIMA | 45 kDa  | 41   | 7    | 112      | 15.6205  | 115.3333 | 9.333333 |
| Putative calmodulin                                          | A4H5R9_LEIBR | 17 kDa  | 0    | 0    | 140.6667 | 12.66667 | 114.6667 | 9.820613 |
| Acetyl-CoA carboxylase                                       | Q4Q5W1_LEIMA | 241 kDa | 66   | 29   | 101.3333 | 17.33333 | 98       | 14.1892  |
| Putative small myristoylated protein-3                       | Q4QFP8_LEIMA | 13 kDa  | 55   | 14   | 109.3333 | 14.52966 | 96.66667 | 4.055175 |
| Prolyl endopeptidase                                         | Q4QHU7_LEIMA | 83 kDa  | 45   | 16   | 110.6667 | 9.614803 | 97.33333 | 7.512952 |
| Glucose-6-phosphate isomerase                                | Q4QGN9_LEIMA | 67 kDa  | 68   | 16   | 110.6667 | 4.807402 | 80.66667 | 15.37675 |
| Putative ribosomal protein L1a                               | E9ADW5_LEIMA | 41 kDa  | 16.5 | 8.5  | 94       | 26       | 120      | 21.93931 |
| Putative calpain-like cysteine peptidase                     | Q4QCS9_LEIMA | 108 kDa | 42.5 | 17.5 | 86.66667 | 10.72898 | 85.33333 | 5.696002 |
| Adenosylhomocysteinase                                       | Q4Q124_LEIMA | 48 kDa  | 49.5 | 4.5  | 121.3333 | 10.41367 | 87.33333 | 11.79454 |
| Malate dehydrogenase                                         | Q4Q3J4_LEIMA | 33 kDa  | 42.5 | 6.5  | 108.6667 | 12.97861 | 102      | 13.85641 |
| Putative pyruvate dehydrogenase E1 component alpha subunit   | Q4QDQ1_LEIMA | 43 kDa  | 10   | 3    | 128      | 7.211103 | 103.3333 | 12.34684 |
| Putative mitotubule-associated protein Gb4                   | Q4Q8Z7_LEIMA | 295 kDa | 17   | 11   | 111.3333 | 21.0502  | 116      | 24.97999 |
| N(1),N(8)-bis(glutathionyl)spermidine reductase              | Q4QJG7_LEIMA | 53 kDa  | 48   | 7    | 113.3333 | 12.34684 | 94.66667 | 12.34684 |
| Leucyl-tRNA synthetase                                       | Q4QG44_LEIMA | 122 kDa | 65.5 | 13.5 | 83.33333 | 6.359595 | 92.66667 | 19.8774  |
| Cysteine protease (Fragment)                                 | A1BLM5_LEIMA | 36 kDa  | 15.5 | 5.5  | 96.66667 | 10.66667 | 136.6667 | 47.83769 |
| Putative proteasome regulatory non-ATPase subunit            | E9ADL4_LEIMA | 99 kDa  | 35.5 | 20.5 | 110.6667 | 9.614803 | 100      | 12.16553 |
| Chaperonin HSP60, mitochondrial                              | Q4Q1M1_LEIMA | 60 kDa  | 41.5 | 18.5 | 114.6667 | 31.54538 | 94       | 5.291503 |
| Nucleoside diphosphate kinase                                | Q9GP00_LEIIN | 17 kDa  | 32.5 | 0.5  | 106.6667 | 11.392   | 111.3333 | 9.333333 |
| Putative translation initiation factor                       | Q4QE62_LEIMA | 81 kDa  | 45.5 | 8.5  | 101.3333 | 7.688375 | 102      | 4.163332 |
| Prolyl endopeptidase                                         | Q4Q080_LEIMA | 78 kDa  | 41.5 | 17.5 | 100.6667 | 4.371626 | 94       | 4        |
| Putative polyubiquitin                                       | A4HPM0_LEIBR | 111 kDa | 52   | 7    | 70.66667 | 10.72898 | 52.66667 | 13.53186 |
| RRM_8 domain-containing protein                              | Q4Q5J8_LEIMA | 57 kDa  | 42   | 11   | 99.33333 | 5.206833 | 102      | 3.05505  |
| Putative long chain fatty Acyl CoA synthetase                | E9ACH0_LEIMA | 79 kDa  | 33   | 4    | 101.3333 | 7.333333 | 98       | 6.928203 |
| Phosphoglycerate mutase (2,3-diphosphoglycerate-independent) | Q4Q090_LEIMA | 61 kDa  | 40   | 16   | 100.6667 | 5.456902 | 95.33333 | 4.371626 |
| Possible 3-ketoacyl-CoA thiolase                             | Q9U0V9_LEIMA | 47 kDa  | 40   | 15   | 96.66667 | 3.527668 | 89.33333 | 10.9138  |
| Adenosine kinase                                             | Q4Q7L5_LEIMA | 37 kDa  | 40   | 6    | 102.6667 | 5.811865 | 91.33333 | 13.38324 |
| Peroxidoxin                                                  | Q4QBH2_LEIMA | 25 kDa  | 48.5 | 19.5 | 81.33333 | 8.353309 | 99.33333 | 20.21001 |
| ATP-dependent RNA helicase eIF4A                             | W5XL77_LEIMA | 45 kDa  | 48   | 1    | 81.33333 | 9.333333 | 102      | 8.082904 |
| Eukaryotic translation initiation factor 5A                  | Q4QA21_LEIMA | 18 kDa  | 49.5 | 22.5 | 91.33333 | 11.85093 | 88       | 11.01514 |

|                                                                                |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial       | Q4QAG8_LEIMA | 67 kDa  | 6    | 1    | 106.6667 | 8.11035  | 102      | 10.06645 |
| C2 DOCK-type domain-containing protein                                         | E9ADL3_LEIMA | 338 kDa | 49   | 30   | 95.33333 | 27.93644 | 71.33333 | 13.77599 |
| Alkyl dihydroxyacetonephosphate synthase                                       | Q7YWB6_LEIMA | 70 kDa  | 28   | 3    | 98.66667 | 16.50589 | 94       | 8.082904 |
| Eukaryotic translation initiation factor 3 subunit L                           | Q4Q253_LEIMA | 73 kDa  | 26   | 6    | 86.66667 | 11.62373 | 90       | 22       |
| Oxoglutarate dehydrogenase (succinyl-transferring)                             | E9AD65_LEIMA | 113 kDa | 25.5 | 9.5  | 94       | 5.291503 | 82       | 10.06645 |
| Eukaryotic translation initiation factor 3 subunit 7-like protein              | Q4Q6Y6_LEIMA | 61 kDa  | 23   | 8    | 81.33333 | 5.811865 | 96       | 11.01514 |
| Uncharacterized protein                                                        | Q4Q3D8_LEIMA | 358 kDa | 19.5 | 6.5  | 106.6667 | 1.763834 | 100.6667 | 5.811865 |
| 60S ribosomal protein L18a                                                     | Q4Q5J4_LEIMA | 21 kDa  | 15.5 | 4.5  | 104.6667 | 8.353309 | 86.66667 | 13.28324 |
| Ribosomal protein L15                                                          | Q9N9V8_LEIIN | 24 kDa  | 21.5 | 7.5  | 94       | 15.53491 | 94.66667 | 15.71977 |
| Putative 60S ribosomal protein L7a                                             | A4HT77_LEIIN | 37 kDa  | 36   | 9    | 79.33333 | 4.666667 | 88       | 12.8582  |
| Putative oxidoreductase                                                        | Q4Q0Z7_LEIMA | 36 kDa  | 41.5 | 8.5  | 74.66667 | 8.969083 | 72       | 10.3923  |
| Alba domain-containing protein                                                 | Q4Q2T7_LEIMA | 23 kDa  | 63.5 | 16.5 | 73.33333 | 30.77517 | 82.66667 | 39.67087 |
| Isocitrate dehydrogenase [NADP]                                                | Q4QHI7_LEIMA | 48 kDa  | 64   | 14   | 66       | 20.29778 | 88       | 25.16611 |
| Putative long-chain-fatty-acid-CoA ligase                                      | A4HRH5_LEIIN | 78 kDa  | 38.5 | 18.5 | 78       | 15.09967 | 74.66667 | 22.4004  |
| Peptidyl-prolyl cis-trans isomerase                                            | A4I935_LEIIN | 29 kDa  | 33.5 | 16.5 | 92       | 10.2632  | 72.66667 | 21.36456 |
| 40S ribosomal protein S7                                                       | E9AC32_LEIMA | 24 kDa  | 13.5 | 7.5  | 74       | 4.618802 | 63.33333 | 7.333333 |
| Putative trypanothione synthetase                                              | Q711P7_LEIMA | 74 kDa  | 38   | 13   | 88.66667 | 15.37675 | 78.66667 | 11.09554 |
| Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex | Q4Q1F5_LEIMA | 49 kDa  | 0    | 0    | 88       | 13.11488 | 92.66667 | 2.403701 |
| Metallo-peptidase, Clan ME, Family M16                                         | Q4QG67_LEIMA | 58 kDa  | 0    | 0    | 103.3333 | 21.0502  | 76.66667 | 16.70662 |
| Dipeptidyl peptidase 3                                                         | Q4QJA6_LEIMA | 76 kDa  | 58.5 | 12.5 | 85.33333 | 2.666667 | 69.33333 | 7.688375 |
| Sacchrp_dh_NADP domain-containing protein                                      | Q4Q8N2_LEIMA | 42 kDa  | 28   | 4    | 92       | 6.429101 | 76       | 1.154701 |
| Metallo-peptidase, Clan MA(E), Family M32                                      | Q4Q3T3_LEIMA | 57 kDa  | 27.5 | 4.5  | 86.66667 | 13.38324 | 73.33333 | 8.743251 |
| Putative surface antigen protein                                               | Q4QGL8_LEIMA | 65 kDa  | 16.5 | 5.5  | 81.33333 | 6.666667 | 72       | 13.31666 |
| Uncharacterized protein                                                        | Q4QGX6_LEIMA | 28 kDa  | 17.5 | 4.5  | 80       | 3.05505  | 72       | 12.70171 |
| Uncharacterized protein                                                        | E9AFU8_LEIMA | 79 kDa  | 18   | 11   | 80.66667 | 17.37175 | 87.33333 | 12.71919 |
| Eukaryotic translation initiation factor 3 subunit I                           | Q4Q127_LEIMA | 45 kDa  | 26.5 | 2.5  | 78.66667 | 1.763834 | 90.66667 | 13.38324 |
| Putative fumarate hydratase                                                    | E9AE57_LEIMA | 63 kDa  | 0    | 0    | 87.33333 | 0.666667 | 81.33333 | 7.688375 |
| Malate dehydrogenase                                                           | Q4QDF0_LEIMA | 34 kDa  | 51.5 | 13.5 | 78       | 5.291503 | 71.33333 | 10.0885  |
| Protein antigen LmSTI1 (Fragment)                                              | P90553_LEIMA | 62 kDa  | 24.5 | 8.5  | 87.33333 | 8.51143  | 62       | 18.90326 |
| Putative NADP-dependent alcohol dehydrogenase                                  | Q4QBD8_LEIMA | 38 kDa  | 42   | 14   | 72       | 11.13553 | 80       | 5.033223 |
| Probable citrate synthase, mitochondrial                                       | CISY_LEIMA   | 52 kDa  | 39.5 | 6.5  | 70       | 16.16581 | 72       | 12.8582  |
| Asparagine--tRNA ligase                                                        | Q4Q2W6_LEIMA | 99 kDa  | 27   | 11   | 86.66667 | 9.261629 | 76       | 6        |
| Inositol-3-phosphate synthase                                                  | Q4QFJ8_LEIMA | 58 kDa  | 15   | 2    | 94.66667 | 10.0885  | 70.66667 | 8.743251 |
| Putative lanosterol 14-alpha-demethylase                                       | Q4QGX0_LEIMA | 54 kDa  | 23   | 9    | 79.33333 | 9.820613 | 80       | 11.13553 |
| Putative eukaryotic release factor 3                                           | Q4QGW5_LEIMA | 84 kDa  | 31.5 | 13.5 | 78.66667 | 4.371626 | 76       | 18.90326 |

|                                                              |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative aminopeptidase P                                    | E9AF59_LEIMA | 54 kDa  | 31.5 | 9.5  | 79.33333 | 8.353309 | 77.33333 | 8.666667 |
| Putative ubiquitin-activating enzyme e1                      | Q9NF77_LEIMA | 115 kDa | 28.5 | 10.5 | 71.33333 | 5.811865 | 82       | 6        |
| Polyadenylate-binding protein                                | E9AFP0_LEIMA | 65 kDa  | 38.5 | 12.5 | 79.33333 | 4.807402 | 68.66667 | 20.40697 |
| Elongation factor 1B gamma                                   | Q66NE0_LEIMA | 46 kDa  | 24   | 4    | 86       | 9.165151 | 69.33333 | 8.666667 |
| Putative acyl-CoA dehydrogenase                              | Q4Q812_LEIMA | 69 kDa  | 27.5 | 8.5  | 84.66667 | 2.403701 | 61.33333 | 7.333333 |
| Nucleosome assembly protein-like protein                     | Q4Q687_LEIMA | 45 kDa  | 25   | 6    | 92       | 4.163332 | 62       | 7.211103 |
| Diadenosine tetraphosphate synthetase                        | Q4Q131_LEIMA | 70 kDa  | 36   | 8    | 72.66667 | 11.62373 | 60       | 16.16581 |
| Glutamine--tRNA ligase                                       | Q4QF36_LEIMA | 66 kDa  | 19   | 9    | 74.66667 | 2.403701 | 71.33333 | 11.56623 |
| Glycerol kinase                                              | E9AFD2_LEIMA | 56 kDa  | 19.5 | 3.5  | 79.33333 | 9.404491 | 75.33333 | 7.423686 |
| Proline dehydrogenase                                        | Q4Q933_LEIMA | 64 kDa  | 28   | 7    | 68       | 12       | 80       | 2        |
| D-lactate dehydrogenase-like protein                         | E9ADI2_LEIMA | 54 kDa  | 25   | 5    | 76       | 4.618802 | 78.66667 | 8.353309 |
| 40S ribosomal protein S24                                    | Q4Q1D2_LEIMA | 16 kDa  | 0    | 0    | 62.66667 | 5.456902 | 64.66667 | 6.359595 |
| CCT-eta                                                      | E9AFL0_LEIMA | 62 kDa  | 17   | 8    | 76       | 9.865766 | 77.33333 | 4.371626 |
| 40S ribosomal protein S8                                     | RS8_LEIMA    | 25 kDa  | 33.5 | 11.5 | 63.33333 | 16.66667 | 84       | 23.69247 |
| Putative dipeptylcarboxypeptidase                            | E9ACE8_LEIMA | 77 kDa  | 72.5 | 16.5 | 58.66667 | 4.807402 | 54       | 10.3923  |
| Uncharacterized protein                                      | Q4Q0Q2_LEIMA | 105 kDa | 61   | 26   | 56       | 27.15388 | 66       | 17.0098  |
| Putative aminopeptidase                                      | Q4QDK5_LEIMA | 43 kDa  | 30.5 | 8.5  | 66.66667 | 1.763834 | 72.66667 | 2.666667 |
| Replication protein A subunit                                | Q4Q884_LEIMA | 52 kDa  | 24.5 | 8.5  | 73.33333 | 4.666667 | 68.66667 | 13.38324 |
| D-lactate dehydrogenase-like protein                         | E9ADN0_LEIMA | 53 kDa  | 27   | 15   | 77.33333 | 5.333333 | 64       | 7.023769 |
| Putative la RNA binding protein                              | Q4QCG1_LEIMA | 37 kDa  | 28.5 | 7.5  | 72.66667 | 2.905933 | 68.66667 | 7.333333 |
| Putative thimet oligopeptidase                               | Q4Q937_LEIMA | 77 kDa  | 43.5 | 6.5  | 67.33333 | 5.925463 | 65.33333 | 12.87547 |
| Putative phosphatase 2C                                      | Q4Q7S1_LEIMA | 42 kDa  | 16.5 | 7.5  | 80       | 6.928203 | 70       | 8.717798 |
| Thiolase protein-like protein                                | Q4Q698_LEIMA | 47 kDa  | 32   | 15   | 72.66667 | 11.21507 | 68       | 2        |
| Uncharacterized protein                                      | Q4QGW6_LEIMA | 122 kDa | 28   | 10   | 72       | 6.110101 | 71.33333 | 14.11067 |
| Arginyl-tRNA synthetase                                      | E9ADB2_LEIMA | 78 kDa  | 41.5 | 7.5  | 61.33333 | 6.359595 | 70       | 18.3303  |
| Peptidyl-prolyl cis-trans isomerase                          | Q4QJ67_LEIMA | 20 kDa  | 27.5 | 4.5  | 67.33333 | 7.512952 | 66.66667 | 2.403701 |
| Actin                                                        | Q9U1E8_LEIMA | 42 kDa  | 47   | 18   | 66       | 17.47379 | 53.33333 | 3.527668 |
| Stress-inducible protein STI1 homolog                        | Q4Q271_LEIMA | 29 kDa  | 24.5 | 6.5  | 78       | 13.11488 | 65.33333 | 16.82591 |
| Uncharacterized protein                                      | Q4Q5I7_LEIMA | 102 kDa | 29.5 | 11.5 | 64.66667 | 8.353309 | 70.66667 | 19.33333 |
| 60S ribosomal protein L11 (L5, L16)                          | A4HS42_LEIIN | 22 kDa  | 18   | 2    | 76       | 2        | 70.66667 | 2.905933 |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QIC8_LEIMA | 149 kDa | 27   | 10   | 63.33333 | 5.696002 | 71.33333 | 9.683893 |
| Seryl-tRNA synthetase                                        | Q4QH70_LEIMA | 53 kDa  | 43   | 7    | 63.33333 | 7.688375 | 60       | 8.326664 |
| Putative small GTP-binding protein Rab1                      | E9AD53_LEIMA | 22 kDa  | 30.5 | 5.5  | 59.33333 | 3.711843 | 56.66667 | 9.333333 |
| Kinesin motor domain-containing protein                      | Q4Q0P7_LEIMA | 71 kDa  | 23.5 | 9.5  | 64.66667 | 6.565905 | 73.33333 | 2.905933 |
| Putative 5'-3' exonuclease                                   | Q4QJ53_LEIMA | 166 kDa | 16.5 | 7.5  | 64.66667 | 9.333333 | 71.33333 | 15.59202 |
| Cathepsin L-like protease                                    | Q4QI61_LEIMA | 48 kDa  | 25   | 3    | 62.66667 | 13.28324 | 75.33333 | 6.359595 |
| Uncharacterized protein                                      | Q4Q9N7_LEIMA | 33 kDa  | 33.5 | 2.5  | 66       | 10       | 66.66667 | 8.819171 |
| Prolyl-tRNA synthetase                                       | Q4QDS0_LEIMA | 82 kDa  | 25   | 6    | 68.66667 | 16.34353 | 66.66667 | 8.666667 |
| 4-coumarate:coa ligase-like protein                          | Q4QDB5_LEIMA | 66 kDa  | 20.5 | 8.5  | 66       | 14       | 64.66667 | 13.96822 |

|                                                                  |              |         |      |      |          |          |          |          |
|------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative ribosomal protein l35a                                  | E9AN13_LEIMU | 16 kDa  | 17   | 6    | 64       | 11.01514 | 74.66667 | 6.765928 |
| Putative RNA binding protein                                     | Q4Q5K7_LEIMA | 25 kDa  | 23   | 8    | 63.33333 | 5.456902 | 75.33333 | 3.527668 |
| Phosphodiesterase                                                | Q6S996_LEIMA | 104 kDa | 30.5 | 9.5  | 64.66667 | 5.206833 | 66       | 5.291503 |
| Superoxide dismutase                                             | Q4Q597_LEIMA | 21 kDa  | 25   | 1    | 68       | 4.163332 | 69.33333 | 4.371626 |
| Uncharacterized protein                                          | E9ADB1_LEIMA | 60 kDa  | 37   | 8    | 60       | 5.291503 | 57.33333 | 5.925463 |
| Thioredoxin-like_fold domain-containing protein                  | Q4QFW6_LEIMA | 22 kDa  | 37   | 10   | 72       | 5.291503 | 51.33333 | 10.9138  |
| Putative 40S ribosomal protein L14                               | Q4QBJ1_LEIMA | 20 kDa  | 14   | 6    | 55.33333 | 9.333333 | 78.66667 | 16.70662 |
| 40S ribosomal protein SA                                         | RSSA_LEIMA   | 27 kDa  | 19.5 | 3.5  | 62.66667 | 2.403701 | 68       | 6.928203 |
| Threonyl-tRNA synthetase                                         | E9AEW4_LEIMA | 89 kDa  | 46.5 | 11.5 | 53.33333 | 11.392   | 62.66667 | 11.56623 |
| Putative 40S ribosomal protein S9                                | Q4Q1V1_LEIMA | 22 kDa  | 14   | 7    | 64       | 11.37248 | 74.66667 | 16.42491 |
| Cytochrome c                                                     | Q4QEN5_LEIMA | 12 kDa  | 17   | 7    | 67.33333 | 4.666667 | 64.66667 | 6.960204 |
| Putative centromere/microtubule binding protein cbf5             | Q4QC07_LEIMA | 48 kDa  | 12.5 | 4.5  | 60.66667 | 4.371626 | 66.66667 | 6.565905 |
| Calreticulin                                                     | Q4Q601_LEIMA | 45 kDa  | 13   | 6    | 65.33333 | 9.404491 | 66.66667 | 2.666667 |
| Succinate--CoA ligase [ADP-forming] subunit alpha, mitochondrial | A4I1R2_LEIIN | 31 kDa  | 58   | 15   | 50       | 4        | 56       | 5.033223 |
| Adenylosuccinate synthetase                                      | PURA_LEIMA   | 78 kDa  | 22.5 | 11.5 | 49.33333 | 7.512952 | 62       | 12.05543 |
| Putative 40S ribosomal protein S11                               | E9AUU6_LEIMU | 16 kDa  | 8    | 5    | 67.33333 | 6.765928 | 68       | 5.773503 |
| Elongation factor 1-beta                                         | Q4Q3C1_LEIMA | 26 kDa  | 29.5 | 10.5 | 56       | 5.033223 | 64.66667 | 7.688375 |
| Putative 40S ribosomal protein S19 protein                       | Q4Q2R6_LEIMA | 19 kDa  | 0    | 0    | 50.66667 | 5.811865 | 73.33333 | 3.333333 |
| Putative 60S ribosomal protein L2                                | A4HL27_LEIBR | 28 kDa  | 15   | 7    | 53.33333 | 7.055337 | 69.33333 | 6.666667 |
| Mevalonate kinase                                                | Q4Q6K7_LEIMA | 35 kDa  | 22   | 2    | 65.33333 | 3.527668 | 58       | 4.163332 |
| Galactokinase-like protein                                       | E9AF98_LEIMA | 53 kDa  | 25.5 | 7.5  | 63.33333 | 5.925463 | 56       | 5.773503 |
| Isoleucyl-tRNA synthetase                                        | Q4Q0J9_LEIMA | 126 kDa | 28   | 9    | 56       | 11.13553 | 56       | 9.451631 |
| 9,11-endoperoxide prostaglandin H2 reductase                     | PGFS_LEIMA   | 32 kDa  | 17.5 | 5.5  | 62.66667 | 8.819171 | 55.33333 | 11.56623 |
| Glutamate dehydrogenase                                          | Q4Q7X1_LEIMA | 49 kDa  | 17   | 8    | 76       | 20.29778 | 43.33333 | 4.371626 |
| Dimethylargininase                                               | Q4QI38_LEIMA | 32 kDa  | 21   | 9    | 59.33333 | 5.696002 | 55.33333 | 12.66667 |
| Uncharacterized protein                                          | Q4QC37_LEIMA | 46 kDa  | 0    | 0    | 63.33333 | 4.666667 | 69.33333 | 4.055175 |
| Glutamate--tRNA ligase                                           | Q4Q6W5_LEIMA | 68 kDa  | 13.5 | 4.5  | 63.33333 | 0.666667 | 62       | 7.211103 |
| Uncharacterized protein L7836.08                                 | Q9U0V4_LEIMA | 63 kDa  | 31.5 | 4.5  | 56.66667 | 5.456902 | 46       | 6.110101 |
| Uncharacterized protein                                          | Q4Q5T1_LEIMA | 36 kDa  | 7    | 1    | 60.66667 | 3.711843 | 62.66667 | 5.696002 |
| S-adenosylmethionine synthase                                    | Q4Q6T6_LEIMA | 43 kDa  | 17   | 5    | 60.66667 | 4.666667 | 56       | 16.16581 |
| Putative cytoskeleton-associated protein CAP5.5                  | Q4Q6L9_LEIMA | 80 kDa  | 0    | 0    | 44       | 4.618802 | 43.33333 | 14.25171 |
| Mitochondrial RNA binding protein 1                              | E9AD89_LEIMA | 27 kDa  | 16.5 | 6.5  | 61.33333 | 7.859884 | 60.66667 | 2.666667 |
| Putative glutamine synthetase                                    | Q4QJ42_LEIMA | 60 kDa  | 15   | 4    | 62.66667 | 1.763834 | 62       | 7.023769 |
| Putative 60S acidic ribosomal protein P2                         | Q4Q6R6_LEIMA | 11 kDa  | 20.5 | 3.5  | 61.33333 | 7.688375 | 58.66667 | 8.819171 |
| Guanine nucleotide-binding protein subunit beta-like protein     | Q4QJ76_LEIMA | 76 kDa  | 23   | 12   | 62.66667 | 8.666667 | 51.33333 | 9.404491 |
| Uncharacterized protein                                          | E9ACC0_LEIMA | 32 kDa  | 0    | 0    | 65.33333 | 4.666667 | 58.66667 | 9.820613 |
| CS domain-containing protein                                     | E9AFS4_LEIMA | 22 kDa  | 22.5 | 6.5  | 44.66667 | 10.9138  | 65.33333 | 18.26959 |
| Nascent polypeptide-associated complex subunit beta              | Q4Q138_LEIMA | 12 kDa  | 24   | 4    | 58.66667 | 3.527668 | 60       | 6.110101 |

|                                                                         |              |         |      |      |          |          |          |          |
|-------------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Coatomer subunit alpha                                                  | Q4Q2B0_LEIMA | 132 kDa | 19.5 | 11.5 | 58.66667 | 5.696002 | 59.33333 | 11.56623 |
| Putative nucleosome assembly protein                                    | Q4QDH7_LEIMA | 40 kDa  | 19   | 3    | 57.33333 | 6.765928 | 53.33333 | 8.11035  |
| Proteasome subunit beta                                                 | Q4QGU1_LEIMA | 30 kDa  | 16.5 | 0.5  | 64.66667 | 3.711843 | 56.66667 | 12.66667 |
| Putative 60S Ribosomal protein L36                                      | A4HZ73_LEIIN | 12 kDa  | 5.5  | 2.5  | 42       | 9.0185   | 42.66667 | 14.62114 |
| Putative reiske iron-sulfur protein                                     | E9AEX6_LEIMA | 34 kDa  | 0    | 0    | 62       | 14.04754 | 66       | 11.01514 |
| 40S ribosomal protein S2                                                | Q4Q5P0_LEIMA | 29 kDa  | 17   | 8    | 48       | 12.49    | 70       | 18.47521 |
| Putative 60S ribosomal protein L27A/L29                                 | E9AFK0_LEIMA | 16 kDa  | 5    | 1    | 53.33333 | 14.71205 | 68.66667 | 27.38207 |
| Inosine-5'-monophosphate dehydrogenase                                  | Q4QD53_LEIMA | 56 kDa  | 49   | 11   | 44       | 7.571878 | 49.33333 | 11.56623 |
| Uncharacterized protein                                                 | Q4QG18_LEIMA | 91 kDa  | 17.5 | 13.5 | 62.66667 | 10.41367 | 53.33333 | 4.666667 |
| 14-3-3 protein I                                                        | Q4Q198_LEIMA | 30 kDa  | 29.5 | 8.5  | 54       | 5.773503 | 52       | 6.429101 |
| Putative elongation factor Tu                                           | Q4QDW8_LEIMA | 52 kDa  | 32   | 14   | 52       | 6.928203 | 52.66667 | 7.688375 |
| Uncharacterized protein                                                 | Q4Q0P9_LEIMA | 30 kDa  | 0    | 0    | 68.66667 | 7.512952 | 57.33333 | 9.333333 |
| Nascent polypeptide associated complex subunit-like protein, copy 2     | O97212_LEIMA | 27 kDa  | 27.5 | 7.5  | 59.33333 | 6.359595 | 51.33333 | 6.960204 |
| Universal minicircle sequence binding protein                           | Q4Q1R3_LEIMA | 19 kDa  | 13   | 2    | 38.66667 | 6.765928 | 81.33333 | 0        |
| Putative 60S ribosomal protein L7                                       | Q4Q9H4_LEIMA | 29 kDa  | 20   | 5    | 53.33333 | 7.688375 | 45.33333 | 2.403701 |
| Putative branched-chain amino acid aminotransferase                     | E9ADI3_LEIMA | 44 kDa  | 23   | 6    | 57.33333 | 2.403701 | 54       | 1.154701 |
| Putative proteasome regulatory non-ATPase subunit 6                     | E9ACB2_LEIMA | 59 kDa  | 26   | 13   | 45.33333 | 6.359595 | 60       | 4.163332 |
| Putative 60S ribosomal protein L9                                       | Q4Q6V5_LEIMA | 22 kDa  | 15   | 8    | 42.66667 | 9.404491 | 54.66667 | 14.34496 |
| Uncharacterized protein                                                 | Q4QFN8_LEIMA | 32 kDa  | 28   | 12   | 54.66667 | 8.353309 | 48       | 9.865766 |
| Putative 40S ribosomal protein S18                                      | Q4Q1Y3_LEIMA | 25 kDa  | 8.5  | 4.5  | 52.66667 | 11.56623 | 66.66667 | 18.55921 |
| Putative N-acyl-L-amino acid amidohydrolase                             | Q4Q6F0_LEIMA | 44 kDa  | 24   | 3    | 48.66667 | 4.371626 | 55.33333 | 5.696002 |
| Putative surface antigen protein 2                                      | Q4QGJ0_LEIMA | 67 kDa  | 0    | 0    | 56.66667 | 4.371626 | 48       | 9.451631 |
| Uncharacterized protein                                                 | Q4QC98_LEIMA | 200 kDa | 30   | 12   | 42       | 5.773503 | 52       | 1.154701 |
| Putative proteasome alpha 7 subunit                                     | E9ACZ6_LEIMA | 26 kDa  | 13   | 5    | 59.33333 | 3.527668 | 53.33333 | 4.666667 |
| Putative 60S ribosomal protein L26                                      | Q4QAC5_LEIMA | 16 kDa  | 0    | 0    | 59.33333 | 12.34684 | 44.66667 | 13.87243 |
| Putative DNAJ domain protein                                            | Q4QAS9_LEIMA | 89 kDa  | 16   | 9    | 53.33333 | 8.666667 | 56.66667 | 8.666667 |
| Uncharacterized protein                                                 | Q4Q428_LEIMA | 36 kDa  | 16.5 | 8.5  | 54.66667 | 12.23837 | 53.33333 | 2.403701 |
| T-complex protein 1 subunit gamma                                       | Q4QB32_LEIMA | 60 kDa  | 0    | 0    | 50.66667 | 12.23837 | 64.66667 | 20.98677 |
| Methyltransferase                                                       | Q4Q1I3_LEIMA | 40 kDa  | 22.5 | 3.5  | 55.33333 | 9.333333 | 44       | 8.326664 |
| Flavoprotein subunit-like protein                                       | Q4QIK9_LEIMA | 71 kDa  | 39   | 12   | 46.66667 | 5.456902 | 43.33333 | 4.666667 |
| Uncharacterized protein                                                 | Q4Q8Z2_LEIMA | 39 kDa  | 13.5 | 3.5  | 50.66667 | 4.371626 | 43.33333 | 5.333333 |
| Proteasome subunit alpha type                                           | Q4QC13_LEIMA | 25 kDa  | 20.5 | 3.5  | 58.66667 | 0.666667 | 46.66667 | 8.666667 |
| Phosphoglucosyltransferase (alpha-D-glucose-1,6-bisphosphate-dependent) | Q4QCF1_LEIMA | 64 kDa  | 17.5 | 6.5  | 50       | 6.928203 | 53.33333 | 4.666667 |
| Putative voltage-dependent anion-selective channel                      | E9ACB9_LEIMA | 30 kDa  | 0    | 0    | 48.66667 | 3.527668 | 65.33333 | 16.42491 |
| 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial         | Q4Q0M6_LEIMA | 32 kDa  | 14.5 | 2.5  | 55.33333 | 1.333333 | 51.33333 | 9.820613 |
| Putative nucleolar protein                                              | Q4QHJ7_LEIMA | 53 kDa  | 16   | 6    | 50.66667 | 4.371626 | 57.33333 | 7.055337 |

|                                                                   |              |         |      |      |          |          |          |          |
|-------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative surface antigen protein                                  | Q4QGI0_LEIMA | 54 kDa  | 0    | 0    | 56.66667 | 2.905933 | 51.33333 | 12.97861 |
| Spermidine synthase                                               | Q95Z84_LEIMA | 33 kDa  | 12   | 2    | 48       | 6.928203 | 54.66667 | 5.811865 |
| Putative dynein heavy chain                                       | Q4QFY9_LEIMA | 529 kDa | 0    | 0    | 46.66667 | 13.28324 | 54       | 29.14332 |
| UTP--glucose-1-phosphate<br>uridylyltransferase                   | Q4QDU3_LEIMA | 55 kDa  | 23   | 11   | 56       | 2        | 39.33333 | 8.192137 |
| Diphosphomevalonate decarboxylase                                 | Q4QE40_LEIMA | 42 kDa  | 27.5 | 1.5  | 52       | 5.033223 | 44       | 6        |
| Uncharacterized protein                                           | Q4Q8V3_LEIMA | 555 kDa | 0    | 0    | 48       | 12.2202  | 47.33333 | 20.07763 |
| 60S ribosomal protein L32                                         | Q4QC11_LEIMA | 15 kDa  | 0    | 0    | 38       | 10.06645 | 74       | 20.13289 |
| Putative 60S ribosomal protein L17                                | A4HD62_LEIBR | 19 kDa  | 3.5  | 0.5  | 36       | 18.3303  | 58       | 24.11086 |
| 14-3-3 protein II                                                 | Q4QH45_LEIMA | 29 kDa  | 13   | 6    | 38       | 5.033223 | 51.33333 | 5.456902 |
| Phosphoglycan beta 1,3<br>galactosyltransferase 5                 | Q4Q5U8_LEIMA | 124 kDa | 20   | 10   | 51.33333 | 8.666667 | 40.66667 | 10.34945 |
| Putative poly-zinc finger protein 2                               | Q4Q1R1_LEIMA | 15 kDa  | 7.5  | 1.5  | 54       | 9.865766 | 46.66667 | 11.85093 |
| Pyruvate dehydrogenase E1<br>component subunit beta               | Q4Q9R8_LEIMA | 38 kDa  | 3    | 0    | 56.66667 | 9.614803 | 50       | 5.291503 |
| Protein-serine/threonine phosphatase                              | Q4QE27_LEIMA | 53 kDa  | 17   | 10   | 52       | 4.618802 | 47.33333 | 1.763834 |
| Putative endoribonuclease L-PSP<br>(Pb5)                          | Q4QBF5_LEIMA | 17 kDa  | 29.5 | 10.5 | 44       | 2        | 50       | 1.154701 |
| Inorganic diphosphatase                                           | Q4QH59_LEIMA | 51 kDa  | 22   | 11   | 52       | 2.309401 | 45.33333 | 9.955456 |
| Uncharacterized protein                                           | Q4QI59_LEIMA | 42 kDa  | 0    | 0    | 58.66667 | 10.34945 | 50       | 2.309401 |
| Uncharacterized protein                                           | Q4Q202_LEIMA | 61 kDa  | 29   | 13   | 38.66667 | 1.333333 | 52.66667 | 6.359595 |
| 60S ribosomal protein L27                                         | Q4Q504_LEIMA | 15 kDa  | 11   | 4    | 30       | 6.429101 | 44.66667 | 5.811865 |
| Uncharacterized protein                                           | Q4QA41_LEIMA | 32 kDa  | 17   | 2    | 50.66667 | 6.666667 | 41.33333 | 9.683893 |
| Phosphomevalonate kinase                                          | Q4QF34_LEIMA | 49 kDa  | 13   | 4    | 43.33333 | 7.688375 | 53.33333 | 11.56623 |
| Putative ATP-dependent zinc<br>metallopeptidase                   | Q4Q5D1_LEIMA | 78 kDa  | 0    | 0    | 54       | 16.77299 | 52.66667 | 7.055337 |
| Putative elongation initiation factor 2<br>alpha subunit          | E9ACP3_LEIMA | 49 kDa  | 27.5 | 6.5  | 34       | 9.237604 | 52.66667 | 1.333333 |
| Trifunctional enzyme alpha subunit,<br>mitochondrial-like protein | Q4Q939_LEIMA | 79 kDa  | 30.5 | 7.5  | 52       | 17.32051 | 32.66667 | 3.527668 |
| ADF/Cofilin                                                       | E9ADQ2_LEIMA | 16 kDa  | 17.5 | 3.5  | 50       | 1.154701 | 49.33333 | 2.666667 |
| mRNA (guanine-N(7))-<br>methyltransferase                         | Q4QH32_LEIMA | 118 kDa | 23.5 | 11.5 | 37.33333 | 3.711843 | 51.33333 | 10.47749 |
| Proliferating cell nuclear antigen                                | Q4QF35_LEIMA | 32 kDa  | 22.5 | 2.5  | 42       | 5.773503 | 41.33333 | 4.055175 |
| Bifunctional dihydrofolate reductase-<br>thymidylate synthase     | DRTS_LEIMA   | 59 kDa  | 8    | 4    | 45.33333 | 7.512952 | 59.33333 | 18.52326 |
| 3-hydroxy-3-methylglutaryl coenzyme<br>A reductase                | Q9Y0F3_LEIMA | 46 kDa  | 15.5 | 5.5  | 51.33333 | 5.206833 | 48.66667 | 10.47749 |
| Cytochrome c oxidase subunit IV                                   | Q4QGM6_LEIMA | 39 kDa  | 0    | 0    | 54       | 10.2632  | 45.33333 | 7.688375 |
| Putative mitochondrial RNA binding<br>protein                     | Q4QB87_LEIMA | 40 kDa  | 23   | 6    | 43.33333 | 3.711843 | 50.66667 | 15.0259  |
| Citrulline--aspartate ligase                                      | Q4QEW0_LEIMA | 206 kDa | 13.5 | 6.5  | 50       | 9.0185   | 42       | 3.464102 |
| Electron transfer flavoprotein-<br>ubiquinone oxidoreductase      | Q4QIN2_LEIMA | 63 kDa  | 12   | 5    | 51.33333 | 5.925463 | 44.66667 | 1.333333 |
| Ribokinase                                                        | RBSK_LEIMA   | 35 kDa  | 27   | 12   | 44       | 6        | 47.33333 | 4.807402 |
| Glutathione peroxidase                                            | Q4Q9B3_LEIMA | 21 kDa  | 9    | 6    | 57.33333 | 11.09554 | 46.66667 | 5.696002 |
| Putative ATP-dependent DEAD/H RNA<br>helicase                     | Q4QIQ9_LEIMA | 64 kDa  | 23.5 | 5.5  | 38       | 11.54701 | 52.66667 | 2.403701 |
| Glucose-6-phosphate 1-epimerase                                   | Q4Q2K6_LEIMA | 33 kDa  | 15   | 9    | 47.33333 | 8.819171 | 44.66667 | 4.371626 |
| Phosphoglycerate kinase                                           | Q4Q6V1_LEIMA | 58 kDa  | 18   | 3    | 47.33333 | 6.960204 | 47.33333 | 6.359595 |

|                                                                 |              |         |      |      |          |          |          |          |
|-----------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative glycosomal membrane protein                            | Q4Q838_LEIMA | 24 kDa  | 0    | 0    | 31.33333 | 9.261629 | 56       | 15.6205  |
| Phosphomannomutase                                              | Q4Q1M7_LEIMA | 28 kDa  | 15.5 | 0.5  | 51.33333 | 8.969083 | 46.66667 | 3.333333 |
| Nodulin-like domain-containing protein                          | E9AE09_LEIMA | 74 kDa  | 0    | 0    | 52.66667 | 5.696002 | 52.66667 | 18.26959 |
| Putative ADP-ribosylation factor                                | Q4Q5Y0_LEIMA | 20 kDa  | 17   | 0    | 46.66667 | 13.4825  | 42       | 8.082904 |
| Putative arginine N-methyltransferase                           | E9ACK5_LEIMA | 44 kDa  | 10.5 | 3.5  | 40.66667 | 2.905933 | 42       | 4.163332 |
| Putative calpain-like cysteine peptidase                        | Q9U0T9_LEIMA | 94 kDa  | 27   | 10   | 44.66667 | 1.763834 | 37.33333 | 4.666667 |
| Uncharacterized protein                                         | Q4Q9J7_LEIMA | 109 kDa | 14.5 | 9.5  | 50.66667 | 6.565905 | 40.66667 | 8.51143  |
| Putative eukaryotic translation initiation factor 2 subunit     | Q4QHR7_LEIMA | 53 kDa  | 31   | 6    | 35.33333 | 5.456902 | 45.33333 | 12.71919 |
| Serine peptidase, Clan SC, Family S9B                           | Q4Q1H9_LEIMA | 94 kDa  | 17.5 | 10.5 | 41.33333 | 10.41367 | 48       | 4        |
| Uncharacterized protein                                         | E9AZ28_LEIMU | 35 kDa  | 23.5 | 6.5  | 40.66667 | 5.925463 | 50       | 8.717798 |
| Uncharacterized protein                                         | Q4QA76_LEIMA | 151 kDa | 22.5 | 13.5 | 44.66667 | 3.333333 | 42.66667 | 3.333333 |
| Aspartate aminotransferase                                      | Q4FX34_LEIMA | 46 kDa  | 23   | 5    | 43.33333 | 4.055175 | 44.66667 | 8.51143  |
| Putative cytochrome c1, heme protein, mitochondrial             | Q4QIT8_LEIMA | 30 kDa  | 5    | 1    | 46.66667 | 6.960204 | 54.66667 | 11.62373 |
| Putative mitochondrial phosphate transporter                    | E9AFS0_LEIMA | 35 kDa  | 0    | 0    | 36.66667 | 2.403701 | 49.33333 | 10.47749 |
| PDZ domain-containing protein                                   | Q4Q7P2_LEIMA | 80 kDa  | 14   | 6    | 42.66667 | 7.688375 | 48.66667 | 11.09554 |
| ER membrane protein complex subunit 1                           | Q4Q2C8_LEIMA | 87 kDa  | 0    | 0    | 60.66667 | 9.820613 | 42       | 5.291503 |
| Putative adenylate kinase                                       | Q4QC71_LEIMA | 30 kDa  | 22.5 | 6.5  | 34.66667 | 11.68094 | 44.66667 | 14.71205 |
| Pyridoxal kinase                                                | Q4Q7H9_LEIMA | 33 kDa  | 41.5 | 11.5 | 29.33333 | 9.955456 | 26.66667 | 5.696002 |
| Putative developmentally regulated GTP-binding protein 1        | Q4Q1J6_LEIMA | 41 kDa  | 13.5 | 7.5  | 45.33333 | 6.565905 | 46       | 9.165151 |
| Tryparedoxin                                                    | E9ADX4_LEIMA | 17 kDa  | 19.5 | 4.5  | 52       | 7.571878 | 37.33333 | 7.512952 |
| Putative carnitine/choline acetyltransferase                    | E9ADY9_LEIMA | 69 kDa  | 20   | 2    | 42.66667 | 11.85093 | 40.66667 | 6.359595 |
| Uncharacterized protein                                         | Q4Q6F8_LEIMA | 39 kDa  | 33.5 | 3.5  | 46.66667 | 5.696002 | 32.66667 | 4.807402 |
| Putative aminopeptidase                                         | Q4Q3T0_LEIMA | 56 kDa  | 15   | 2    | 40.66667 | 9.261629 | 44.66667 | 2.666667 |
| Glycosomal membrane protein                                     | E9AFJ4_LEIMA | 25 kDa  | 0    | 0    | 52.66667 | 5.206833 | 46       | 4.163332 |
| Obg-like ATPase 1                                               | E9ACV1_LEIMA | 44 kDa  | 11   | 2    | 40.66667 | 3.527668 | 44       | 6.110101 |
| Uncharacterized protein                                         | Q4QGD8_LEIMA | 56 kDa  | 16.5 | 7.5  | 38.66667 | 8.11035  | 36       | 6.928203 |
| Macrophage migration inhibitory factor-like protein             | Q4Q412_LEIMA | 13 kDa  | 13   | 4    | 50       | 4.163332 | 35.33333 | 8.969083 |
| Sucrose-phosphate synthase-like protein                         | Q4QES5_LEIMA | 52 kDa  | 19.5 | 7.5  | 42       | 6.429101 | 35.33333 | 1.763834 |
| Putative high mobility group protein homolog tdp-1              | A4I4D9_LEIIN | 34 kDa  | 22.5 | 10.5 | 46.66667 | 5.925463 | 34       | 8.326664 |
| 4-coumarate:coa ligase-like protein                             | Q4QDB7_LEIMA | 66 kDa  | 13   | 7    | 42.66667 | 11.85093 | 42.66667 | 9.820613 |
| Fructose-bisphosphatase                                         | O97193_LEIMA | 39 kDa  | 14.5 | 8.5  | 40.66667 | 8.666667 | 42.66667 | 4.666667 |
| Uncharacterized protein                                         | Q4Q6Z3_LEIMA | 49 kDa  | 6.5  | 1.5  | 46       | 4.163332 | 46.66667 | 1.333333 |
| Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial | Q4Q1C4_LEIMA | 44 kDa  | 30   | 8    | 41.33333 | 5.206833 | 36       | 4.163332 |
| Uncharacterized protein                                         | Q4Q9T7_LEIMA | 81 kDa  | 10   | 5    | 40.66667 | 1.763834 | 48.66667 | 1.333333 |
| Uncharacterized protein                                         | Q4Q350_LEIMA | 17 kDa  | 27.5 | 8.5  | 37.33333 | 7.688375 | 42       | 8.717798 |
| Acetylornithine deacetylase                                     | Q4QIR7_LEIMA | 43 kDa  | 16.5 | 3.5  | 42.66667 | 3.527668 | 42.66667 | 2.905933 |
| Prohibitin                                                      | E9AEI4_LEIMA | 32 kDa  | 0    | 0    | 45.33333 | 9.820613 | 48       | 4.163332 |

|                                                                                |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| 5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase        | Q4Q6R3_LEIMA | 86 kDa  | 33   | 13   | 36       | 4        | 35.33333 | 6.359595 |
| Putative 40S ribosomal protein S16                                             | Q4Q9A5_LEIMA | 17 kDa  | 0    | 0    | 39.33333 | 8.192137 | 50.66667 | 13.33333 |
| Putative ATP synthase F1 subunit gamma protein                                 | Q4QC06_LEIMA | 34 kDa  | 6.5  | 1.5  | 42       | 4.618802 | 38.66667 | 5.811865 |
| Uncharacterized protein                                                        | Q4QH88_LEIMA | 36 kDa  | 0    | 0    | 48.66667 | 7.423686 | 34       | 9.0185   |
| Putative 60S ribosomal protein L35                                             | A4I2G1_LEIIN | 15 kDa  | 0    | 0    | 32.66667 | 18.48723 | 20.66667 | 0        |
| Putative chaperone protein DNAj                                                | Q4Q0D3_LEIMA | 38 kDa  | 28   | 9    | 40.66667 | 2.905933 | 34       | 2        |
| Putative inosine-guanine nucleoside hydrolase                                  | Q4QFX2_LEIMA | 39 kDa  | 13   | 7    | 39.33333 | 2.905933 | 40.66667 | 1.763834 |
| GTP-binding nuclear protein                                                    | Q4Q9V1_LEIMA | 24 kDa  | 11.5 | 3.5  | 40.66667 | 5.456902 | 44       | 6.110101 |
| Profilin                                                                       | Q4Q5N1_LEIMA | 16 kDa  | 12.5 | 2.5  | 38       | 6.110101 | 45.33333 | 7.512952 |
| Formate--tetrahydrofolate ligase                                               | Q4Q735_LEIMA | 67 kDa  | 18.5 | 6.5  | 36       | 3.464102 | 40.66667 | 2.905933 |
| 2-deoxy-D-ribose 5-phosphate aldolase                                          | Q4QIW9_LEIMA | 30 kDa  | 14.5 | 4.5  | 44       | 4        | 40       | 4.163332 |
| Putative 60S ribosomal protein L19                                             | Q4QJ37_LEIMA | 29 kDa  | 8    | 4    | 19.33333 | 0.666667 | 42.66667 | 18.98537 |
| Putative 60S ribosomal protein L23                                             | E9AFK3_LEIMA | 15 kDa  | 0    | 0    | 33.33333 | 14.62114 | 52       | 8        |
| Vacuolar proton pump subunit B                                                 | Q4Q821_LEIMA | 56 kDa  | 22.5 | 9.5  | 38       | 3.05505  | 38       | 4        |
| Putative ATP-binding cassette protein subfamily E, member 1                    | Q4QCE4_LEIMA | 74 kDa  | 8.5  | 3.5  | 43.33333 | 7.423686 | 41.33333 | 10.47749 |
| Regulatory subunit of protein kinase a-like protein                            | Q4Q2R2_LEIMA | 72 kDa  | 21.5 | 9.5  | 42       | 7.023769 | 35.33333 | 6.359595 |
| 40S ribosomal protein S6                                                       | RS6_LEIMA    | 28 kDa  | 0    | 0    | 32       | 5.033223 | 56.66667 | 6.960204 |
| Uncharacterized protein                                                        | Q4QFB6_LEIMA | 54 kDa  | 18.5 | 8.5  | 38.66667 | 7.055337 | 38.66667 | 3.711843 |
| Rab GDP dissociation inhibitor                                                 | A4I4S5_LEIIN | 50 kDa  | 22   | 6    | 36.66667 | 3.527668 | 36.66667 | 2.666667 |
| Uncharacterized protein                                                        | Q4QCD3_LEIMA | 249 kDa | 34   | 17   | 29.33333 | 7.688375 | 32.66667 | 4.371626 |
| Peptidyl-prolyl cis-trans isomerase                                            | Q4QBH1_LEIMA | 31 kDa  | 16.5 | 12.5 | 32       | 7.023769 | 40.66667 | 3.333333 |
| Putative heat shock protein 70                                                 | E9B0A4_LEIMU | 72 kDa  | 0    | 0    | 0        | 0        | 34       | 17.0098  |
| 40S ribosomal protein S12                                                      | Q4QG97_LEIMA | 16 kDa  | 14.5 | 4.5  | 41.33333 | 4.371626 | 41.33333 | 8.666667 |
| Uncharacterized protein L7845.03                                               | Q9GRP8_LEIMA | 63 kDa  | 10.5 | 5.5  | 40       | 4.618802 | 39.33333 | 2.403701 |
| Alba domain-containing protein                                                 | Q4QGA9_LEIMA | 13 kDa  | 25   | 9    | 25.33333 | 9.404491 | 43.33333 | 24.50397 |
| Uncharacterized protein                                                        | Q4Q3K8_LEIMA | 34 kDa  | 0    | 0    | 47.33333 | 5.811865 | 40.66667 | 4.807402 |
| Uncharacterized protein                                                        | Q4Q2U2_LEIMA | 477 kDa | 11.5 | 4.5  | 39.33333 | 3.711843 | 44.66667 | 1.763834 |
| Mitochondrial RNA binding protein 2                                            | Q4QHR3_LEIMA | 27 kDa  | 0    | 0    | 45.33333 | 2.905933 | 39.33333 | 1.763834 |
| Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex | Q4Q822_LEIMA | 42 kDa  | 21   | 3    | 44.66667 | 2.905933 | 28.66667 | 3.711843 |
| 6-phosphogluconate dehydrogenase, decarboxylating (Fragment)                   | Q9NGR0_LEIMA | 52 kDa  | 28.5 | 4.5  | 37.33333 | 11.09554 | 30       | 4.163332 |
| Malic enzyme                                                                   | E9AWR7_LEIMU | 63 kDa  | 40.5 | 9.5  | 31.33333 | 7.512952 | 22.66667 | 12.71919 |
| Putative 2,4-dienoyl-coa reductase fadh1                                       | Q4Q4A9_LEIMA | 79 kDa  | 23   | 8    | 39.33333 | 9.820613 | 32       | 7.023769 |
| Glucose-6-phosphate 1-dehydrogenase                                            | Q4Q3K1_LEIMA | 63 kDa  | 19.5 | 10.5 | 40.66667 | 1.763834 | 32       | 3.05505  |
| Tyrosyl or methionyl-tRNA synthetase-like protein                              | Q4QEQ4_LEIMA | 20 kDa  | 19   | 10   | 36.66667 | 5.696002 | 34.66667 | 0.666667 |
| Putative calpain-like cysteine peptidase                                       | Q5SDH5_LEIMA | 15 kDa  | 8    | 2    | 42       | 4.163332 | 42.66667 | 4.055175 |
| Putative chaperone protein DNAj                                                | E9AFC2_LEIMA | 51 kDa  | 0    | 0    | 40.66667 | 4.055175 | 37.33333 | 1.333333 |

|                                                     |              |         |      |      |          |          |          |          |
|-----------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative 40S ribosomal protein S13                  | Q4Q3M1_LEIMA | 17 kDa  | 0    | 0    | 38.66667 | 6.765928 | 50.66667 | 4.666667 |
| Putative 60S ribosomal protein L28                  | Q4QGW7_LEIMA | 16 kDa  | 0    | 0    | 35.33333 | 8.743251 | 45.33333 | 20.17699 |
| Putative 40S ribosomal protein S17                  | Q4Q806_LEIMA | 17 kDa  | 12.5 | 6.5  | 29.33333 | 6.359595 | 48       | 11.01514 |
| Putative nima-related protein kinase                | Q4Q1S4_LEIMA | 56 kDa  | 12   | 7    | 44.66667 | 3.711843 | 30.66667 | 2.905933 |
| Mannose-6-phosphate isomerase                       | Q4Q5C3_LEIMA | 46 kDa  | 19   | 6    | 39.33333 | 2.403701 | 32       | 4.163332 |
| UDP-galactopyranose mutase                          | Q5EEK0_LEIMA | 55 kDa  | 12   | 6    | 35.33333 | 6.765928 | 40.66667 | 4.807402 |
| Putative fucose kinase                              | Q4QEX2_LEIMA | 127 kDa | 14   | 8    | 41.33333 | 4.371626 | 33.33333 | 2.403701 |
| Uncharacterized protein                             | Q4Q079_LEIMA | 29 kDa  | 0    | 0    | 42       | 5.773503 | 38       | 4.618802 |
| Putative fumarate hydratase                         | Q4QAU9_LEIMA | 61 kDa  | 0    | 0    | 32.66667 | 3.711843 | 38       | 7.211103 |
| Putative centrin                                    | A4I000_LEIIN | 16 kDa  | 0    | 0    | 41.33333 | 2.403701 | 39.33333 | 5.925463 |
| Putative cation-transporting ATPase                 | Q4QII2_LEIMA | 139 kDa | 5.5  | 2.5  | 34       | 12.49    | 45.33333 | 5.811865 |
| Putative pretranslocation protein, alpha subunit    | Q4QGX4_LEIMA | 54 kDa  | 0    | 0    | 36       | 4.163332 | 36.66667 | 2.403701 |
| Uncharacterized protein                             | Q4Q8X5_LEIMA | 298 kDa | 12.5 | 4.5  | 35.33333 | 2.905933 | 40.66667 | 5.811865 |
| Uncharacterized protein                             | Q4Q9C8_LEIMA | 41 kDa  | 17   | 11   | 37.33333 | 4.807402 | 34       | 7.571878 |
| Putative ubiquitin/ribosomal protein S27a           | A4ICS4_LEIIN | 17 kDa  | 6    | 0    | 0        | 0        | 0        | 0        |
| GMP reductase                                       | GMPR_LEIMA   | 52 kDa  | 24.5 | 10.5 | 36       | 6.429101 | 28       | 5.291503 |
| Serine palmitoyltransferase 1                       | Q6XFB3_LEIMA | 54 kDa  | 10   | 4    | 37.33333 | 6.765928 | 36       | 2.309401 |
| Putative heat shock protein 20                      | E9AEA7_LEIMA | 17 kDa  | 10   | 2    | 36       | 4.618802 | 38       | 9.865766 |
| CCT-theta                                           | Q4Q063_LEIMA | 58 kDa  | 13.5 | 4.5  | 35.33333 | 11.62373 | 36       | 6.928203 |
| Uncharacterized protein                             | A4HTE9_LEIIN | 38 kDa  | 23   | 5    | 30       | 4        | 34       | 3.05505  |
| Putative RNA binding protein rbp16                  | Q4Q8I6_LEIMA | 15 kDa  | 14   | 10   | 45.33333 | 18.7735  | 22.66667 | 6.666667 |
| Putative ubiquitin-activating enzyme e1             | E9AFD0_LEIMA | 126 kDa | 16   | 4    | 32.66667 | 8.819171 | 34.66667 | 5.925463 |
| CS domain-containing protein                        | Q4Q848_LEIMA | 71 kDa  | 16.5 | 10.5 | 35.33333 | 4.371626 | 30       | 5.773503 |
| Uncharacterized protein                             | Q4Q111_LEIMA | 47 kDa  | 12.5 | 5.5  | 36       | 3.464102 | 35.33333 | 4.371626 |
| CCT-epsilon                                         | Q4Q5I2_LEIMA | 59 kDa  | 7.5  | 3.5  | 30       | 7.023769 | 39.33333 | 10.41367 |
| Uncharacterized protein                             | Q4Q6X5_LEIMA | 23 kDa  | 10   | 2    | 38       | 2        | 36.66667 | 4.371626 |
| Metallo-peptidase, Clan ME, Family M16              | Q4QCI1_LEIMA | 51 kDa  | 6    | 1    | 41.33333 | 3.333333 | 30.66667 | 5.696002 |
| Calcium-transporting ATPase                         | Q4QIM8_LEIMA | 122 kDa | 0    | 0    | 36.66667 | 5.456902 | 34       | 3.05505  |
| Uncharacterized protein                             | Q4QEH5_LEIMA | 94 kDa  | 9    | 7    | 37.33333 | 5.206833 | 31.33333 | 9.820613 |
| Putative vacuolar ATP synthase catalytic subunit A  | Q4Q2H7_LEIMA | 68 kDa  | 39   | 8    | 23.33333 | 0        | 26.66667 | 4.666667 |
| 60S acidic ribosomal protein P0                     | E9ADB9_LEIMA | 35 kDa  | 8    | 3    | 33.33333 | 7.859884 | 38       | 12.05543 |
| Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase  | Q4QJH5_LEIMA | 25 kDa  | 0    | 0    | 36.66667 | 7.423686 | 41.33333 | 3.711843 |
| Mannose-1-phosphate guanylyltransferase             | Q4QBG5_LEIMA | 42 kDa  | 17.5 | 8.5  | 31.33333 | 5.206833 | 32.66667 | 2.666667 |
| Putative small GTP-binding protein Rab11            | Q4QHC3_LEIMA | 23 kDa  | 17   | 6    | 27.33333 | 4.807402 | 40       | 5.291503 |
| Putative 40S ribosomal protein S15A                 | Q4QGW3_LEIMA | 15 kDa  | 0    | 0    | 32       | 7.571878 | 45.33333 | 3.711843 |
| Coatomer subunit beta'                              | Q4Q3L5_LEIMA | 98 kDa  | 18.5 | 4.5  | 28       | 4.163332 | 34.66667 | 4.666667 |
| Succinyl-diaminopimelate desuccinylase-like protein | Q4Q660_LEIMA | 62 kDa  | 14.5 | 6.5  | 30       | 4.163332 | 28.66667 | 7.688375 |
| Ankyrin/TPR repeat protein                          | Q4QAW7_LEIMA | 43 kDa  | 15   | 4    | 32       | 6.110101 | 19.33333 | 6.359595 |

|                                                      |              |         |      |      |          |          |          |          |
|------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Peptidyl-prolyl cis-trans isomerase                  | Q4Q6Q9_LEIMA | 25 kDa  | 7    | 4    | 39.33333 | 1.333333 | 32       | 6.429101 |
| Putative ribosomal protein S20                       | Q4Q8G4_LEIMA | 13 kDa  | 0    | 0    | 26       | 13.01281 | 42       | 21.00794 |
| Uncharacterized protein                              | Q4QCR7_LEIMA | 31 kDa  | 17   | 6    | 36.66667 | 6.960204 | 26.66667 | 4.371626 |
| Putative 60S ribosomal protein L12                   | E9AHW0_LEIIN | 18 kDa  | 0    | 0    | 26       | 3.464102 | 33.33333 | 3.333333 |
| Uncharacterized protein                              | Q4QDK7_LEIMA | 68 kDa  | 9.5  | 4.5  | 26       | 4.163332 | 38       | 4.618802 |
| Succinyl-CoA:3-ketoacid-coenzyme A transferase       | Q4Q7A2_LEIMA | 54 kDa  | 22   | 9    | 35.33333 | 4.371626 | 24       | 5.291503 |
| Putative isovaleryl-coA dehydrogenase                | E9AD70_LEIMA | 45 kDa  | 21   | 6    | 30       | 3.05505  | 27.33333 | 2.905933 |
| Elongation of fatty acids protein                    | Q4QFR8_LEIMA | 33 kDa  | 0    | 0    | 36.66667 | 6.765928 | 36       | 9.237604 |
| Intraflagellar transport protein 122 homolog         | Q4Q1X6_LEIMA | 145 kDa | 12.5 | 5.5  | 28.66667 | 3.527668 | 34       | 4.163332 |
| Putative ATP synthase, epsilon chain                 | Q4Q6S8_LEIMA | 20 kDa  | 12.5 | 2.5  | 37.33333 | 4.666667 | 28.66667 | 2.905933 |
| 3-methylcrotonyl-CoA carboxylase 2                   | Q4QH21_LEIMA | 58 kDa  | 0    | 0    | 34.66667 | 8.353309 | 34.66667 | 15.59202 |
| Fibrillarin                                          | Q4Q1B2_LEIMA | 26 kDa  | 12   | 4    | 18       | 9.0185   | 36.66667 | 3.527668 |
| Putative glycoprotein 96-92                          | Q4Q843_LEIMA | 87 kDa  | 13.5 | 2.5  | 38       | 1.154701 | 25.33333 | 5.206833 |
| Putative proteasome regulatory non-ATP-ase subunit 2 | Q4Q893_LEIMA | 106 kDa | 19.5 | 4.5  | 28.66667 | 0.666667 | 27.33333 | 3.527668 |
| Putative RNA-binding protein                         | A4I2Y0_LEIIN | 50 kDa  | 14   | 4    | 17.33333 | 2.905933 | 29.33333 | 7.423686 |
| Uncharacterized protein                              | Q4Q6U6_LEIMA | 90 kDa  | 9.5  | 2.5  | 30.66667 | 4.371626 | 37.33333 | 6.565905 |
| Elongation of fatty acids protein                    | Q4QFR7_LEIMA | 33 kDa  | 0    | 0    | 36.66667 | 4.371626 | 34.66667 | 3.711843 |
| Ribosomal protein L37                                | E9AEH2_LEIMA | 10 kDa  | 0    | 0    | 15.33333 | 5.696002 | 20       | 0        |
| Phenylalanyl-tRNA synthetase beta subunit            | Q4QDB1_LEIMA | 70 kDa  | 14.5 | 5.5  | 27.33333 | 2.905933 | 30.66667 | 8.51143  |
| Kinesin-like protein                                 | Q9NF78_LEIMA | 75 kDa  | 19   | 10   | 22.66667 | 5.456902 | 32.66667 | 1.333333 |
| Putative beta eliminating lyase                      | E9AC39_LEIMA | 39 kDa  | 21.5 | 2.5  | 30       | 4.163332 | 23.33333 | 6.960204 |
| Putative acyl-CoA dehydrogenase                      | E9AF97_LEIMA | 45 kDa  | 6.5  | 1.5  | 27.33333 | 6.960204 | 31.33333 | 9.955456 |
| Proteasome subunit beta type                         | E9AFK8_LEIMA | 28 kDa  | 9    | 2    | 37.33333 | 2.905933 | 28.66667 | 5.925463 |
| Aldehyde dehydrogenase, mitochondrial                | Q4Q9Y1_LEIMA | 54 kDa  | 39.5 | 12.5 | 25.33333 | 1.763834 | 21.33333 | 5.206833 |
| T-complex protein 1 subunit delta                    | Q4QC87_LEIMA | 60 kDa  | 11   | 6    | 32       | 13.11488 | 30.66667 | 8.666667 |
| FACT complex subunit                                 | E9ADK4_LEIMA | 115 kDa | 10.5 | 6.5  | 33.33333 | 4.055175 | 32       | 4        |
| Malate dehydrogenase                                 | Q4Q7X6_LEIMA | 34 kDa  | 21.5 | 7.5  | 31.33333 | 2.403701 | 25.33333 | 4.666667 |
| Electron transfer flavoprotein subunit alpha         | Q4Q8F2_LEIMA | 34 kDa  | 26.5 | 7.5  | 25.33333 | 5.456902 | 28       | 7.023769 |
| Coronin                                              | Q4QB38_LEIMA | 57 kDa  | 11.5 | 3.5  | 22       | 3.464102 | 23.33333 | 2.403701 |
| Histone H3                                           | Q4QHB5_LEIMA | 15 kDa  | 0    | 0    | 33.33333 | 4.055175 | 34.66667 | 1.763834 |
| Kinetoplast membrane protein 11                      | E9AF45_LEIMA | 11 kDa  | 12.5 | 6.5  | 30       | 5.291503 | 24       | 0        |
| Polyprenol reductase                                 | Q4Q9R2_LEIMA | 34 kDa  | 0    | 0    | 38       | 10.2632  | 26.66667 | 7.512952 |
| Uncharacterized protein                              | A4I5Z0_LEIIN | 36 kDa  | 9.5  | 2.5  | 25.33333 | 2.905933 | 30.66667 | 3.527668 |
| Proteasome subunit beta                              | Q4QJ65_LEIMA | 28 kDa  | 7.5  | 1.5  | 32.66667 | 5.456902 | 23.33333 | 3.711843 |
| Kinesin-like protein                                 | Q4Q7S4_LEIMA | 68 kDa  | 14.5 | 7.5  | 24.66667 | 13.67886 | 35.33333 | 0        |
| Fer2_3 domain-containing protein                     | Q4QB10_LEIMA | 27 kDa  | 3.5  | 0.5  | 37.33333 | 2.403701 | 29.33333 | 5.925463 |
| Uncharacterized protein                              | Q4Q3M7_LEIMA | 158 kDa | 0    | 0    | 32       | 8        | 33.33333 | 6.765928 |
| Cysteine desulphydrase                               | Q4Q511_LEIMA | 44 kDa  | 7    | 0    | 34.66667 | 3.527668 | 28       | 8.717798 |

|                                            |               |         |      |     |          |          |          |          |
|--------------------------------------------|---------------|---------|------|-----|----------|----------|----------|----------|
| Biotin/lipoate protein ligase-like protein | Q4Q6F6_LEIMA  | 28 kDa  | 11.5 | 0.5 | 30.66667 | 0.666667 | 30.66667 | 1.763834 |
| Putative serine/threonine-protein kinase   | Q4Q9K2_LEIMA  | 44 kDa  | 16   | 9   | 26.66667 | 4.666667 | 28       | 6        |
| Uncharacterized protein                    | Q4Q9T0_LEIMA  | 37 kDa  | 15.5 | 4.5 | 31.33333 | 5.456902 | 27.33333 | 0.666667 |
| Putative 60S ribosomal protein L10a        | Q4QDX9_LEIMA  | 25 kDa  | 0    | 0   | 26       | 6.110101 | 32       | 9.865766 |
| Uncharacterized protein                    | E9AE81_LEIMA  | 155 kDa | 0    | 0   | 34.66667 | 1.333333 | 30       | 6        |
| Proteasome subunit alpha type              | Q4Q1R5_LEIMA  | 30 kDa  | 14.5 | 2.5 | 29.33333 | 2.666667 | 24.66667 | 4.666667 |
| GrpE protein homolog                       | Q4Q7N4_LEIMA  | 24 kDa  | 13   | 7   | 30       | 3.464102 | 31.33333 | 4.371626 |
| Glycine cleavage system P protein          | Q4Q9I8_LEIMA  | 106 kDa | 24   | 15  | 29.33333 | 1.333333 | 20       | 2        |
| Ribosome biogenesis protein BOP1 homolog   | BOP1_LEIMA    | 91 kDa  | 0    | 0   | 25.33333 | 5.206833 | 31.33333 | 5.696002 |
| Ribonucleoside-diphosphate reductase       | Q4Q8H8_LEIMA  | 91 kDa  | 9.5  | 6.5 | 30       | 8        | 26       | 9.0185   |
| NADH-cytochrome b5 reductase               | Q4QG48_LEIMA  | 35 kDa  | 5    | 1   | 36.66667 | 6.666667 | 27.33333 | 7.055337 |
| Uncharacterized protein                    | E9ACC9_LEIMA  | 18 kDa  | 17.5 | 3.5 | 32       | 4.163332 | 24       | 6.429101 |
| Putative 60S ribosomal subunit protein L31 | A4IBL9_LEIIN  | 21 kDa  | 9    | 6   | 28.66667 | 10.72898 | 31.33333 | 14.34496 |
| MRH domain-containing protein              | Q4QBV4_LEIMA  | 128 kDa | 0    | 0   | 32.66667 | 10.41367 | 25.33333 | 3.711843 |
| Uncharacterized protein                    | Q4QGZ7_LEIMA  | 37 kDa  | 19.5 | 8.5 | 28.66667 | 3.711843 | 25.33333 | 4.371626 |
| Proteasome subunit alpha type              | E9AFW0_LEIMA  | 27 kDa  | 17.5 | 2.5 | 28.66667 | 1.763834 | 24       | 2        |
| Uncharacterized protein                    | Q4Q137_LEIMA  | 36 kDa  | 19   | 5   | 24       | 5.033223 | 26       | 4.618802 |
| Farnesyl pyrophosphate synthase            | Q4QBL1_LEIMA  | 41 kDa  | 8.5  | 0.5 | 24       | 5.773503 | 34       | 3.05505  |
| 40S ribosomal protein S5                   | Q868B1_LEIMA  | 21 kDa  | 6.5  | 0.5 | 18.66667 | 0        | 34.66667 | 15.37675 |
| Proteasome subunit beta                    | Q4Q289_LEIMA  | 25 kDa  | 12   | 4   | 25.33333 | 1.333333 | 14       | 2.309401 |
| Alanine transaminase                       | Q4QGN0_LEIMA  | 55 kDa  | 13.5 | 5.5 | 22.66667 | 0.666667 | 22.66667 | 5.456902 |
| ATP-dependent RNA helicase eIF4A           | E9AEL4_LEIMA  | 46 kDa  | 8    | 6   | 33.33333 | 5.696002 | 28       | 4.163332 |
| Uncharacterized protein                    | Q4QH89_LEIMA  | 37 kDa  | 3    | 0   | 33.33333 | 3.527668 | 30       | 1.154701 |
| Uncharacterized protein                    | Q4Q0W8_LEIMA  | 17 kDa  | 4.5  | 1.5 | 30       | 1.154701 | 31.33333 | 5.333333 |
| Enoyl-CoA hydratase/isomerase-like protein | E9AE93_LEIMA  | 29 kDa  | 11.5 | 1.5 | 27.33333 | 9.333333 | 28.66667 | 4.666667 |
| Aspartyl putative aminopeptidase           | E9AE98_LEIMA  | 50 kDa  | 12   | 0   | 27.33333 | 5.206833 | 27.33333 | 4.055175 |
| Uncharacterized protein                    | Q4QB62_LEIMA  | 114 kDa | 13   | 7   | 21.33333 | 3.527668 | 30.66667 | 4.371626 |
| RuvB-like helicase                         | Q4Q2T4_LEIMA  | 54 kDa  | 27   | 12  | 22.66667 | 5.456902 | 23.33333 | 7.423686 |
| CCT-alpha                                  | Q4Q4U5_LEIMA  | 59 kDa  | 14.5 | 2.5 | 22       | 5.033223 | 28.66667 | 5.811865 |
| Cysteinyl-tRNA synthetase                  | Q4QGR8_LEIMA  | 89 kDa  | 8    | 3   | 28       | 3.464102 | 29.33333 | 5.696002 |
| Uncharacterized protein                    | Q4QEM4_LEIMA  | 40 kDa  | 4.5  | 0.5 | 30       | 2        | 29.33333 | 5.696002 |
| Putative ribosomal protein L24             | A4HNNW7_LEIBR | 15 kDa  | 6    | 3   | 13.33333 | 4.055175 | 14.66667 | 3.333333 |
| Triosephosphate isomerase                  | Q4QAP8_LEIMA  | 27 kDa  | 12.5 | 1.5 | 28.66667 | 4.055175 | 26       | 6.110101 |
| Putative RNA helicase                      | Q4QC38_LEIMA  | 49 kDa  | 10   | 5   | 27.33333 | 7.423686 | 28.66667 | 6.666667 |
| Putative mitogen-activated protein kinase  | Q4QDK6_LEIMA  | 31 kDa  | 15.5 | 5.5 | 26       | 3.05505  | 23.33333 | 4.807402 |
| Valyl-tRNA synthetase                      | Q4Q6X7_LEIMA  | 110 kDa | 21.5 | 0.5 | 26       | 3.05505  | 22       | 3.05505  |
| Aspartyl-tRNA synthetase                   | Q4Q7R2_LEIMA  | 62 kDa  | 16.5 | 5.5 | 23.33333 | 3.527668 | 26.66667 | 4.666667 |
| Putative RNA-binding protein, UPB2         | Q4QA45_LEIMA  | 19 kDa  | 12.5 | 2.5 | 25.33333 | 6.565905 | 30.66667 | 9.683893 |
| Uncharacterized protein                    | Q4QHD6_LEIMA  | 46 kDa  | 6.5  | 2.5 | 27.33333 | 1.763834 | 26       | 4.163332 |

|                                                              |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative short chain dehydrogenase                           | E9AEU6_LEIMA | 28 kDa  | 18   | 8    | 28       | 4.163332 | 20.66667 | 6.765928 |
| Serine/threonine-protein phosphatase                         | A4HSD9_LEIIN | 72 kDa  | 0    | 0    | 26       | 3.05505  | 28       | 2        |
| Cleavage and polyadenylation specificity factor-like protein | Q4Q5E8_LEIMA | 168 kDa | 9    | 5    | 28       | 5.033223 | 26.66667 | 7.688375 |
| Elongation of fatty acids protein                            | E9APR0_LEIMU | 32 kDa  | 0    | 0    | 27.33333 | 4.055175 | 30.66667 | 6.359595 |
| Myosin XXI                                                   | Q4Q4N5_LEIMA | 119 kDa | 14.5 | 7.5  | 24       | 4.163332 | 23.33333 | 7.333333 |
| ADP-ribosylation factor 1                                    | Q6IMM3_LEIMA | 21 kDa  | 7.5  | 2.5  | 23.33333 | 5.925463 | 28.66667 | 5.206833 |
| Putative aldehyde dehydrogenase                              | Q4Q702_LEIMA | 64 kDa  | 14.5 | 4.5  | 26.66667 | 7.859884 | 20.66667 | 6.565905 |
| Uncharacterized protein                                      | Q4QJ16_LEIMA | 111 kDa | 11   | 8    | 21.33333 | 5.333333 | 17.33333 | 5.206833 |
| Methionine--tRNA ligase                                      | Q4QCD2_LEIMA | 84 kDa  | 17   | 6    | 24.66667 | 5.333333 | 23.33333 | 2.666667 |
| Uncharacterized protein                                      | Q4QH84_LEIMA | 35 kDa  | 9.5  | 5.5  | 30       | 2        | 22       | 5.033223 |
| Putative proteasome regulatory ATPase subunit 1              | A4HZT4_LEIIN | 49 kDa  | 9.5  | 3.5  | 16.66667 | 3.527668 | 25.33333 | 4.055175 |
| Glucosamine-6-phosphate isomerase                            | Q4Q4U6_LEIMA | 32 kDa  | 11   | 1    | 20.66667 | 7.688375 | 18       | 8.082904 |
| Putative aspartate--ammonia ligase                           | Q4Q9B1_LEIMA | 40 kDa  | 8.5  | 0.5  | 25.33333 | 5.696002 | 26.66667 | 1.763834 |
| Methionine aminopeptidase                                    | Q4QDG7_LEIMA | 45 kDa  | 11   | 6    | 26       | 3.05505  | 23.33333 | 4.055175 |
| Putative 10 kDa heat shock protein                           | Q4Q9D2_LEIMA | 11 kDa  | 13.5 | 2.5  | 18.66667 | 9.333333 | 25.33333 | 3.711843 |
| RanBP2-type domain-containing protein                        | Q4Q6E2_LEIMA | 548 kDa | 17   | 14   | 22.66667 | 2.905933 | 21.33333 | 3.333333 |
| Uncharacterized protein                                      | Q4QH86_LEIMA | 35 kDa  | 10.5 | 6.5  | 30       | 4.163332 | 21.33333 | 2.403701 |
| Transmembrane 9 superfamily member                           | E9AE21_LEIMA | 76 kDa  | 0    | 0    | 26.66667 | 4.371626 | 28.66667 | 1.333333 |
| Putative fibrillarin                                         | Q4QDL1_LEIMA | 31 kDa  | 6    | 2    | 20.66667 | 5.811865 | 27.33333 | 0.666667 |
| Putative folate/biopterin transporter                        | Q4QH10_LEIMA | 75 kDa  | 0    | 0    | 28.66667 | 5.206833 | 29.33333 | 12.71919 |
| Uncharacterized protein                                      | Q4QCD1_LEIMA | 150 kDa | 7.5  | 0.5  | 21.33333 | 6.666667 | 22       | 2.309401 |
| Uncharacterized protein                                      | Q4Q353_LEIMA | 38 kDa  | 0    | 0    | 30       | 3.464102 | 25.33333 | 7.055337 |
| Putative rab1 small GTP-binding protein                      | Q4QH98_LEIMA | 25 kDa  | 8    | 3    | 23.33333 | 1.333333 | 28.66667 | 2.403701 |
| Putative immunodominant antigen                              | Q4QFP2_LEIMA | 92 kDa  | 9.5  | 4.5  | 27.33333 | 2.905933 | 19.33333 | 1.763834 |
| Mkiaa0324 protein-like protein                               | Q4Q0H9_LEIMA | 57 kDa  | 0    | 0    | 32       | 8.717798 | 16.66667 | 0        |
| Putative RNA-binding protein                                 | Q4Q7J2_LEIMA | 68 kDa  | 18   | 7    | 17.33333 | 3.711843 | 26       | 2        |
| Putative glutamamyl carboxypeptidase                         | E9AE16_LEIMA | 44 kDa  | 21.5 | 11.5 | 22       | 4.618802 | 20.66667 | 5.811865 |
| 2-oxoisovalerate dehydrogenase subunit alpha                 | Q4QC52_LEIMA | 53 kDa  | 7.5  | 0.5  | 24       | 2.309401 | 22.66667 | 4.055175 |
| Glutathione synthetase                                       | Q4QFP4_LEIMA | 67 kDa  | 14.5 | 6.5  | 21.33333 | 1.763834 | 18       | 4.163332 |
| Putative peptidase M20/M25/M40                               | Q4Q426_LEIMA | 52 kDa  | 16.5 | 5.5  | 20       | 3.05505  | 24       | 5.291503 |
| CS domain-containing protein                                 | Q4QFT9_LEIMA | 37 kDa  | 10   | 3    | 23.33333 | 1.763834 | 26       | 6.110101 |
| Ubiquitin-conjugating enzyme-like protein                    | Q4QFZ6_LEIMA | 16 kDa  | 0    | 0    | 26       | 4        | 24.66667 | 3.527668 |
| Putative p450 reductase                                      | Q4Q8E2_LEIMA | 72 kDa  | 6.5  | 2.5  | 30       | 5.291503 | 20.66667 | 2.403701 |
| DNA topoisomerase I                                          | Q9GPZ9_LEIIN | 74 kDa  | 14.5 | 8.5  | 19.33333 | 5.333333 | 14       | 0        |
| Proteasome regulatory ATPase subunit                         | Q4Q0X8_LEIMA | 46 kDa  | 10.5 | 5.5  | 18       | 9.165151 | 27.33333 | 4.055175 |
| 40S ribosomal protein S14                                    | Q4Q8H1_LEIMA | 16 kDa  | 8    | 4    | 22       | 12.16553 | 25.33333 | 6.765928 |
| Membrane-bound acid phosphatase                              | Q4Q1G4_LEIMA | 57 kDa  | 0    | 0    | 28       | 4.163332 | 26       | 3.05505  |
| Amidase domain-containing protein                            | Q4QEN1_LEIMA | 66 kDa  | 6.5  | 1.5  | 29.33333 | 1.763834 | 18.66667 | 1.333333 |

|                                                              |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Peptidylprolyl isomerase                                     | Q4QHC5_LEIMA | 17 kDa  | 0    | 0    | 27.33333 | 2.403701 | 19.33333 | 7.055337 |
| Putative malic enzyme                                        | Q4QAQ6_LEIMA | 63 kDa  | 42   | 11   | 10       | 5.291503 | 12.66667 | 4.666667 |
| Proteasome subunit alpha type                                | Q4QC00_LEIMA | 27 kDa  | 17.5 | 0.5  | 22.66667 | 1.763834 | 18.66667 | 4.371626 |
| DNA-directed RNA polymerase                                  | Q4QEN2_LEIMA | 200 kDa | 6.5  | 3.5  | 18       | 4.618802 | 28.66667 | 9.820613 |
| Vesicle-fusing ATPase                                        | Q4QCW5_LEIMA | 81 kDa  | 6.5  | 3.5  | 18.66667 | 6.359595 | 27.33333 | 3.711843 |
| Putative small myristoylated protein 4                       | Q5SDH3_LEIMA | 17 kDa  | 8.5  | 0.5  | 22       | 5.291503 | 22.66667 | 5.456902 |
| ACB domain-containing protein                                | Q4QED0_LEIMA | 11 kDa  | 7    | 3    | 26       | 5.033223 | 23.33333 | 5.696002 |
| Uncharacterized protein                                      | Q4Q5Q4_LEIMA | 181 kDa | 9.5  | 6.5  | 21.33333 | 1.763834 | 24.66667 | 4.371626 |
| Uncharacterized protein                                      | Q4QJB3_LEIMA | 208 kDa | 11   | 6    | 20.66667 | 3.711843 | 24       | 7.211103 |
| 60S acidic ribosomal protein P2                              | Q4QF62_LEIMA | 11 kDa  | 18   | 6    | 21.33333 | 6.565905 | 20       | 4.163332 |
| Putative DnaJ protein                                        | Q4QCG6_LEIMA | 49 kDa  | 6.5  | 4.5  | 26       | 7.023769 | 21.33333 | 5.696002 |
| C3H1-type domain-containing protein                          | Q4Q6Q6_LEIMA | 26 kDa  | 9.5  | 1.5  | 30.66667 | 4.807402 | 0        | 0        |
| Putative ATP-binding cassette protein subfamily B, member 1  | Q4QA42_LEIMA | 72 kDa  | 0    | 0    | 27.33333 | 7.688375 | 22.66667 | 2.403701 |
| Aha1_N domain-containing protein                             | Q4QE21_LEIMA | 38 kDa  | 17   | 8    | 20       | 6.928203 | 19.33333 | 2.403701 |
| Putative zinc transporter                                    | Q4Q873_LEIMA | 44 kDa  | 0    | 0    | 25.33333 | 2.905933 | 20.66667 | 10.72898 |
| Putative mitogen activated protein kinase                    | Q4Q449_LEIMA | 47 kDa  | 8.5  | 4.5  | 20       | 3.464102 | 25.33333 | 1.763834 |
| Putative kinesin                                             | E9AF32_LEIMA | 77 kDa  | 12   | 5    | 26       | 11.13553 | 14.66667 | 4.055175 |
| Probable ubiquitin-conjugating enzyme e2-17 kDa              | Q9GNC1_LEIMA | 17 kDa  | 3    | 1    | 24.66667 | 4.666667 | 19.33333 | 2.403701 |
| Uncharacterized protein                                      | Q4QE89_LEIMA | 72 kDa  | 2.5  | 0.5  | 22.66667 | 4.807402 | 27.33333 | 12.45436 |
| Uncharacterized protein                                      | Q4Q4E4_LEIMA | 22 kDa  | 6.5  | 0.5  | 23.33333 | 2.666667 | 22       | 2.309401 |
| Uncharacterized protein                                      | Q4Q4F0_LEIMA | 64 kDa  | 7    | 5    | 17.33333 | 2.403701 | 26       | 5.291503 |
| Putative calcium motive P-type ATPase                        | E9AF31_LEIMA | 122 kDa | 0    | 0    | 24       | 4.163332 | 22       | 6        |
| Guanine nucleotide-binding protein subunit beta-like protein | A4HAA4_LEIBR | 42 kDa  | 0    | 0    | 22       | 3.05505  | 16.66667 | 8.51143  |
| Reticulon-like protein                                       | Q4Q737_LEIMA | 22 kDa  | 0    | 0    | 25.33333 | 1.763834 | 21.33333 | 1.333333 |
| Ubiquitin carboxyl-terminal hydrolase                        | E9AE92_LEIMA | 82 kDa  | 9.5  | 3.5  | 18       | 2.309401 | 23.33333 | 3.527668 |
| Putative 26S proteasome regulatory subunit                   | Q4Q5P6_LEIMA | 40 kDa  | 9.5  | 1.5  | 22       | 3.05505  | 22       | 3.464102 |
| Pyridoxal phosphate homeostasis protein                      | Q4QAZ0_LEIMA | 27 kDa  | 11.5 | 2.5  | 19.33333 | 5.811865 | 20       | 4.163332 |
| Putative hydroxyacylglutathione hydrolase                    | Q4QGS1_LEIMA | 33 kDa  | 6.5  | 0.5  | 24       | 3.464102 | 16.66667 | 8.51143  |
| Uncharacterized protein                                      | Q4Q561_LEIMA | 118 kDa | 0    | 0    | 23.33333 | 4.055175 | 20.66667 | 2.905933 |
| CS domain-containing protein                                 | Q4Q549_LEIMA | 20 kDa  | 0    | 0    | 22       | 5.033223 | 24.66667 | 2.403701 |
| Putative lectin                                              | Q4QG87_LEIMA | 55 kDa  | 7    | 4    | 22.66667 | 2.403701 | 22       | 4.163332 |
| Uncharacterized protein                                      | Q4Q5Y6_LEIMA | 51 kDa  | 0    | 0    | 20       | 0        | 20       | 3.464102 |
| 40S ribosomal protein S25                                    | Q4Q3G4_LEIMA | 13 kDa  | 0    | 0    | 17.33333 | 0        | 26.66667 | 13.53186 |
| Putative 60S ribosomal protein L34                           | A4HY10_LEIIN | 19 kDa  | 0    | 0    | 19.33333 | 5.456902 | 28.66667 | 7.512952 |
| Uncharacterized protein                                      | Q4QDY7_LEIMA | 41 kDa  | 0    | 0    | 22.66667 | 2.403701 | 23.33333 | 2.905933 |
| Proteasome subunit alpha type                                | Q4QFV4_LEIMA | 32 kDa  | 11   | 3    | 20.66667 | 3.711843 | 18.66667 | 2.666667 |
| Alanine--tRNA ligase                                         | Q4QBJ3_LEIMA | 106 kDa | 17.5 | 10.5 | 12.66667 | 3.527668 | 21.33333 | 5.696002 |
| META domain containing protein                               | Q4QEA3_LEIMA | 48 kDa  | 21   | 5    | 17.33333 | 3.711843 | 13.33333 | 7.055337 |

|                                                                |              |         |      |     |          |          |          |          |
|----------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Putative small glutamine-rich tetratricopeptide repeat protein | Q4Q720_LEIMA | 46 kDa  | 0    | 0   | 19.33333 | 3.333333 | 20.66667 | 8.353309 |
| Uncharacterized protein                                        | Q4Q5Q8_LEIMA | 140 kDa | 0    | 0   | 24.66667 | 1.333333 | 21.33333 | 2.403701 |
| Uncharacterized protein                                        | Q4QDI8_LEIMA | 67 kDa  | 7.5  | 1.5 | 14       | 2        | 20.66667 | 6.359595 |
| Superoxide dismutase                                           | Q4QIE0_LEIMA | 26 kDa  | 12   | 4   | 20       | 2        | 20       | 1.154701 |
| Glycerol-3-phosphate dehydrogenase                             | Q4Q8P6_LEIMA | 67 kDa  | 7    | 2   | 20.66667 | 7.688375 | 19.33333 | 7.512952 |
| Peptidase_M28 domain-containing protein                        | Q4Q990_LEIMA | 60 kDa  | 0    | 0   | 24       | 9.451631 | 20.66667 | 1.763834 |
| 6-phosphogluconolactonase                                      | Q4Q8S1_LEIMA | 28 kDa  | 11.5 | 4.5 | 19.33333 | 2.403701 | 20.66667 | 2.905933 |
| Uncharacterized protein                                        | Q4QGZ6_LEIMA | 145 kDa | 0    | 0   | 19.33333 | 2.403701 | 23.33333 | 3.711843 |
| Uncharacterized protein                                        | E9AFG1_LEIMA | 117 kDa | 10.5 | 6.5 | 17.33333 | 3.711843 | 19.33333 | 4.666667 |
| Importin subunit alpha                                         | Q4Q7J1_LEIMA | 58 kDa  | 8    | 1   | 19.33333 | 1.333333 | 22       | 3.464102 |
| Histone H2B                                                    | A4I394_LEIIN | 16 kDa  | 0    | 0   | 18.66667 | 2.666667 | 24.66667 | 4.807402 |
| Putative NADH-cytochrome b5 reductase                          | Q4QBS0_LEIMA | 32 kDa  | 4.5  | 1.5 | 19.33333 | 4.807402 | 22       | 1.154701 |
| Non-specific serine/threonine protein kinase                   | Q9NHE1_LEIMA | 40 kDa  | 16   | 8   | 13.33333 | 6.765928 | 16       | 8.717798 |
| Uncharacterized protein                                        | Q4Q1P9_LEIMA | 59 kDa  | 0    | 0   | 19.33333 | 4.807402 | 22.66667 | 1.763834 |
| Proteasome subunit beta                                        | Q4Q1Q9_LEIMA | 34 kDa  | 7    | 1   | 19.33333 | 1.333333 | 22       | 2        |
| Dihydrolipoamide acetyltransferase                             | Q4QCG0_LEIMA | 40 kDa  | 0    | 0   | 22       | 1.154701 | 22.66667 | 2.403701 |
| DNA-directed RNA polymerase subunit                            | Q4Q600_LEIMA | 185 kDa | 0    | 0   | 18       | 2.309401 | 18.66667 | 2.666667 |
| Adenine phosphoribosyltransferase                              | Q4Q9H8_LEIMA | 26 kDa  | 7.5  | 3.5 | 18       | 3.464102 | 18       | 3.05505  |
| Uncharacterized protein                                        | E9AE73_LEIMA | 192 kDa | 7.5  | 3.5 | 22       | 6.110101 | 15.33333 | 5.206833 |
| Isopentenyl-pyrophosphate isomerase                            | Q5QQ43_LEIMA | 40 kDa  | 5.5  | 1.5 | 20       | 5.033223 | 12.66667 | 4.055175 |
| Transmembrane 9 superfamily member                             | Q4Q2H8_LEIMA | 71 kDa  | 0    | 0   | 22.66667 | 0.666667 | 19.33333 | 3.527668 |
| ATPase ASNA1 homolog                                           | ASNA_LEIMA   | 44 kDa  | 9.5  | 0.5 | 17.33333 | 4.807402 | 19.33333 | 0.666667 |
| Putative phosphoribosyl transferase                            | Q4Q3Z4_LEIMA | 39 kDa  | 0    | 0   | 20.66667 | 7.512952 | 20       | 5.033223 |
| Inosine-guanosine transporter                                  | Q4Q1M9_LEIMA | 54 kDa  | 0    | 0   | 23.33333 | 5.333333 | 11.33333 | 3.527668 |
| CAP/Srv2p                                                      | Q4Q0K2_LEIMA | 29 kDa  | 0    | 0   | 22.66667 | 2.905933 | 19.33333 | 2.905933 |
| Pre-mRNA-processing factor 19                                  | E9ACW8_LEIMA | 53 kDa  | 0    | 0   | 21.33333 | 1.763834 | 18.66667 | 1.333333 |
| RNA binding protein-like protein                               | Q4QII7_LEIMA | 35 kDa  | 6    | 2   | 20       | 2.309401 | 21.33333 | 2.905933 |
| Protein SEY1 homolog                                           | SEY1_LEIMA   | 98 kDa  | 3.5  | 1.5 | 18       | 3.464102 | 22       | 3.05505  |
| Putative 2-hydroxy-3-oxopropionate reductase                   | Q4Q7T9_LEIMA | 31 kDa  | 0    | 0   | 22       | 3.05505  | 16       | 3.05505  |
| Uncharacterized protein                                        | Q4QD20_LEIMA | 29 kDa  | 0    | 0   | 18.66667 | 4.055175 | 22.66667 | 3.711843 |
| Uncharacterized protein                                        | Q4QDM4_LEIMA | 13 kDa  | 10.5 | 2.5 | 15.33333 | 1.333333 | 19.33333 | 2.403701 |
| Uncharacterized protein                                        | Q4QFI9_LEIMA | 104 kDa | 0    | 0   | 20.66667 | 5.206833 | 21.33333 | 10.9138  |
| Uncharacterized protein                                        | E9AD42_LEIMA | 20 kDa  | 0    | 0   | 20       | 2        | 19.33333 | 3.527668 |
| Putative chaperone protein DNAj                                | O97016_LEIMA | 51 kDa  | 0    | 0   | 17.33333 | 3.527668 | 24       | 2        |
| Coatomer subunit beta                                          | Q4QCR1_LEIMA | 110 kDa | 6.5  | 3.5 | 17.33333 | 3.333333 | 18       | 3.05505  |
| Glycine cleavage system H protein                              | E9AFU9_LEIMA | 12 kDa  | 5    | 2   | 18.66667 | 0.666667 | 0        | 0        |
| Putative fatty acyl CoA syntetase 1                            | E9AC38_LEIMA | 77 kDa  | 0    | 0   | 16.66667 | 3.711843 | 18       | 9.0185   |
| Aminopeptidase                                                 | E9AE86_LEIMA | 96 kDa  | 18   | 7   | 18       | 5.291503 | 11.33333 | 0.666667 |

|                                                                     |                  |         |      |     |          |          |          |          |
|---------------------------------------------------------------------|------------------|---------|------|-----|----------|----------|----------|----------|
| Metallo-peptidase, Clan ME, Family M16                              | Q4Q3S5_LEIMA     | 53 kDa  | 11   | 4   | 13.33333 | 2.905933 | 20.66667 | 7.859884 |
| Putative short chain 3-hydroxyacyl-CoA dehydrogenase                | Q4Q1W2_LEIMA     | 33 kDa  | 9.5  | 2.5 | 19.33333 | 5.456902 | 11.33333 | 1.763834 |
| Prohibitin                                                          | Q4QEK4_LEIMA     | 30 kDa  | 0    | 0   | 25.33333 | 4.055175 | 16.66667 | 1.763834 |
| Uncharacterized protein                                             | Q4Q4T2_LEIMA     | 67 kDa  | 0    | 0   | 30       | 14       | 12       | 5.033223 |
| Ubiquitin carboxyl-terminal hydrolase                               | Q4Q2D5_LEIMA     | 103 kDa | 12   | 7   | 21.33333 | 2.666667 | 14       | 2        |
| Uncharacterized protein                                             | Q4QFU5_LEIMA     | 34 kDa  | 7    | 2   | 19.33333 | 1.333333 | 16       | 2        |
| Uncharacterized protein                                             | Q4Q9D1_LEIMA     | 45 kDa  | 7.5  | 3.5 | 17.33333 | 0.666667 | 20       | 3.05505  |
| Putative CAS/CSE/importin domain protein                            | Q4Q6V0_LEIMA     | 108 kDa | 0    | 0   | 16.66667 | 2.403701 | 20.66667 | 0.666667 |
| Putative kinesin K39                                                | Q4QFM2_LEIMA     | 341 kDa | 15   | 5   | 20.66667 | 10.47749 | 0        | 0        |
| Nucleolar protein family a member-like protein                      | Q4Q2E3_LEIMA     | 17 kDa  | 6    | 0   | 18       | 3.464102 | 17.33333 | 2.403701 |
| AMPK1_CBM domain-containing protein                                 | Q4Q4X1_LEIMA     | 97 kDa  | 0    | 0   | 12.66667 | 0        | 18       | 9.451631 |
| Uncharacterized protein                                             | E9ADU3_LEIMA     | 53 kDa  | 0    | 0   | 21.33333 | 6.666667 | 18       | 3.464102 |
| Uncharacterized protein                                             | Q4Q8H4_LEIMA     | 46 kDa  | 0    | 0   | 20       | 6        | 21.33333 | 4.666667 |
| Uncharacterized protein                                             | Q4QG66_LEIMA     | 77 kDa  | 8.5  | 4.5 | 13.33333 | 1.763834 | 13.33333 | 3.711843 |
| Uncharacterized protein                                             | Q4QBX5_LEIMA     | 171 kDa | 0    | 0   | 9.333333 | 4.807402 | 25.33333 | 10.47749 |
| Aromatic amino acid hydroxylase-like                                | Q6WRI4_LEIMA     | 51 kDa  | 0    | 0   | 22       | 4        | 18.66667 | 0.666667 |
| Putative Tob55                                                      | E9AE43_LEIMA     | 53 kDa  | 0    | 0   | 19.33333 | 3.527668 | 22       | 3.05505  |
| Arginase                                                            | A0A145YEM0_LEIMA | 36 kDa  | 4.5  | 1.5 | 17.33333 | 4.055175 | 18.66667 | 2.403701 |
| Uncharacterized protein                                             | Q4QEF3_LEIMA     | 114 kDa | 0    | 0   | 15.33333 | 6.565905 | 13.33333 | 3.527668 |
| Uncharacterized protein                                             | E9AFW3_LEIMA     | 43 kDa  | 0    | 0   | 13.33333 | 3.333333 | 16.66667 | 2.905933 |
| Putative N-acetyltransferase subunit Nat1                           | Q4Q1U2_LEIMA     | 81 kDa  | 0    | 0   | 18.66667 | 5.925463 | 18       | 5.291503 |
| Uncharacterized protein                                             | Q4QCC9_LEIMA     | 347 kDa | 12.5 | 9.5 | 15.33333 | 4.055175 | 14       | 3.05505  |
| Putative 40S ribosomal protein S10                                  | Q4Q1X7_LEIMA     | 19 kDa  | 17   | 4   | 7.333333 | 2.403701 | 10.66667 | 0        |
| ADP/ATP translocase                                                 | Q4QFN6_LEIMA     | 41 kDa  | 0    | 0   | 19.33333 | 6.565905 | 21.33333 | 3.711843 |
| Putative 40S ribosomal protein S23                                  | Q4QC89_LEIMA     | 16 kDa  | 0    | 0   | 12       | 6.110101 | 27.33333 | 11.09554 |
| Putative chaperonin TCP20                                           | Q4QFY8_LEIMA     | 59 kDa  | 0    | 0   | 19.33333 | 6.765928 | 18.66667 | 0        |
| CSN8_PSD8_EIF3K domain-containing protein                           | Q4Q557_LEIMA     | 26 kDa  | 8    | 3   | 16.66667 | 2.905933 | 17.33333 | 5.456902 |
| Uncharacterized protein                                             | Q4Q2J7_LEIMA     | 39 kDa  | 9    | 1   | 18       | 2        | 13.33333 | 1.763834 |
| Protein transport protein SEC23                                     | Q4QHI5_LEIMA     | 88 kDa  | 4.5  | 1.5 | 16.66667 | 5.811865 | 17.33333 | 5.925463 |
| Uncharacterized protein                                             | Q4QD36_LEIMA     | 73 kDa  | 0    | 0   | 17.33333 | 1.763834 | 18       | 3.05505  |
| CDP-diacylglycerol--inositol 3-phosphatidyltransferase              | Q4Q8U3_LEIMA     | 25 kDa  | 0    | 0   | 16       | 8.082904 | 21.33333 | 7.688375 |
| Putative cytochrome c oxidase VII                                   | Q4Q9Y0_LEIMA     | 19 kDa  | 0    | 0   | 20       | 8.326664 | 16.66667 | 8.353309 |
| Putative nucleolar RNA binding protein                              | Q4QF42_LEIMA     | 66 kDa  | 6.5  | 2.5 | 14.66667 | 0        | 20       | 2.309401 |
| Putative ATP synthase                                               | Q4Q1A9_LEIMA     | 25 kDa  | 10.5 | 1.5 | 19.33333 | 1.763834 | 14       | 1.154701 |
| Putative proteasome regulatory non-ATP-ase subunit                  | Q4QIH4_LEIMA     | 36 kDa  | 14   | 7   | 14       | 4        | 12.66667 | 6.359595 |
| Putative 2-oxoisovalerate dehydrogenase beta subunit, mitochondrial | E9AEI2_LEIMA     | 40 kDa  | 4.5  | 0.5 | 19.33333 | 1.763834 | 14       | 2        |
| Uncharacterized protein                                             | Q4Q3G9_LEIMA     | 62 kDa  | 0    | 0   | 18.66667 | 4.807402 | 17.33333 | 2.666667 |

|                                                             |              |         |      |     |          |          |          |          |
|-------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Putative 3-hydroxyacyl-ACP dehydratase                      | Q4QIQ0_LEIMA | 17 kDa  | 10   | 6   | 0        | 0        | 0        | 0        |
| Ribosome production factor 2 homolog                        | Q4Q9J0_LEIMA | 40 kDa  | 6    | 2   | 13.33333 | 2.403701 | 20.66667 | 4.807402 |
| Uncharacterized protein                                     | Q4QEP4_LEIMA | 200 kDa | 0    | 0   | 14       | 4        | 14.66667 | 6.359595 |
| Putative casein kinase II                                   | A4IB73_LEIIN | 43 kDa  | 0    | 0   | 18       | 6.110101 | 18       | 1.154701 |
| Uncharacterized protein                                     | E9ADY2_LEIMA | 79 kDa  | 21   | 6   | 10.66667 | 5.811865 | 12       | 1.154701 |
| Putative ATP-binding cassette protein subfamily F, member 2 | Q4QDE1_LEIMA | 68 kDa  | 9    | 5   | 15.33333 | 3.527668 | 16       | 4.163332 |
| Glutaredoxin domain-containing protein                      | Q4Q940_LEIMA | 43 kDa  | 0    | 0   | 16.66667 | 1.763834 | 13.33333 | 2.666667 |
| Uncharacterized protein                                     | A4I0A2_LEIIN | 16 kDa  | 3.5  | 1.5 | 16.66667 | 5.696002 | 14       | 7.023769 |
| Putative lanosterol synthase                                | Q4QJ13_LEIMA | 113 kDa | 0    | 0   | 16.66667 | 3.711843 | 16       | 6.110101 |
| 40S ribosomal protein S26                                   | Q4Q8L6_LEIMA | 13 kDa  | 0    | 0   | 0        | 0        | 22.66667 | 0        |
| DUF4110 domain-containing protein                           | Q4QF12_LEIMA | 83 kDa  | 11   | 6   | 16.66667 | 4.371626 | 15.33333 | 0.666667 |
| Uncharacterized protein                                     | E9AE96_LEIMA | 21 kDa  | 5    | 3   | 11.33333 | 5.925463 | 14.66667 | 2.403701 |
| Proteasome subunit beta                                     | Q4Q8Q9_LEIMA | 22 kDa  | 9    | 3   | 18       | 2.309401 | 15.33333 | 2.905933 |
| Putative adenylate kinase                                   | Q9U1D3_LEIMA | 23 kDa  | 0    | 0   | 16.66667 | 2.905933 | 16       | 2.309401 |
| Inhibitor of cysteine peptidase                             | Q868G9_LEIMA | 13 kDa  | 7    | 1   | 16       | 1.154701 | 15.33333 | 2.905933 |
| Translation initiation factor-like protein                  | Q4QIB4_LEIMA | 38 kDa  | 14.5 | 3.5 | 13.33333 | 2.403701 | 10.66667 | 2.403701 |
| ANAPC4_WD40 domain-containing protein                       | Q4QI50_LEIMA | 139 kDa | 8.5  | 3.5 | 12       | 1.154701 | 12       | 1.154701 |
| Metallo-peptidase, Clan MA(E), family 32                    | Q4QGE5_LEIMA | 57 kDa  | 12   | 5   | 14       | 3.05505  | 15.33333 | 5.206833 |
| Carboxypeptidase                                            | Q4QDZ7_LEIMA | 52 kDa  | 0    | 0   | 14.66667 | 3.711843 | 14.66667 | 1.763834 |
| Uncharacterized protein                                     | Q4Q4R1_LEIMA | 17 kDa  | 0    | 0   | 18.66667 | 3.333333 | 18.66667 | 2.403701 |
| Uncharacterized protein                                     | E9AEW9_LEIMA | 36 kDa  | 0    | 0   | 14.66667 | 7.688375 | 20.66667 | 4.666667 |
| Pitrilysin-like metalloprotease                             | Q4QIT4_LEIMA | 116 kDa | 6    | 4   | 16.66667 | 3.711843 | 16.66667 | 2.403701 |
| Lysine--tRNA ligase                                         | Q4QFF7_LEIMA | 67 kDa  | 10.5 | 3.5 | 14.66667 | 4.371626 | 14.66667 | 4.055175 |
| START domain-containing protein                             | Q4QGZ1_LEIMA | 37 kDa  | 0    | 0   | 16.66667 | 1.763834 | 16.66667 | 0.666667 |
| AP-1 complex subunit gamma                                  | Q4Q2E4_LEIMA | 89 kDa  | 0    | 0   | 12       | 0        | 17.33333 | 2.905933 |
| Methenyltetrahydrofolate cyclohydrolase                     | Q4Q9F9_LEIMA | 32 kDa  | 5    | 0   | 18       | 2        | 14       | 1.154701 |
| Uncharacterized protein                                     | Q4QDQ7_LEIMA | 160 kDa | 10   | 4   | 14       | 1.154701 | 13.33333 | 4.666667 |
| Putative nuclear transport factor 2                         | A4HUJ7_LEIIN | 14 kDa  | 7    | 0   | 16.66667 | 2.905933 | 14.66667 | 1.763834 |
| Polyadenylate-binding protein                               | E9AFX7_LEIMA | 63 kDa  | 7.5  | 3.5 | 18       | 2.309401 | 14       | 1.154701 |
| Putative acyl-coenzyme A dehydrogenase                      | Q4QIY9_LEIMA | 45 kDa  | 7.5  | 2.5 | 14       | 3.05505  | 12.66667 | 5.206833 |
| Uncharacterized protein                                     | Q4QD99_LEIMA | 41 kDa  | 0    | 0   | 19.33333 | 0.666667 | 17.33333 | 8.969083 |
| Cysteine synthase                                           | Q4Q159_LEIMA | 35 kDa  | 16   | 5   | 14.66667 | 1.763834 | 11.33333 | 2.403701 |
| NADH-cytochrome b5 reductase                                | Q4QFH9_LEIMA | 32 kDa  | 9    | 6   | 15.33333 | 4.371626 | 14       | 1.154701 |
| CTP synthase                                                | A4HYQ5_LEIIN | 66 kDa  | 6.5  | 1.5 | 12       | 3.05505  | 18       | 2        |
| Uncharacterized protein                                     | Q4QHU0_LEIMA | 42 kDa  | 4.5  | 0.5 | 12       | 4.163332 | 15.33333 | 5.206833 |
| Coatomer subunit delta                                      | Q4QEP9_LEIMA | 58 kDa  | 8    | 6   | 14.66667 | 4.807402 | 10.66667 | 5.456902 |
| Peptide-methionine (S)-S-oxide reductase                    | Q4QIH2_LEIMA | 20 kDa  | 0    | 0   | 18       | 3.05505  | 15.33333 | 2.666667 |
| Putative nucleolar RNA-binding protein                      | A4HTC3_LEIIN | 22 kDa  | 9.5  | 2.5 | 0        | 0        | 0        | 0        |

|                                                          |              |         |      |      |          |          |          |          |
|----------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Aldose 1-epimerase-like protein                          | Q4QBD1_LEIMA | 44 kDa  | 9    | 5    | 13.33333 | 0.666667 | 16.66667 | 5.206833 |
| Putative heat shock protein DNAJ                         | Q4QBY6_LEIMA | 37 kDa  | 0    | 0    | 15.33333 | 3.527668 | 18       | 4        |
| LmRab7 GTP-binding protein                               | Q9N2P5_LEIMA | 24 kDa  | 8    | 2    | 14.66667 | 1.333333 | 17.33333 | 3.527668 |
| GP-PDE domain-containing protein                         | Q4QAB4_LEIMA | 38 kDa  | 0    | 0    | 18       | 2        | 11.33333 | 4.055175 |
| NTF2 domain-containing protein                           | Q4QCH2_LEIMA | 45 kDa  | 32.5 | 10.5 | 7.333333 | 1.333333 | 5.333333 | 2.666667 |
| Uncharacterized protein                                  | E9ADG8_LEIMA | 133 kDa | 0    | 0    | 18       | 2        | 15.33333 | 3.333333 |
| CCT-beta                                                 | E9ADA4_LEIMA | 58 kDa  | 0    | 0    | 15.33333 | 0.666667 | 15.33333 | 6.359595 |
| tRNA-binding domain-containing protein                   | Q4QBU9_LEIMA | 45 kDa  | 10   | 4    | 14.66667 | 1.333333 | 14       | 3.464102 |
| Putative alpha glucosidase II subunit                    | Q4QE33_LEIMA | 91 kDa  | 0    | 0    | 12       | 4        | 17.33333 | 6.359595 |
| Uncharacterized protein                                  | Q9NF96_LEIMA | 49 kDa  | 7.5  | 1.5  | 11.33333 | 5.696002 | 16       | 1.154701 |
| Putative ras-related protein rab-5                       | A4HXY7_LEIIN | 25 kDa  | 0    | 0    | 16       | 5.033223 | 17.33333 | 2.905933 |
| Cell division protein kinase 2                           | E9AGX6_LEIIN | 34 kDa  | 0    | 0    | 16.66667 | 1.763834 | 18       | 3.05505  |
| Calcium-transporting ATPase                              | Q95Z93_LEIMA | 112 kDa | 0    | 0    | 21.33333 | 10.34945 | 12       | 3.464102 |
| Putative replication Factor A 28 kDa subunit             | Q4QFF3_LEIMA | 28 kDa  | 8    | 3    | 14       | 3.05505  | 14.66667 | 4.666667 |
| GMP_PDE_delta domain-containing protein                  | E9ADI0_LEIMA | 22 kDa  | 5    | 1    | 16       | 4.618802 | 15.33333 | 3.711843 |
| C-8 sterol isomerase-like protein                        | E9AE76_LEIMA | 25 kDa  | 0    | 0    | 17.33333 | 2.403701 | 16       | 2.309401 |
| Uncharacterized protein                                  | E9AF39_LEIMA | 48 kDa  | 0    | 0    | 18.66667 | 3.333333 | 14       | 4.163332 |
| Putative cleavage and polyadenylation specificity factor | Q4Q2K1_LEIMA | 84 kDa  | 0    | 0    | 15.33333 | 2.403701 | 11.33333 | 5.696002 |
| Putative cytochrome c oxidase subunit V                  | Q4Q922_LEIMA | 22 kDa  | 0    | 0    | 20       | 4        | 16.66667 | 0.666667 |
| Putative NADH:flavin oxidoreductase/NADH oxidase         | Q4QGH6_LEIMA | 42 kDa  | 6.5  | 2.5  | 17.33333 | 2.403701 | 13.33333 | 3.711843 |
| Putative short chain dehydrogenase                       | E9AEU5_LEIMA | 25 kDa  | 0    | 0    | 18.66667 | 3.711843 | 14.66667 | 4.807402 |
| RuvB-like helicase                                       | Q4Q2J4_LEIMA | 50 kDa  | 13   | 8    | 8.666667 | 4.371626 | 14       | 1.154701 |
| Putative acid phosphatase                                | Q4Q1H4_LEIMA | 51 kDa  | 0    | 0    | 13.33333 | 4.055175 | 16       | 3.464102 |
| Uncharacterized protein                                  | E9AE06_LEIMA | 75 kDa  | 15.5 | 7.5  | 12.66667 | 3.527668 | 11.33333 | 0.666667 |
| Mitogen-activated protein kinase                         | Q4QHG6_LEIMA | 44 kDa  | 8.5  | 3.5  | 12       | 1.154701 | 13.33333 | 2.666667 |
| Uncharacterized protein                                  | E9AER5_LEIMA | 28 kDa  | 8    | 4    | 17.33333 | 4.666667 | 9.333333 | 0        |
| Putative monoglyceride lipase                            | Q4Q6E9_LEIMA | 35 kDa  | 0    | 0    | 20       | 5.773503 | 11.33333 | 1.763834 |
| Uncharacterized protein                                  | Q4QEW8_LEIMA | 37 kDa  | 0    | 0    | 17.33333 | 4.371626 | 14       | 7.211103 |
| Cyclic nucleotide-binding domain-containing protein      | Q4Q279_LEIMA | 89 kDa  | 8.5  | 2.5  | 14       | 6.110101 | 15.33333 | 2.905933 |
| Ribulose-phosphate 3-epimerase                           | Q4Q431_LEIMA | 28 kDa  | 10.5 | 0.5  | 14       | 2.309401 | 12.66667 | 2.905933 |
| AAA domain-containing protein                            | Q4Q470_LEIMA | 73 kDa  | 0    | 0    | 12.66667 | 6.565905 | 20       | 3.05505  |
| Putative 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase | Q4Q9N8_LEIMA | 30 kDa  | 5.5  | 1.5  | 13.33333 | 2.403701 | 12.66667 | 6.359595 |
| Putative paraflagellar rod protein 1D                    | E9AE36_LEIMA | 69 kDa  | 0    | 0    | 14.66667 | 2.905933 | 10.66667 | 2.905933 |
| Uncharacterized protein                                  | Q4Q4T3_LEIMA | 66 kDa  | 0    | 0    | 12       | 6.429101 | 17.33333 | 6.960204 |
| Putative prostaglandin f synthase                        | Q4Q5N9_LEIMA | 32 kDa  | 0    | 0    | 14       | 4.618802 | 16.66667 | 4.055175 |
| Uncharacterized protein                                  | Q4QIW6_LEIMA | 33 kDa  | 0    | 0    | 15.33333 | 4.371626 | 19.33333 | 3.527668 |
| S-methyl-5'-thioadenosine phosphorylase                  | Q4QJB9_LEIMA | 33 kDa  | 9    | 2    | 16       | 2        | 12       | 1.154701 |
| Putative ATP-dependent RNA helicase                      | Q4Q1B9_LEIMA | 108 kDa | 10   | 5    | 9.333333 | 0.666667 | 14       | 7.211103 |

|                                                                                 |              |         |      |      |          |          |          |          |
|---------------------------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Putative sterol C-24 reductase                                                  | A4I8T4_LEIIN | 58 kDa  | 0    | 0    | 13.33333 | 6.666667 | 0        | 0        |
| Cytochrome b5 heme-binding domain-containing protein                            | Q4Q088_LEIMA | 16 kDa  | 0    | 0    | 21.33333 | 0.666667 | 12.66667 | 4.666667 |
| Putative proteasome regulatory ATPase subunit 5                                 | Q4QBT5_LEIMA | 50 kDa  | 10   | 4    | 14       | 2        | 11.33333 | 3.333333 |
| Peptidyl-prolyl cis-trans isomerase                                             | Q4QII4_LEIMA | 13 kDa  | 7    | 3    | 17.33333 | 2.905933 | 12       | 2.309401 |
| Putative eukaryotic translation initiation factor 5                             | Q4Q3H3_LEIMA | 43 kDa  | 0    | 0    | 14       | 4.163332 | 14       | 4        |
| Eukaryotic translation initiation factor 5B                                     | Q4Q3R1_LEIMA | 92 kDa  | 21   | 6    | 7.333333 | 3.711843 | 8        | 4        |
| Putative succinate dehydrogenase                                                | A4HWJ7_LEIIN | 22 kDa  | 0    | 0    | 21.33333 | 3.711843 | 12.66667 | 2.905933 |
| Putative RNA-binding protein                                                    | A4HMP2_LEIBR | 30 kDa  | 51   | 11   | 0        | 0        | 0        | 0        |
| Putative ubiquitin hydrolase                                                    | Q4QF52_LEIMA | 155 kDa | 9.5  | 7.5  | 10.66667 | 1.763834 | 10.66667 | 5.333333 |
| Putative N-acyl-L-amino acid amidohydrolase                                     | Q4QCM5_LEIMA | 43 kDa  | 9    | 4    | 12       | 3.05505  | 12       | 6.110101 |
| Putative Ran-binding protein 1                                                  | A4HVS6_LEIIN | 18 kDa  | 0    | 0    | 18.66667 | 2.403701 | 13.33333 | 2.905933 |
| Uncharacterized protein                                                         | A4HM49_LEIBR | 24 kDa  | 0    | 0    | 18.66667 | 1.763834 | 0        | 0        |
| BRO1 domain-containing protein                                                  | E9ADE3_LEIMA | 102 kDa | 8    | 3    | 14       | 3.464102 | 13.33333 | 5.811865 |
| Glutamine amidotransferase                                                      | Q4QBY3_LEIMA | 71 kDa  | 14   | 4    | 6.666667 | 3.333333 | 12       | 2.309401 |
| Vesicle-fusing ATPase                                                           | E9AD85_LEIMA | 108 kDa | 16.5 | 10.5 | 8        | 0        | 9.333333 | 2.403701 |
| 1-alkyl-2-acetylglycerophosphocholine esterase                                  | E9AFC6_LEIMA | 50 kDa  | 0    | 0    | 15.33333 | 3.711843 | 12.66667 | 1.763834 |
| DAO domain-containing protein                                                   | Q4Q0L5_LEIMA | 57 kDa  | 0    | 0    | 15.33333 | 5.206833 | 15.33333 | 4.666667 |
| Putative casein kinase II, alpha chain                                          | E9ACB1_LEIMA | 44 kDa  | 0    | 0    | 8.666667 | 2.905933 | 12       | 2        |
| Oxygen-dependent coproporphyrinogen-III oxidase                                 | HEM6_LEIMA   | 34 kDa  | 6    | 2    | 15.33333 | 2.403701 | 9.333333 | 0.666667 |
| Putative aspartate aminotransferase                                             | Q4QAU4_LEIMA | 48 kDa  | 0    | 0    | 16       | 2        | 11.33333 | 3.527668 |
| Uncharacterized protein                                                         | Q4QBL5_LEIMA | 228 kDa | 0    | 0    | 15.33333 | 3.333333 | 9.333333 | 4.807402 |
| Guanine nucleotide-binding protein subunit beta-like protein                    | Q4Q4L7_LEIMA | 47 kDa  | 0    | 0    | 11.33333 | 2.666667 | 14.66667 | 4.055175 |
| Serine/threonine-protein phosphatase                                            | A4I3E3_LEIIN | 34 kDa  | 0    | 0    | 14.66667 | 0.666667 | 12       | 3.464102 |
| DUF4139 domain-containing protein                                               | Q4Q7L0_LEIMA | 63 kDa  | 0    | 0    | 16       | 3.464102 | 12       | 3.05505  |
| Putative RNA-binding protein                                                    | A4HXP8_LEIIN | 43 kDa  | 0    | 0    | 10.66667 | 2.403701 | 0        | 0        |
| Uncharacterized protein                                                         | A4IC22_LEIIN | 39 kDa  | 0    | 0    | 12       | 6.110101 | 15.33333 | 2.666667 |
| Uncharacterized protein                                                         | Q4Q4S1_LEIMA | 56 kDa  | 0    | 0    | 15.33333 | 2.905933 | 10.66667 | 2.403701 |
| Paraflagellar rod protein 2C                                                    | Q4QEM2_LEIMA | 69 kDa  | 0    | 0    | 16       | 4.163332 | 11.33333 | 5.696002 |
| Putative ubiquitin hydrolase                                                    | Q4Q7T4_LEIMA | 126 kDa | 5    | 2    | 14.66667 | 5.811865 | 11.33333 | 1.333333 |
| 2-oxoglutarate dehydrogenase, e3 component, lipoamidedehydrogenase-like protein | Q4Q5Z7_LEIMA | 67 kDa  | 0    | 0    | 14.66667 | 3.333333 | 15.33333 | 2.403701 |
| GRIP domain-containing protein                                                  | Q4QH73_LEIMA | 148 kDa | 0    | 0    | 16.66667 | 5.206833 | 0        | 0        |
| Uncharacterized protein                                                         | Q4QHK7_LEIMA | 79 kDa  | 14   | 8    | 9.333333 | 5.206833 | 10       | 2.309401 |
| Uncharacterized protein                                                         | Q4Q1W0_LEIMA | 41 kDa  | 0    | 0    | 16.66667 | 1.763834 | 12       | 0        |
| Uridine 5'-monophosphate synthase                                               | Q4QEW5_LEIMA | 50 kDa  | 0    | 0    | 13.33333 | 1.763834 | 12       | 1.154701 |
| Uncharacterized protein                                                         | Q4Q9S9_LEIMA | 17 kDa  | 0    | 0    | 10       | 5.291503 | 16       | 5.033223 |
| Putative dynein heavy chain                                                     | Q4Q9Z6_LEIMA | 535 kDa | 0    | 0    | 10       | 5.291503 | 12       | 6.429101 |
| Uncharacterized protein                                                         | Q4QDF6_LEIMA | 203 kDa | 6.5  | 3.5  | 12.66667 | 3.711843 | 7.333333 | 3.711843 |

|                                                              |              |         |      |      |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|------|----------|----------|----------|----------|
| Uncharacterized protein                                      | Q4Q5E5_LEIMA | 86 kDa  | 4.5  | 0.5  | 11.33333 | 2.905933 | 15.33333 | 2.905933 |
| Putative intraflagellar transport protein IFT88              | E9AD91_LEIMA | 91 kDa  | 10   | 3    | 12       | 2.309401 | 10       | 1.154701 |
| Squalene monooxygenase                                       | Q4QFZ2_LEIMA | 64 kDa  | 0    | 0    | 12       | 5.291503 | 10.66667 | 3.711843 |
| Peptidyl-prolyl cis-trans isomerase                          | Q4Q1A6_LEIMA | 29 kDa  | 0    | 0    | 12       | 2.309401 | 15.33333 | 1.763834 |
| Uncharacterized protein                                      | E9AC37_LEIMA | 150 kDa | 14.5 | 11.5 | 8        | 2        | 8.666667 | 4.666667 |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4Q8F5_LEIMA | 68 kDa  | 0    | 0    | 16.66667 | 4.055175 | 13.33333 | 3.711843 |
| Uncharacterized protein                                      | Q4Q7J9_LEIMA | 29 kDa  | 0    | 0    | 16       | 1.154701 | 12.66667 | 4.055175 |
| Uncharacterized protein                                      | E9ADV8_LEIMA | 67 kDa  | 0    | 0    | 18.66667 | 6.565905 | 11.33333 | 5.696002 |
| Uncharacterized protein                                      | Q4Q0G9_LEIMA | 41 kDa  | 12   | 0    | 8        | 4.163332 | 10.66667 | 0.666667 |
| CS domain-containing protein                                 | Q4Q3I7_LEIMA | 23 kDa  | 0    | 0    | 12.66667 | 1.763834 | 10.66667 | 5.333333 |
| NADH-cytochrome b5 reductase                                 | Q4QBR9_LEIMA | 32 kDa  | 4.5  | 1.5  | 9.333333 | 4.807402 | 16       | 3.05505  |
| Uncharacterized protein                                      | Q4QBP8_LEIMA | 139 kDa | 11.5 | 2.5  | 11.33333 | 2.666667 | 9.333333 | 4.807402 |
| Uncharacterized protein                                      | O97200_LEIMA | 167 kDa | 14.5 | 8.5  | 8.666667 | 4.371626 | 10.66667 | 2.905933 |
| HASPA1                                                       | P90552_LEIMA | 8 kDa   | 4    | 1    | 0        | 0        | 0        | 0        |
| V-type proton ATPase subunit a                               | Q4QAY7_LEIMA | 88 kDa  | 6    | 3    | 16       | 0        | 5.333333 | 2.905933 |
| Protein transport protein SEC23                              | Q4Q0B5_LEIMA | 94 kDa  | 0    | 0    | 14       | 1.154701 | 11.33333 | 2.905933 |
| Uncharacterized protein                                      | Q4Q553_LEIMA | 154 kDa | 0    | 0    | 15.33333 | 3.711843 | 11.33333 | 1.333333 |
| Putative proteasome regulatory ATPase subunit tcc118.3       | E9AGB0_LEIIN | 46 kDa  | 4.5  | 1.5  | 11.33333 | 5.696002 | 14       | 2.309401 |
| Putative cysteine desulfurase                                | E9AD84_LEIMA | 48 kDa  | 0    | 0    | 17.33333 | 5.333333 | 8.666667 | 1.763834 |
| Uncharacterized protein                                      | Q4Q7P8_LEIMA | 54 kDa  | 0    | 0    | 12       | 2.309401 | 15.33333 | 2.403701 |
| Hypothetical STT3 ortholog                                   | Q9U5N8_LEIMA | 92 kDa  | 0    | 0    | 8.666667 | 4.666667 | 12.66667 | 1.333333 |
| H/ACA ribonucleoprotein complex subunit                      | Q4Q2B2_LEIMA | 22 kDa  | 0    | 0    | 14.66667 | 2.905933 | 12       | 2        |
| Uncharacterized protein                                      | Q4Q3H0_LEIMA | 62 kDa  | 0    | 0    | 14       | 7.023769 | 9.333333 | 2.666667 |
| Putative tyrosyl-tRNA synthetase                             | Q4QFJ7_LEIMA | 75 kDa  | 11.5 | 1.5  | 9.333333 | 1.763834 | 7.333333 | 3.711843 |
| Electron transfer flavoprotein subunit beta                  | Q4QA84_LEIMA | 28 kDa  | 9.5  | 4.5  | 14       | 2.309401 | 8.666667 | 2.403701 |
| Uncharacterized protein                                      | Q4Q1W3_LEIMA | 50 kDa  | 0    | 0    | 16       | 5.773503 | 10       | 3.05505  |
| Uncharacterized protein                                      | E9AFT2_LEIMA | 161 kDa | 0    | 0    | 10.66667 | 2.403701 | 14       | 0        |
| UDP-glucose 4-epimerase                                      | Q4Q3V7_LEIMA | 44 kDa  | 0    | 0    | 16       | 2.309401 | 10       | 4        |
| Putative ornithine decarboxylase                             | Q4QGR5_LEIMA | 77 kDa  | 0    | 0    | 14       | 3.05505  | 12.66667 | 3.527668 |
| Uncharacterized protein                                      | Q4Q109_LEIMA | 75 kDa  | 0    | 0    | 12       | 1.154701 | 13.33333 | 4.055175 |
| Uncharacterized protein                                      | A4I239_LEIIN | 15 kDa  | 0    | 0    | 16.66667 | 5.925463 | 11.33333 | 3.527668 |
| Uncharacterized protein                                      | Q4QDX5_LEIMA | 77 kDa  | 12.5 | 6.5  | 0        | 0        | 11.33333 | 0        |
| Uncharacterized protein                                      | Q4Q2F3_LEIMA | 35 kDa  | 0    | 0    | 11.33333 | 5.925463 | 12.66667 | 0.666667 |
| Putative pantothenate kinase subunit                         | Q4Q8Q6_LEIMA | 162 kDa | 0    | 0    | 9.333333 | 2.905933 | 14.66667 | 5.456902 |
| Uncharacterized protein                                      | Q4QBV1_LEIMA | 32 kDa  | 4    | 2    | 11.33333 | 2.403701 | 12       | 6.110101 |
| Pyrroline-5-carboxylate reductase                            | Q4QFY6_LEIMA | 29 kDa  | 10.5 | 2.5  | 14       | 3.05505  | 6        | 3.05505  |
| Uncharacterized protein                                      | E9AF04_LEIMA | 82 kDa  | 0    | 0    | 10       | 0        | 8.666667 | 4.371626 |
| Uncharacterized protein                                      | E9AEK6_LEIMA | 151 kDa | 0    | 0    | 18       | 5.033223 | 8.666667 | 4.666667 |
| Uncharacterized protein                                      | Q4Q483_LEIMA | 28 kDa  | 0    | 0    | 12.66667 | 3.711843 | 16       | 4.163332 |

|                                                                          |              |         |     |     |          |          |          |          |
|--------------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative mitochondrial carrier protein                                   | E9ACE1_LEIMA | 34 kDa  | 0   | 0   | 13.33333 | 3.333333 | 13.33333 | 1.333333 |
| Putative epsin                                                           | Q4QA28_LEIMA | 66 kDa  | 0   | 0   | 12       | 4        | 12       | 3.05505  |
| Putative CCR4 associated factor                                          | Q4QBI4_LEIMA | 38 kDa  | 0   | 0   | 11.33333 | 1.763834 | 14       | 4        |
| Uncharacterized protein                                                  | Q4QH54_LEIMA | 66 kDa  | 0   | 0   | 10       | 4        | 13.33333 | 2.403701 |
| Putative serine-threonine dehydratase                                    | A4HSW5_LEIIN | 37 kDa  | 10  | 4   | 10.66667 | 0.666667 | 10       | 1.154701 |
| Ubiquitin carboxyl-terminal hydrolase                                    | Q4QAT9_LEIMA | 34 kDa  | 0   | 0   | 11.33333 | 2.403701 | 13.33333 | 4.666667 |
| Putative serine/threonine-protein kinase                                 | Q4Q6T0_LEIMA | 85 kDa  | 6.5 | 3.5 | 10.66667 | 1.763834 | 10       | 0        |
| Casein kinase II subunit beta                                            | Q4Q0Q3_LEIMA | 31 kDa  | 0   | 0   | 11.33333 | 1.763834 | 14       | 2        |
| Putative p22 protein                                                     | Q4Q7K6_LEIMA | 22 kDa  | 0   | 0   | 17.33333 | 5.456902 | 0        | 0        |
| Excreted/secreted protein 27                                             | Q1X7K5_LEIMA | 13 kDa  | 0   | 0   | 12       | 6.429101 | 14       | 3.05505  |
| Uncharacterized protein                                                  | E9AHP6_LEIIN | 56 kDa  | 7.5 | 2.5 | 12       | 2.309401 | 8        | 0        |
| Poly(A)-binding protein 3                                                | Q4QA88_LEIMA | 61 kDa  | 7.5 | 3.5 | 8        | 1.154701 | 11.33333 | 4.371626 |
| Peptidase_M24 domain-containing protein                                  | Q4QG86_LEIMA | 62 kDa  | 0   | 0   | 10.66667 | 4.666667 | 11.33333 | 5.696002 |
| Transaldolase                                                            | Q4QEU3_LEIMA | 37 kDa  | 9.5 | 1.5 | 12       | 6.429101 | 5.333333 | 1.333333 |
| Kinetoplast-associated protein-like protein                              | E9AD01_LEIMA | 79 kDa  | 7   | 3   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                                  | Q4QF65_LEIMA | 138 kDa | 7   | 2   | 11.33333 | 1.763834 | 8.666667 | 0.666667 |
| Uncharacterized protein                                                  | O97220_LEIMA | 39 kDa  | 0   | 0   | 10       | 0        | 8        | 4.163332 |
| DUF4139 domain-containing protein                                        | E9AE72_LEIMA | 69 kDa  | 0   | 0   | 10       | 3.464102 | 6.666667 | 3.527668 |
| Uncharacterized protein                                                  | Q4QB63_LEIMA | 26 kDa  | 0   | 0   | 13.33333 | 4.807402 | 12       | 1.154701 |
| Uncharacterized protein                                                  | Q4Q4D2_LEIMA | 59 kDa  | 0   | 0   | 12       | 4.163332 | 12       | 0        |
| Uncharacterized protein                                                  | Q4Q5J9_LEIMA | 51 kDa  | 0   | 0   | 10       | 5.033223 | 12.66667 | 1.763834 |
| Conserved CBS domain protein                                             | E9AEQ1_LEIMA | 52 kDa  | 7.5 | 3.5 | 10.66667 | 2.666667 | 10       | 2.309401 |
| Uncharacterized protein                                                  | Q4Q3Z6_LEIMA | 65 kDa  | 0   | 0   | 12.66667 | 3.527668 | 10.66667 | 2.905933 |
| Prefoldin subunit 3                                                      | A4I267_LEIIN | 22 kDa  | 0   | 0   | 12.66667 | 2.403701 | 9.333333 | 4.666667 |
| Uncharacterized protein                                                  | Q4QAQ0_LEIMA | 94 kDa  | 0   | 0   | 8.666667 | 4.371626 | 14       | 1.154701 |
| Uncharacterized protein                                                  | Q4Q8J8_LEIMA | 65 kDa  | 0   | 0   | 12       | 1.154701 | 10.66667 | 2.403701 |
| SURF1-like protein                                                       | Q4QGE3_LEIMA | 40 kDa  | 0   | 0   | 11.33333 | 3.527668 | 11.33333 | 0.666667 |
| Putative glycogen synthase kinase                                        | Q4QE15_LEIMA | 41 kDa  | 0   | 0   | 8.666667 | 4.666667 | 0        | 0        |
| Putative signal recognition particle receptor like protein               | Q4Q161_LEIMA | 66 kDa  | 0   | 0   | 10       | 1.154701 | 7.333333 | 3.711843 |
| Putative DNA-directed RNA polymerase, alpha subunit                      | Q4QDF5_LEIMA | 47 kDa  | 0   | 0   | 10       | 5.291503 | 12.66667 | 3.527668 |
| FYVE-type domain-containing protein                                      | Q4Q952_LEIMA | 257 kDa | 6   | 1   | 6.666667 | 3.527668 | 12       | 1.154701 |
| DNA topoisomerase                                                        | Q4Q888_LEIMA | 96 kDa  | 8   | 2   | 8.666667 | 0.666667 | 10.66667 | 2.403701 |
| Putative OSM3-like kinesin                                               | Q4Q5L5_LEIMA | 127 kDa | 8   | 3   | 9.333333 | 2.905933 | 0        | 0        |
| Uncharacterized protein                                                  | Q4Q760_LEIMA | 37 kDa  | 0   | 0   | 10       | 1.154701 | 6.666667 | 0.666667 |
| Phosphomannomutase-like protein                                          | Q4Q2G5_LEIMA | 65 kDa  | 0   | 0   | 7.333333 | 3.711843 | 10       | 5.291503 |
| Methylcrotonoyl-coa carboxylase biotinylated subunitprotein-like protein | Q4Q5U3_LEIMA | 76 kDa  | 0   | 0   | 8        | 4.163332 | 15.33333 | 7.859884 |
| Uncharacterized protein                                                  | A4I5W4_LEIIN | 13 kDa  | 0   | 0   | 12.66667 | 1.333333 | 8        | 4        |
| DNA-(apurinic or apyrimidinic site) endonuclease                         | O15922_LEIMA | 49 kDa  | 0   | 0   | 11.33333 | 0.666667 | 10.66667 | 3.333333 |

|                                                                                                                           |              |         |     |     |          |          |          |          |
|---------------------------------------------------------------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative proteasome regulatory non-ATP-ase subunit                                                                        | A4HYC9_LEIIN | 47 kDa  | 6   | 2   | 10       | 0        | 4.666667 | 0        |
| Hs1vu complex proteolytic subunit-like,hs1vu complex proteolytic subunit-like, threonine peptidase, Clan T(1), family T1B | E9AT11_LEIMU | 25 kDa  | 0   | 0   | 13.33333 | 0.666667 | 8.666667 | 4.371626 |
| Uncharacterized protein                                                                                                   | Q4Q5R6_LEIMA | 82 kDa  | 3.5 | 0.5 | 11.33333 | 4.371626 | 9.333333 | 1.333333 |
| Fatty acyl-CoA reductase                                                                                                  | Q4QAG7_LEIMA | 65 kDa  | 4.5 | 0.5 | 10.66667 | 1.763834 | 10       | 2        |
| Uncharacterized protein                                                                                                   | Q4QH87_LEIMA | 36 kDa  | 8.5 | 3.5 | 7.333333 | 3.711843 | 6        | 3.05505  |
| Uncharacterized protein                                                                                                   | Q4QHQ8_LEIMA | 22 kDa  | 0   | 0   | 11.33333 | 1.333333 | 7.333333 | 0.666667 |
| Uncharacterized protein                                                                                                   | Q4QI34_LEIMA | 19 kDa  | 0   | 0   | 13.33333 | 5.206833 | 11.33333 | 0.666667 |
| PITH domain-containing protein                                                                                            | Q4QGU2_LEIMA | 23 kDa  | 6.5 | 2.5 | 9.333333 | 2.403701 | 10.66667 | 1.333333 |
| Putative eukaryotic initiation factor 4a                                                                                  | Q4Q0F4_LEIMA | 85 kDa  | 5.5 | 1.5 | 10       | 3.05505  | 9.333333 | 1.763834 |
| Uncharacterized protein                                                                                                   | Q4QC59_LEIMA | 38 kDa  | 0   | 0   | 10.66667 | 2.666667 | 8.666667 | 1.763834 |
| Uncharacterized protein                                                                                                   | Q4Q9Q7_LEIMA | 90 kDa  | 0   | 0   | 12.66667 | 1.333333 | 10       | 5.291503 |
| Uncharacterized protein                                                                                                   | Q4Q425_LEIMA | 101 kDa | 0   | 0   | 8.666667 | 1.763834 | 9.333333 | 2.905933 |
| Cystathionine beta-synthase                                                                                               | Q4QEG9_LEIMA | 39 kDa  | 8   | 5   | 10       | 1.154701 | 8        | 1.154701 |
| Isocitrate dehydrogenase [NADP]                                                                                           | Q4Q3T2_LEIMA | 46 kDa  | 6.5 | 2.5 | 10       | 3.05505  | 7.333333 | 4.055175 |
| NLE domain-containing protein                                                                                             | Q4Q6P4_LEIMA | 52 kDa  | 0   | 0   | 11.33333 | 0.666667 | 11.33333 | 2.403701 |
| Uncharacterized protein                                                                                                   | E9ADE7_LEIMA | 59 kDa  | 10  | 6   | 6        | 0        | 4.666667 | 2.403701 |
| Acetyltransferase-like protein                                                                                            | Q53HX3_LEIMA | 18 kDa  | 0   | 0   | 14       | 2        | 0        | 0        |
| Protein kinase A catalytic subunit                                                                                        | Q27687_LEIMA | 38 kDa  | 5.5 | 2.5 | 7.333333 | 3.711843 | 10       | 1.154701 |
| Putative pumilio protein 6                                                                                                | Q4Q475_LEIMA | 93 kDa  | 0   | 0   | 7.333333 | 1.333333 | 4.666667 | 0        |
| Putative chaperone protein DNAj                                                                                           | Q4Q4U2_LEIMA | 45 kDa  | 0   | 0   | 10.66667 | 2.403701 | 11.33333 | 0.666667 |
| Putative haloacid dehalogenase-like hydrolase                                                                             | Q4Q8C9_LEIMA | 32 kDa  | 5.5 | 2.5 | 12.66667 | 1.763834 | 0        | 0        |
| Epimerase domain-containing protein                                                                                       | Q4Q2M5_LEIMA | 55 kDa  | 0   | 0   | 8.666667 | 2.403701 | 12.66667 | 2.905933 |
| Uncharacterized protein                                                                                                   | E9AEL2_LEIMA | 76 kDa  | 0   | 0   | 10.66667 | 2.905933 | 10       | 3.464102 |
| Putative propionyl-coa carboxylase beta chain                                                                             | Q4Q8M1_LEIMA | 57 kDa  | 0   | 0   | 10       | 3.05505  | 5.333333 | 2.905933 |
| PH domain-containing protein                                                                                              | Q4Q7E6_LEIMA | 93 kDa  | 0   | 0   | 8        | 4        | 9.333333 | 1.763834 |
| Uncharacterized protein                                                                                                   | E9AFY0_LEIMA | 19 kDa  | 0   | 0   | 11.33333 | 1.333333 | 10       | 1.154701 |
| Uncharacterized protein                                                                                                   | Q4Q3K4_LEIMA | 66 kDa  | 0   | 0   | 10.66667 | 2.666667 | 10       | 2        |
| Uncharacterized protein                                                                                                   | Q4Q1B0_LEIMA | 89 kDa  | 0   | 0   | 10       | 0        | 10.66667 | 5.456902 |
| Putative sodium stibogluconate resistance protein                                                                         | Q4Q6H0_LEIMA | 69 kDa  | 0   | 0   | 8.666667 | 0        | 9.333333 | 0        |
| Proteasome subunit alpha type                                                                                             | A4HUT6_LEIIN | 28 kDa  | 8   | 2   | 9.333333 | 2.905933 | 6        | 3.05505  |
| Putative proteasome regulatory non-ATP-ase subunit 5                                                                      | A4HZ77_LEIIN | 54 kDa  | 0   | 0   | 10       | 1.154701 | 7.333333 | 1.333333 |
| Putative calpain-like cysteine peptidase                                                                                  | Q4Q6M4_LEIMA | 97 kDa  | 0   | 0   | 12       | 0        | 0        | 0        |
| Uncharacterized protein                                                                                                   | Q4Q450_LEIMA | 45 kDa  | 0   | 0   | 10.66667 | 2.403701 | 10.66667 | 0.666667 |
| DNA-directed RNA polymerase subunit beta                                                                                  | A4I6A8_LEIIN | 133 kDa | 0   | 0   | 10       | 1.154701 | 9.333333 | 4.807402 |
| 60S ribosomal protein L37a                                                                                                | A4HLC4_LEIBR | 11 kDa  | 0   | 0   | 8.666667 | 4.666667 | 0        | 0        |
| Uncharacterized protein                                                                                                   | Q4Q6B2_LEIMA | 120 kDa | 0   | 0   | 8.666667 | 1.763834 | 9.333333 | 1.763834 |
| Putative eukaryotic translation initiation factor 1A                                                                      | A4H8E1_LEIBR | 19 kDa  | 5   | 1   | 8        | 0        | 8.666667 | 4.371626 |

|                                                              |              |         |     |     |           |          |          |          |
|--------------------------------------------------------------|--------------|---------|-----|-----|-----------|----------|----------|----------|
| Cleavage and polyadenylation specificity factor subunit 2    | Q4QA79_LEIMA | 90 kDa  | 0   | 0   | 8.666667  | 0.666667 | 6.666667 | 3.527668 |
| Nucleoside phosphorylase-like protein                        | Q4QHB3_LEIMA | 37 kDa  | 0   | 0   | 11.333333 | 1.333333 | 10       | 2.309401 |
| 60S ribosomal protein L29                                    | Q4Q178_LEIMA | 8 kDa   | 0   | 0   | 8.666667  | 4.371626 | 12.66667 | 6.565905 |
| Uncharacterized protein                                      | Q4QH85_LEIMA | 35 kDa  | 3.5 | 0.5 | 10.66667  | 2.403701 | 7.333333 | 1.333333 |
| Putative eukaryotic translation release factor               | A4I2W3_LEIIN | 51 kDa  | 8.5 | 2.5 | 6         | 3.05505  | 8.666667 | 0.666667 |
| Uncharacterized protein                                      | Q4QAZ1_LEIMA | 24 kDa  | 4.5 | 0.5 | 7.333333  | 3.711843 | 6.666667 | 3.333333 |
| FCP1 homology domain-containing protein                      | E9AEJ6_LEIMA | 27 kDa  | 0   | 0   | 0         | 0        | 9.333333 | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QA52_LEIMA | 110 kDa | 0   | 0   | 5.333333  | 2.905933 | 10       | 5.033223 |
| Uncharacterized protein                                      | E9AFI0_LEIMA | 44 kDa  | 0   | 0   | 9.333333  | 4.666667 | 9.333333 | 1.763834 |
| Uncharacterized protein                                      | A4HN11_LEIBR | 13 kDa  | 0   | 0   | 8.666667  | 4.371626 | 12       | 1.154701 |
| Putative ADP ribosylation factor 3                           | E9ADU4_LEIMA | 20 kDa  | 13  | 5   | 0         | 0        | 0        | 0        |
| Hypothetical predicted multi-pass transmembrane protein      | Q4QAE6_LEIMA | 44 kDa  | 0   | 0   | 9.333333  | 1.333333 | 8        | 0        |
| Actin interacting protein-like protein                       | Q4QB82_LEIMA | 57 kDa  | 8.5 | 3.5 | 7.333333  | 3.711843 | 0        | 0        |
| PPM-type phosphatase domain-containing protein               | Q4QFG7_LEIMA | 44 kDa  | 0   | 0   | 9.333333  | 4.807402 | 10.66667 | 5.333333 |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QAE1_LEIMA | 88 kDa  | 0   | 0   | 10.66667  | 2.403701 | 10       | 3.05505  |
| Uncharacterized protein                                      | Q4Q2P0_LEIMA | 64 kDa  | 0   | 0   | 8         | 1.154701 | 12       | 3.464102 |
| Putative ribose 5-phosphate isomerase                        | Q4Q869_LEIMA | 19 kDa  | 0   | 0   | 10        | 2        | 7.333333 | 3.711843 |
| PHB domain-containing protein                                | Q4QD86_LEIMA | 31 kDa  | 0   | 0   | 8.666667  | 4.371626 | 10       | 2.309401 |
| Delta-12 fatty acid desaturase                               | Q4Q3K9_LEIMA | 46 kDa  | 0   | 0   | 0         | 0        | 10.66667 | 5.333333 |
| Major vault protein                                          | MVP_LEIMA    | 93 kDa  | 0   | 0   | 9.333333  | 0        | 6        | 0        |
| Putative ribosomal protein S6 (Nph2/rs6-like protein)        | E9AQE3_LEIMU | 14 kDa  | 0   | 0   | 0         | 0        | 9.333333 | 4.807402 |
| Uncharacterized protein                                      | E9AE74_LEIMA | 32 kDa  | 0   | 0   | 10        | 1.154701 | 11.33333 | 1.763834 |
| Putative ER--golgi transport protein p24                     | Q4Q574_LEIMA | 18 kDa  | 0   | 0   | 10        | 1.154701 | 8.666667 | 1.763834 |
| AMMECR1 domain-containing protein                            | Q4Q5M0_LEIMA | 21 kDa  | 5.5 | 2.5 | 8.666667  | 2.905933 | 6.666667 | 1.333333 |
| 60S ribosomal export protein NMD3                            | A4I226_LEIIN | 64 kDa  | 0   | 0   | 7.333333  | 1.333333 | 8.666667 | 1.763834 |
| Uncharacterized protein                                      | Q4Q0E2_LEIMA | 30 kDa  | 0   | 0   | 8         | 1.154701 | 9.333333 | 0.666667 |
| Eukaryotic translation initiation factor 3 subunit E         | Q4Q833_LEIMA | 46 kDa  | 0   | 0   | 8.666667  | 1.763834 | 9.333333 | 3.527668 |
| Uncharacterized protein                                      | Q4QCX2_LEIMA | 141 kDa | 0   | 0   | 8         | 4        | 11.33333 | 0.666667 |
| Putative elongation factor                                   | Q4QA83_LEIMA | 70 kDa  | 7.5 | 4.5 | 6.666667  | 3.333333 | 8        | 2        |
| Protein tyrosine phosphatase-like protein                    | A4HWS2_LEIIN | 19 kDa  | 3   | 1   | 9.333333  | 4.371626 | 8        | 4        |
| Putative 60S ribosomal protein L23a                          | E9AKT6_LEIMU | 16 kDa  | 0   | 0   | 0         | 0        | 12       | 2        |
| Uncharacterized protein                                      | Q4Q214_LEIMA | 21 kDa  | 0   | 0   | 8         | 1.154701 | 0        | 0        |
| Uncharacterized protein                                      | A4I3N4_LEIIN | 27 kDa  | 0   | 0   | 12.66667  | 3.711843 | 7.333333 | 2.403701 |
| Uncharacterized protein                                      | Q4QIW4_LEIMA | 30 kDa  | 0   | 0   | 5.333333  | 2.905933 | 6.666667 | 3.527668 |
| Putative amino acid transporter aATP11                       | Q4Q6M9_LEIMA | 52 kDa  | 0   | 0   | 7.333333  | 3.711843 | 0        | 0        |
| Uncharacterized protein                                      | Q4QC22_LEIMA | 40 kDa  | 0   | 0   | 10.66667  | 2.905933 | 9.333333 | 0.666667 |

|                                                                                                                                    |              |         |     |     |          |          |          |          |
|------------------------------------------------------------------------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative ribonucleoside-diphosphate reductase small chain                                                                          | E9ADI5_LEIMA | 44 kDa  | 0   | 0   | 6.666667 | 0.666667 | 12.66667 | 3.711843 |
| SAM domain-containing protein                                                                                                      | E9AEC5_LEIMA | 132 kDa | 7.5 | 5.5 | 6        | 3.05505  | 7.333333 | 3.711843 |
| Uncharacterized protein                                                                                                            | Q4Q3Q5_LEIMA | 17 kDa  | 0   | 0   | 6.666667 | 3.333333 | 7.333333 | 0        |
| Uncharacterized protein                                                                                                            | E9ACP0_LEIMA | 71 kDa  | 0   | 0   | 7.333333 | 4.055175 | 8.666667 | 0.666667 |
| Putative dynein heavy chain                                                                                                        | Q4Q8K9_LEIMA | 478 kDa | 0   | 0   | 10       | 0        | 7.333333 | 4.055175 |
| Putative U-box domain protein                                                                                                      | E9ADT0_LEIMA | 67 kDa  | 7.5 | 2.5 | 7.333333 | 1.763834 | 6        | 0        |
| Putative proteasome regulatory non-ATP-ase subunit                                                                                 | A4I8A3_LEIIN | 46 kDa  | 0   | 0   | 10       | 5.033223 | 8.666667 | 0.666667 |
| RRM domain-containing protein                                                                                                      | E9ACJ7_LEIMA | 91 kDa  | 15  | 10  | 0        | 0        | 0        | 0        |
| Putative cAMP-specific phosphodiesterase                                                                                           | Q95Z92_LEIMA | 79 kDa  | 0   | 0   | 10       | 1.154701 | 9.333333 | 1.763834 |
| Putative cytochrome b5-like protein                                                                                                | Q4QHM6_LEIMA | 13 kDa  | 0   | 0   | 9.333333 | 0.666667 | 10       | 1.154701 |
| Uncharacterized protein                                                                                                            | Q4Q369_LEIMA | 46 kDa  | 0   | 0   | 10       | 0        | 8        | 1.154701 |
| Cytochrome c oxidase assembly factor-like protein                                                                                  | Q4QCW2_LEIMA | 26 kDa  | 0   | 0   | 9.333333 | 1.763834 | 9.333333 | 2.905933 |
| Protein disulfide isomerase                                                                                                        | Q4QIX1_LEIMA | 15 kDa  | 0   | 0   | 12       | 0        | 0        | 0        |
| Uncharacterized protein L7845.02                                                                                                   | Q9GRP9_LEIMA | 23 kDa  | 0   | 0   | 14.66667 | 5.925463 | 3.333333 | 1.763834 |
| Uncharacterized protein                                                                                                            | Q4Q190_LEIMA | 132 kDa | 0   | 0   | 8.666667 | 4.666667 | 7.333333 | 0        |
| Putative Serine-threonin protein phosphatase                                                                                       | Q4QBJ8_LEIMA | 40 kDa  | 0   | 0   | 9.333333 | 2.905933 | 9.333333 | 1.333333 |
| Cytosolic leucyl aminopeptidase                                                                                                    | Q7KF27_LEIMA | 60 kDa  | 0   | 0   | 6        | 3.05505  | 13.33333 | 2.905933 |
| Putative NADH-dependent fumarate reductase                                                                                         | E9AEU2_LEIMA | 52 kDa  | 0   | 0   | 10.66667 | 5.456902 | 0        | 0        |
| Putative 40S ribosomal protein S29                                                                                                 | E9AIT2_LEIBR | 7 kDa   | 0   | 0   | 10       | 5.291503 | 0        | 0        |
| Glycerol-3-phosphate dehydrogenase [NAD(+)], glycosomal                                                                            | GPDA_LEIMA   | 39 kDa  | 3.5 | 1.5 | 6        | 0        | 0        | 0        |
| Uncharacterized protein                                                                                                            | E9ADM4_LEIMA | 107 kDa | 0   | 0   | 7.333333 | 1.333333 | 8.666667 | 1.763834 |
| RRM domain-containing protein                                                                                                      | Q4Q2S5_LEIMA | 29 kDa  | 0   | 0   | 4.666667 | 2.403701 | 0        | 0        |
| Putative phosphoribosylpyrophosphate synthetase                                                                                    | Q4Q0M2_LEIMA | 39 kDa  | 6   | 2   | 4        | 0        | 9.333333 | 2.666667 |
| Uncharacterized protein                                                                                                            | Q4QGN3_LEIMA | 55 kDa  | 0   | 0   | 9.333333 | 2.403701 | 9.333333 | 1.333333 |
| Uncharacterized protein                                                                                                            | Q4QJF7_LEIMA | 22 kDa  | 0   | 0   | 5.333333 | 2.666667 | 8        | 4.163332 |
| Proteasome regulatory non-ATP-ase subunit 11, putative (19s proteasome regulatory subunit, metallo-peptidase, clan mp, family m67) | E9B4N4_LEIMU | 35 kDa  | 0   | 0   | 4        | 0        | 8        | 0        |
| 3-keto-dihydrosphingosine reductase                                                                                                | E9AEL0_LEIMA | 36 kDa  | 0   | 0   | 12       | 6        | 4.666667 | 0        |
| Uncharacterized protein                                                                                                            | Q4QIR5_LEIMA | 27 kDa  | 0   | 0   | 10.66667 | 2.905933 | 8        | 4.163332 |
| Uncharacterized protein                                                                                                            | Q4Q3B4_LEIMA | 20 kDa  | 0   | 0   | 8        | 4        | 8.666667 | 1.763834 |
| Nucleobase transporter                                                                                                             | Q4QH25_LEIMA | 60 kDa  | 0   | 0   | 2.666667 | 1.333333 | 7.333333 | 4.055175 |
| Phosphatase-like protein                                                                                                           | Q4QCN8_LEIMA | 24 kDa  | 0   | 0   | 8.666667 | 2.905933 | 8        | 2        |
| RRM domain-containing protein                                                                                                      | E9AEE5_LEIMA | 24 kDa  | 8   | 2   | 7.333333 | 0.666667 | 6        | 0        |
| Putative ribonuclease II-like protein                                                                                              | Q4Q8M6_LEIMA | 105 kDa | 0   | 0   | 8        | 0        | 8        | 1.154701 |
| SUI1 domain-containing protein                                                                                                     | Q4QBR8_LEIMA | 74 kDa  | 0   | 0   | 6.666667 | 0        | 10       | 1.154701 |
| Protein phosphatase 2C-like protein                                                                                                | Q4Q225_LEIMA | 33 kDa  | 0   | 0   | 9.333333 | 2.403701 | 8.666667 | 2.905933 |
| Adenine aminohydrolase                                                                                                             | E9AF40_LEIMA | 41 kDa  | 10  | 1   | 4.666667 | 2.403701 | 6        | 0        |

|                                                              |               |         |      |     |          |          |          |          |
|--------------------------------------------------------------|---------------|---------|------|-----|----------|----------|----------|----------|
| Thioredoxin-like protein                                     | E9AEU8_LEIMA  | 25 kDa  | 0    | 0   | 10       | 3.05505  | 0        | 0        |
| Uncharacterized protein                                      | Q4QC32_LEIMA  | 52 kDa  | 13.5 | 5.5 | 4.666667 | 2.403701 | 0        | 0        |
| Eukaryotic translation initiation factor 6                   | A4HNU9_LEIBR  | 27 kDa  | 0    | 0   | 0        | 0        | 9.333333 | 1.763834 |
| Symplekin_C domain-containing protein                        | Q4Q626_LEIMA  | 170 kDa | 6    | 4   | 8.666667 | 1.763834 | 6        | 3.05505  |
| ATP binding protein-like protein                             | Q9U1D8_LEIMA  | 22 kDa  | 0    | 0   | 7.333333 | 0.666667 | 9.333333 | 2.666667 |
| Serine hydroxymethyltransferase                              | Q4QFK2_LEIMA  | 51 kDa  | 0    | 0   | 10       | 0        | 4.666667 | 2.403701 |
| Uncharacterized protein                                      | Q4QD70_LEIMA  | 26 kDa  | 0    | 0   | 8        | 4.163332 | 8.666667 | 1.763834 |
| Infective insect stage-specific protein                      | Q25307_LEIMA  | 12 kDa  | 13   | 1   | 5.333333 | 0        | 4        | 2        |
| Kinesin-like protein                                         | Q4QDJ5_LEIMA  | 93 kDa  | 15   | 6   | 6        | 3.05505  | 0        | 0        |
| Uncharacterized protein                                      | Q4QAH4_LEIMA  | 73 kDa  | 5    | 2   | 4        | 0        | 8.666667 | 1.763834 |
| Putative nucleolar protein                                   | Q4Q6P5_LEIMA  | 70 kDa  | 0    | 0   | 6.666667 | 1.333333 | 5.333333 | 2.905933 |
| Uncharacterized protein                                      | Q4Q3U3_LEIMA  | 56 kDa  | 0    | 0   | 7.333333 | 0.666667 | 10       | 2        |
| Uncharacterized protein                                      | Q4Q2Z9_LEIMA  | 68 kDa  | 0    | 0   | 8.666667 | 4.371626 | 6.666667 | 3.333333 |
| Putative small GTP-binding protein Rab18                     | Q4Q404_LEIMA  | 24 kDa  | 0    | 0   | 0        | 0        | 7.333333 | 3.711843 |
| Uncharacterized protein                                      | Q4Q8H2_LEIMA  | 14 kDa  | 0    | 0   | 9.333333 | 0.666667 | 8.666667 | 1.763834 |
| Uncharacterized protein                                      | Q4Q8E4_LEIMA  | 50 kDa  | 0    | 0   | 8        | 0        | 10       | 1.154701 |
| Methionine aminopeptidase 2                                  | Q4QCC5_LEIMA  | 51 kDa  | 0    | 0   | 6        | 0        | 8        | 0        |
| Adenylosuccinate lyase                                       | Q9U0T7_LEIMA  | 54 kDa  | 0    | 0   | 0        | 0        | 4        | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QCL0_LEIMA  | 47 kDa  | 0    | 0   | 8        | 1.154701 | 8.666667 | 0.666667 |
| Uncharacterized protein                                      | E9AE30_LEIMA  | 39 kDa  | 0    | 0   | 6        | 3.05505  | 4.666667 | 2.403701 |
| Uncharacterized protein                                      | Q4Q7B7_LEIMA  | 40 kDa  | 8    | 5   | 4.666667 | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4QBBQ3_LEIMA | 80 kDa  | 0    | 0   | 6        | 1.154701 | 7.333333 | 2.403701 |
| Zinc transporter-like protein                                | Q4Q622_LEIMA  | 49 kDa  | 0    | 0   | 10       | 1.154701 | 0        | 0        |
| p21                                                          | Q9U8C2_LEIMA  | 21 kDa  | 3    | 0   | 0        | 0        | 8.666667 | 1.333333 |
| Serine/threonine-protein phosphatase                         | A4I400_LEIIN  | 35 kDa  | 0    | 0   | 9.333333 | 0.666667 | 6        | 3.05505  |
| Uncharacterized protein                                      | Q4QI25_LEIMA  | 66 kDa  | 0    | 0   | 8.666667 | 1.763834 | 6.666667 | 0        |
| Putative mitogen-activated protein kinase                    | Q4QHJ8_LEIMA  | 46 kDa  | 5.5  | 0.5 | 6        | 0        | 5.333333 | 2.905933 |
| Putative lysophospholipase                                   | Q4QAE7_LEIMA  | 30 kDa  | 4    | 0   | 7.333333 | 4.055175 | 0        | 0        |
| D-isomer specific 2-hydroxyacid dehydrogenase-like protein   | Q4Q361_LEIMA  | 37 kDa  | 0    | 0   | 7.333333 | 2.403701 | 6.666667 | 3.527668 |
| Uncharacterized protein                                      | Q4QDB4_LEIMA  | 85 kDa  | 9    | 4   | 6        | 3.05505  | 4        | 0        |
| Putative RNA helicase                                        | Q4QGA4_LEIMA  | 225 kDa | 0    | 0   | 5.333333 | 0        | 6.666667 | 0.666667 |
| Uncharacterized protein                                      | Q4Q708_LEIMA  | 12 kDa  | 0    | 0   | 9.333333 | 1.333333 | 0        | 0        |
| Putative cytochrome c oxidase copper chaperone               | E9ACL9_LEIMA  | 9 kDa   | 0    | 0   | 8        | 4.163332 | 0        | 0        |
| Excreted/secreted protein 66                                 | Q1X7K0_LEIMA  | 52 kDa  | 0    | 0   | 5.333333 | 2.666667 | 9.333333 | 2.905933 |
| 60S ribosomal protein L30                                    | E9AEK1_LEIMA  | 11 kDa  | 0    | 0   | 0        | 0        | 12       | 3.464102 |
| Uncharacterized protein                                      | Q4Q3U0_LEIMA  | 43 kDa  | 0    | 0   | 8        | 1.154701 | 5.333333 | 2.905933 |
| Putative eukaryotic translation initiation factor            | Q4Q813_LEIMA  | 38 kDa  | 6    | 2   | 7.333333 | 1.763834 | 3.333333 | 1.763834 |
| Uncharacterized protein                                      | Q4Q6C2_LEIMA  | 138 kDa | 0    | 0   | 6.666667 | 3.527668 | 5.333333 | 2.905933 |

|                                                               |              |         |     |     |          |          |          |          |
|---------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Uncharacterized protein                                       | Q4Q9B0_LEIMA | 100 kDa | 9.5 | 5.5 | 0        | 0        | 4.666667 | 2.403701 |
| Uncharacterized protein                                       | Q4Q2T9_LEIMA | 313 kDa | 0   | 0   | 5.333333 | 2.905933 | 9.333333 | 4.666667 |
| Uncharacterized protein                                       | E9AD47_LEIMA | 49 kDa  | 0   | 0   | 6.666667 | 0.666667 | 8.666667 | 0        |
| Protein kinase domain-containing protein                      | Q4Q9T5_LEIMA | 89 kDa  | 0   | 0   | 7.333333 | 4.055175 | 0        | 0        |
| Putative kinesin                                              | E9AS20_LEIMU | 126 kDa | 0   | 0   | 0        | 0        | 9.333333 | 0.666667 |
| Putative cytochrome b5                                        | E9ANK3_LEIMU | 16 kDa  | 0   | 0   | 8.666667 | 0.666667 | 6        | 3.05505  |
| Uncharacterized protein                                       | Q4Q2Q3_LEIMA | 49 kDa  | 0   | 0   | 7.333333 | 4.055175 | 8        | 1.154701 |
| Uncharacterized protein                                       | Q4QEH6_LEIMA | 36 kDa  | 0   | 0   | 8.666667 | 0.666667 | 0        | 0        |
| Uncharacterized protein                                       | Q4Q9C6_LEIMA | 26 kDa  | 0   | 0   | 8.666667 | 0        | 0        | 0        |
| Putative 40S ribosomal protein S27-1                          | Q4Q140_LEIMA | 10 kDa  | 0   | 0   | 6.666667 | 3.527668 | 9.333333 | 0        |
| Uncharacterized protein                                       | E9ACI5_LEIMA | 33 kDa  | 0   | 0   | 8        | 2        | 0        | 0        |
| PARP-type domain-containing protein                           | Q4Q1U1_LEIMA | 31 kDa  | 0   | 0   | 8        | 2        | 6        | 3.05505  |
| Putative intraflagellar transport protein component           | Q4QDI9_LEIMA | 73 kDa  | 11  | 7   | 0        | 0        | 3.333333 | 1.763834 |
| Uncharacterized protein                                       | Q4QIA5_LEIMA | 31 kDa  | 5.5 | 3.5 | 7.333333 | 3.711843 | 0        | 0        |
| Uncharacterized protein                                       | Q4Q2R5_LEIMA | 67 kDa  | 6.5 | 2.5 | 5.333333 | 2.905933 | 4        | 2        |
| Uncharacterized protein                                       | E9ADF0_LEIMA | 38 kDa  | 0   | 0   | 6        | 0        | 6.666667 | 3.527668 |
| Uncharacterized protein                                       | Q4QIY5_LEIMA | 49 kDa  | 0   | 0   | 6        | 0        | 8        | 1.154701 |
| Protein kinase domain-containing protein                      | Q4QIV8_LEIMA | 51 kDa  | 0   | 0   | 7.333333 | 3.711843 | 4.666667 | 2.403701 |
| Sphingolipid 4-desaturase                                     | A4I2A2_LEIIN | 42 kDa  | 0   | 0   | 6.666667 | 1.333333 | 7.333333 | 3.711843 |
| Peptidase C51 domain-containing protein                       | Q4Q9R0_LEIMA | 35 kDa  | 0   | 0   | 9.333333 | 1.333333 | 0        | 0        |
| Putative ubiquitin carrier protein 4                          | Q4Q5L3_LEIMA | 26 kDa  | 4   | 0   | 5.333333 | 2.905933 | 0        | 0        |
| Uncharacterized protein                                       | E9AF57_LEIMA | 99 kDa  | 0   | 0   | 6.666667 | 0.666667 | 8.666667 | 2.905933 |
| I/6 autoantigen-like protein                                  | Q4QBK1_LEIMA | 23 kDa  | 0   | 0   | 8.666667 | 4.371626 | 6        | 0        |
| Uncharacterized protein                                       | Q4QBS4_LEIMA | 50 kDa  | 0   | 0   | 6        | 3.05505  | 0        | 0        |
| Uncharacterized protein                                       | Q4Q902_LEIMA | 134 kDa | 0   | 0   | 0        | 0        | 8.666667 | 0.666667 |
| Putative 1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase | Q4Q281_LEIMA | 33 kDa  | 3   | 0   | 6.666667 | 1.333333 | 6.666667 | 1.333333 |
| Tubulin-folding cofactor B                                    | Q4QDZ6_LEIMA | 26 kDa  | 0   | 0   | 7.333333 | 0.666667 | 6        | 1.154701 |
| Ubiquitin-like protein                                        | Q4Q2Y8_LEIMA | 9 kDa   | 5   | 1   | 6.666667 | 1.763834 | 0        | 0        |
| Tcp10_C domain-containing protein                             | Q4QFZ5_LEIMA | 83 kDa  | 0   | 0   | 5.333333 | 2.905933 | 7.333333 | 4.055175 |
| Putative polyprenyl synthase                                  | Q4QDJ9_LEIMA | 83 kDa  | 2.5 | 0.5 | 9.333333 | 0        | 4        | 0        |
| RING-type E3 ubiquitin transferase                            | Q4QDS7_LEIMA | 39 kDa  | 0   | 0   | 6        | 0        | 0        | 0        |
| Putative importin beta-1 subunit                              | Q4Q3F9_LEIMA | 96 kDa  | 0   | 0   | 0        | 0        | 7.333333 | 0        |
| Proteasome subunit beta                                       | Q4Q246_LEIMA | 23 kDa  | 0   | 0   | 6.666667 | 3.333333 | 0        | 0        |
| C2 domain-containing protein                                  | Q4Q2U1_LEIMA | 100 kDa | 0   | 0   | 6.666667 | 3.333333 | 6.666667 | 3.527668 |
| Beta-ketoacyl-[acyl-carrier-protein] synthase I               | Q4Q3R4_LEIMA | 47 kDa  | 5   | 1   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                       | Q4Q6G3_LEIMA | 121 kDa | 6   | 2   | 4.666667 | 2.403701 | 4.666667 | 2.403701 |
| Putative ATP-binding cassette protein subfamily G, member 6   | Q4Q1D0_LEIMA | 73 kDa  | 0   | 0   | 0        | 0        | 8.666667 | 1.333333 |
| Putative R-SNARE protein                                      | Q4QC67_LEIMA | 28 kDa  | 0   | 0   | 6        | 3.05505  | 5.333333 | 2.666667 |
| Elongation factor Ts, mitochondrial                           | EFTS_LEIMA   | 30 kDa  | 0   | 0   | 0        | 0        | 8.666667 | 4.666667 |

|                                                              |              |         |     |     |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative proteasome regulatory non-ATP-ase subunit 3         | A4I372_LEIIN | 38 kDa  | 0   | 0   | 8        | 2        | 4.666667 | 0        |
| Uncharacterized protein                                      | Q4QDU8_LEIMA | 140 kDa | 0   | 0   | 0        | 0        | 5.333333 | 0        |
| Uncharacterized protein                                      | Q4QEC4_LEIMA | 50 kDa  | 0   | 0   | 7.333333 | 3.333333 | 8        | 2        |
| Uncharacterized protein                                      | E9AEJ1_LEIMA | 14 kDa  | 0   | 0   | 6.666667 | 3.333333 | 0        | 0        |
| Uncharacterized protein                                      | E9AFK1_LEIMA | 71 kDa  | 0   | 0   | 7.333333 | 0        | 6.666667 | 0.666667 |
| Uncharacterized protein                                      | Q4QHX2_LEIMA | 87 kDa  | 0   | 0   | 5.333333 | 2.666667 | 0        | 0        |
| Elongation of fatty acids protein                            | Q4QFS0_LEIMA | 32 kDa  | 0   | 0   | 0        | 0        | 10       | 2        |
| 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase         | MTND_LEIMA   | 39 kDa  | 6.5 | 1.5 | 5.333333 | 2.905933 | 0        | 0        |
| Uncharacterized protein                                      | Q4Q599_LEIMA | 17 kDa  | 0   | 0   | 8        | 0        | 6.666667 | 3.333333 |
| Small nuclear ribonucleoprotein component-like protein       | Q4Q555_LEIMA | 110 kDa | 0   | 0   | 4        | 0        | 5.333333 | 2.666667 |
| Uncharacterized protein                                      | Q4Q9Y7_LEIMA | 80 kDa  | 10  | 6   | 0        | 0        | 2.666667 | 1.333333 |
| Putative rrp44p homologue                                    | Q4Q8N7_LEIMA | 104 kDa | 0   | 0   | 4        | 0        | 3.333333 | 1.763834 |
| Dihydroorotate dehydrogenase (fumarate)                      | Q4QEW7_LEIMA | 35 kDa  | 0   | 0   | 3.333333 | 1.763834 | 7.333333 | 3.711843 |
| Uncharacterized protein                                      | E9ALE8_LEIMU | 65 kDa  | 0   | 0   | 6.666667 | 0.666667 | 0        | 0        |
| Putative kinesin                                             | Q4Q7F9_LEIMA | 133 kDa | 0   | 0   | 6.666667 | 0.666667 | 4.666667 | 2.403701 |
| Uncharacterized protein                                      | Q4Q3I8_LEIMA | 57 kDa  | 8   | 4   | 0        | 0        | 5.333333 | 2.666667 |
| Amino acid permease                                          | Q4Q682_LEIMA | 64 kDa  | 0   | 0   | 4        | 2        | 0        | 0        |
| Uncharacterized protein                                      | Q4Q2Y7_LEIMA | 18 kDa  | 0   | 0   | 6.666667 | 0        | 8        | 1.154701 |
| Uncharacterized protein                                      | Q4Q8T2_LEIMA | 88 kDa  | 0   | 0   | 5.333333 | 0        | 5.333333 | 2.666667 |
| Uncharacterized protein                                      | Q4QBJ6_LEIMA | 52 kDa  | 0   | 0   | 6.666667 | 1.763834 | 0        | 0        |
| Uncharacterized protein                                      | Q4QCD8_LEIMA | 78 kDa  | 0   | 0   | 7.333333 | 0        | 2.666667 | 1.333333 |
| Tryptophanyl-tRNA synthetase                                 | E9ADK8_LEIMA | 55 kDa  | 0   | 0   | 5.333333 | 2.905933 | 0        | 0        |
| Putative ribosome biogenesis regulatory protein (RRS1)       | Q4Q7P4_LEIMA | 28 kDa  | 0   | 0   | 4.666667 | 2.403701 | 5.333333 | 2.666667 |
| Acetoin dehydrogenase e3 component-like protein              | Q4Q5Z6_LEIMA | 61 kDa  | 0   | 0   | 6        | 0        | 0        | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4QBM2_LEIMA | 47 kDa  | 0   | 0   | 0        | 0        | 6        | 3.05505  |
| Uncharacterized protein                                      | Q4QEE6_LEIMA | 60 kDa  | 0   | 0   | 7.333333 | 2.403701 | 6        | 3.05505  |
| Putative dihydroorotase                                      | Q4QEW1_LEIMA | 44 kDa  | 0   | 0   | 8        | 1.154701 | 5.333333 | 2.666667 |
| ER membrane protein complex subunit 3                        | Q4Q242_LEIMA | 30 kDa  | 0   | 0   | 5.333333 | 2.905933 | 8        | 2        |
| ATP pyrophosphate-lyase                                      | Q4Q1A1_LEIMA | 150 kDa | 0   | 0   | 5.333333 | 2.905933 | 4        | 0        |
| Uncharacterized protein                                      | Q4QFC7_LEIMA | 36 kDa  | 0   | 0   | 5.333333 | 2.666667 | 8        | 0        |
| Uncharacterized protein                                      | Q4Q5A4_LEIMA | 54 kDa  | 0   | 0   | 5.333333 | 2.905933 | 6.666667 | 3.527668 |
| Uncharacterized protein                                      | E9B3A4_LEIMU | 24 kDa  | 0   | 0   | 3.333333 | 1.763834 | 6.666667 | 3.333333 |
| Uncharacterized protein                                      | E9ACY4_LEIMA | 34 kDa  | 0   | 0   | 4        | 0        | 6        | 3.05505  |
| Putative endoplasmic reticulum oxidoreductin                 | E9AQW6_LEIMU | 48 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Putative ATP-dependent zinc metalloproteinase                | Q4QDY0_LEIMA | 65 kDa  | 0   | 0   | 6.666667 | 0        | 0        | 0        |
| STI1 domain-containing protein                               | E9ADN3_LEIMA | 37 kDa  | 0   | 0   | 4        | 0        | 5.333333 | 2.905933 |
| Uncharacterized protein                                      | Q4QEE7_LEIMA | 381 kDa | 0   | 0   | 0        | 0        | 8        | 4.163332 |
| LsmAD domain-containing protein                              | Q4QHA3_LEIMA | 56 kDa  | 15  | 6   | 0        | 0        | 0        | 0        |

|                                                              |              |         |      |     |          |          |          |          |
|--------------------------------------------------------------|--------------|---------|------|-----|----------|----------|----------|----------|
| Major vault protein-like protein                             | Q4QCJ5_LEIMA | 105 kDa | 0    | 0   | 8        | 4        | 5.333333 | 0        |
| Uncharacterized protein                                      | E9AGL7_LEIIN | 154 kDa | 0    | 0   | 6.666667 | 1.333333 | 0        | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4Q6U8_LEIMA | 67 kDa  | 0    | 0   | 4        | 0        | 2.666667 | 1.333333 |
| Putative intergrin alpha chain protein                       | Q4Q6Q3_LEIMA | 86 kDa  | 0    | 0   | 6        | 3.05505  | 0        | 0        |
| Uncharacterized protein                                      | Q4QA48_LEIMA | 119 kDa | 0    | 0   | 0        | 0        | 8        | 1.154701 |
| Protein phosphatase type 1 regulator-like protein            | Q4QJ81_LEIMA | 45 kDa  | 0    | 0   | 4        | 0        | 8.666667 | 4.666667 |
| Uncharacterized protein                                      | Q4Q867_LEIMA | 81 kDa  | 0    | 0   | 4        | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4QCY2_LEIMA | 45 kDa  | 0    | 0   | 5.333333 | 2.666667 | 5.333333 | 0        |
| Uncharacterized protein                                      | E9ACG2_LEIMA | 35 kDa  | 0    | 0   | 6        | 3.05505  | 5.333333 | 2.905933 |
| Putative ribosomal protein L38                               | Q4Q8X0_LEIMA | 9 kDa   | 0    | 0   | 0        | 0        | 7.333333 | 4.055175 |
| Putative kinetoplast-associated protein p18-2                | Q4Q4P5_LEIMA | 15 kDa  | 9    | 4   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4QDL2_LEIMA | 47 kDa  | 0    | 0   | 0        | 0        | 8.666667 | 1.333333 |
| Uncharacterized protein                                      | Q4Q4M2_LEIMA | 92 kDa  | 0    | 0   | 6.666667 | 1.333333 | 5.333333 | 2.905933 |
| Putative pumilio protein 8                                   | Q4Q9K0_LEIMA | 65 kDa  | 0    | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Uncharacterized protein L8530.05                             | Q9GRN1_LEIMA | 23 kDa  | 0    | 0   | 5.333333 | 2.905933 | 6.666667 | 3.527668 |
| Uncharacterized protein                                      | Q4QIC7_LEIMA | 200 kDa | 11.5 | 9.5 | 0        | 0        | 3.333333 | 1.763834 |
| Ubiquitin carboxyl-terminal hydrolase                        | Q4Q6Q0_LEIMA | 51 kDa  | 6    | 2   | 5.333333 | 0.666667 | 0        | 0        |
| Pteridine reductase 1                                        | PTR1_LEIMA   | 30 kDa  | 4.5  | 1.5 | 4.666667 | 2.403701 | 0        | 0        |
| Putative zinc-finger protein ZPR1                            | Q4QE48_LEIMA | 56 kDa  | 5    | 1   | 0        | 0        | 7.333333 | 1.763834 |
| TPK_B1_binding domain-containing protein                     | Q4QG30_LEIMA | 62 kDa  | 0    | 0   | 4.666667 | 2.403701 | 0        | 0        |
| Uncharacterized protein                                      | Q4QBI6_LEIMA | 76 kDa  | 0    | 0   | 4        | 2        | 5.333333 | 2.666667 |
| Putative ribosomal RNA processing protein                    | E9ACA7_LEIMA | 34 kDa  | 0    | 0   | 3.333333 | 1.763834 | 6        | 1.154701 |
| Uncharacterized protein                                      | A4I0Y7_LEIIN | 47 kDa  | 0    | 0   | 6        | 1.154701 | 6        | 3.05505  |
| CRAL-TRIO domain-containing protein                          | Q4QFC4_LEIMA | 60 kDa  | 0    | 0   | 2.666667 | 1.333333 | 7.333333 | 0        |
| tRNA (adenine(58)-N(1))-methyltransferase                    | Q4Q824_LEIMA | 38 kDa  | 0    | 0   | 3.333333 | 1.763834 | 8.666667 | 2.403701 |
| Uncharacterized protein                                      | Q4QEV0_LEIMA | 60 kDa  | 0    | 0   | 6        | 3.05505  | 6        | 3.05505  |
| Uncharacterized protein                                      | Q4Q5L0_LEIMA | 55 kDa  | 2    | 0   | 5.333333 | 2.666667 | 2.666667 | 1.333333 |
| Elongation factor G, mitochondrial                           | Q4Q219_LEIMA | 84 kDa  | 0    | 0   | 5.333333 | 2.666667 | 3.333333 | 1.763834 |
| Kinesin-like protein                                         | A4HVT9_LEIIN | 81 kDa  | 0    | 0   | 6        | 3.05505  | 4        | 2        |
| Phosphoglycan beta 1,2 arabinosyltransferase,(SCA like)      | Q4Q3F6_LEIMA | 100 kDa | 0    | 0   | 0        | 0        | 5.333333 | 2.666667 |
| Uncharacterized protein                                      | Q4Q835_LEIMA | 90 kDa  | 0    | 0   | 0        | 0        | 6        | 0        |
| Putative ubiquitin carboxy-terminal hydrolase                | Q9U1E5_LEIMA | 148 kDa | 0    | 0   | 5.333333 | 2.666667 | 5.333333 | 1.333333 |
| Uncharacterized protein                                      | Q4QC63_LEIMA | 18 kDa  | 0    | 0   | 6.666667 | 0.666667 | 4        | 2        |
| Uncharacterized protein                                      | Q4Q3D9_LEIMA | 139 kDa | 9.5  | 2.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4Q283_LEIMA | 35 kDa  | 0    | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Uncharacterized protein                                      | Q4Q3H8_LEIMA | 81 kDa  | 0    | 0   | 4.666667 | 0.666667 | 0        | 0        |
| Serine/threonine-protein phosphatase                         | Q4QGM7_LEIMA | 109 kDa | 0    | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Ascorbate peroxidase                                         | Q4Q3K2_LEIMA | 34 kDa  | 0    | 0   | 6        | 0        | 0        | 0        |

|                                                                            |              |         |     |     |          |          |          |          |
|----------------------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Glutamate--cysteine ligase                                                 | Q4QDM2_LEIMA | 78 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Putative nucleoporin                                                       | E9AD15_LEIMA | 159 kDa | 0   | 0   | 4.666667 | 2.403701 | 4.666667 | 0        |
| Uncharacterized protein                                                    | Q4QDQ9_LEIMA | 33 kDa  | 0   | 0   | 0        | 0        | 7.333333 | 0.666667 |
| rRNA biogenesis protein-like protein                                       | Q4QCX6_LEIMA | 81 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 2.666667 |
| Putative RNA binding protein                                               | Q4QDJ1_LEIMA | 35 kDa  | 0   | 0   | 4.666667 | 0        | 0        | 0        |
| Putative autophagocytosis protein                                          | Q4Q4J0_LEIMA | 31 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |
| Cleavage stimulation factor 50 kDa subunit                                 | Q4Q4J2_LEIMA | 56 kDa  | 0   | 0   | 0        | 0        | 7.333333 | 3.711843 |
| Uncharacterized protein                                                    | Q4QHW8_LEIMA | 29 kDa  | 5.5 | 2.5 | 8        | 2.309401 | 0        | 0        |
| Putative RNA-binding protein                                               | A4H432_LEIBR | 35 kDa  | 4   | 0   | 4        | 2        | 0        | 0        |
| Kinesin-like protein                                                       | Q4QAR9_LEIMA | 63 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 2.905933 |
| DNA-directed RNA polymerase subunit beta                                   | Q4QA33_LEIMA | 182 kDa | 0   | 0   | 4.666667 | 0.666667 | 0        | 0        |
| Protein kinase domain-containing protein                                   | Q4QJG3_LEIMA | 77 kDa  | 0   | 0   | 4.666667 | 2.403701 | 6        | 1.154701 |
| DUF1935 domain-containing protein                                          | Q4QCR9_LEIMA | 20 kDa  | 0   | 0   | 0        | 0        | 6.666667 | 2.666667 |
| Putative extracellular receptor                                            | Q4QDF7_LEIMA | 94 kDa  | 0   | 0   | 4.666667 | 0        | 5.333333 | 0.666667 |
| Nuclear pore protein                                                       | Q4Q1H0_LEIMA | 97 kDa  | 0   | 0   | 5.333333 | 2.905933 | 4        | 2        |
| Uncharacterized protein                                                    | E9ADW8_LEIMA | 40 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 0        |
| Uncharacterized protein                                                    | E9AE90_LEIMA | 35 kDa  | 0   | 0   | 4.666667 | 2.403701 | 4.666667 | 2.403701 |
| Uncharacterized protein                                                    | Q4Q3D3_LEIMA | 23 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| ABC-thiol transporter                                                      | Q4QBE9_LEIMA | 174 kDa | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| ADP-ribosylation factor-like protein 1                                     | A4HX72_LEIIN | 21 kDa  | 0   | 0   | 4.666667 | 2.403701 | 4.666667 | 2.403701 |
| Guanine nucleotide-binding protein subunit beta-like protein               | E9AM04_LEIMU | 41 kDa  | 0   | 0   | 4        | 2        | 5.333333 | 2.905933 |
| Uncharacterized protein                                                    | E9AEL6_LEIMA | 57 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein               | Q4Q0Z3_LEIMA | 106 kDa | 0   | 0   | 4        | 2        | 0        | 0        |
| Putative mitochondrial processing peptide beta subunit                     | E9AC56_LEIMA | 55 kDa  | 0   | 0   | 5.333333 | 1.333333 | 0        | 0        |
| Phosphatidylinositol:ceramide inositolphosphotransferase                   | SLS_LEIMA    | 38 kDa  | 0   | 0   | 5.333333 | 1.333333 | 4.666667 | 2.403701 |
| Putative proteasome activator protein pa26                                 | E9AEQ0_LEIMA | 24 kDa  | 0   | 0   | 5.333333 | 2.666667 | 0        | 0        |
| Thiol-dependent reductase 1                                                | Q70GE8_LEIMA | 50 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Putative R-SNARE protein                                                   | Q4Q559_LEIMA | 141 kDa | 0   | 0   | 5.333333 | 2.666667 | 0        | 0        |
| Putative 26S protease regulatory subunit                                   | E9ACJ9_LEIMA | 44 kDa  | 4   | 1   | 0        | 0        | 0        | 0        |
| C-CAP/cofactor C-like domain-containing protein                            | E9ADF3_LEIMA | 61 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Amidohydro_3 domain-containing protein                                     | E9ACN8_LEIMA | 76 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Uncharacterized protein                                                    | Q4Q5T9_LEIMA | 48 kDa  | 3   | 1   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                                    | Q4Q7V4_LEIMA | 87 kDa  | 0   | 0   | 4        | 0        | 3.333333 | 1.763834 |
| Uncharacterized protein                                                    | Q4QBP9_LEIMA | 75 kDa  | 3   | 0   | 0        | 0        | 4.666667 | 0        |
| Uncharacterized protein                                                    | Q4QDV7_LEIMA | 303 kDa | 0   | 0   | 3.333333 | 1.763834 | 7.333333 | 1.333333 |
| Putative X-pro, dipeptidyl-peptidase,serine peptidase, Clan SC, family S15 | Q4Q871_LEIMA | 76 kDa  | 0   | 0   | 4.666667 | 2.403701 | 0        | 0        |
| Uncharacterized protein                                                    | Q4QD92_LEIMA | 38 kDa  | 0   | 0   | 4        | 2        | 4        | 0        |

|                                                              |               |         |     |     |          |          |          |          |
|--------------------------------------------------------------|---------------|---------|-----|-----|----------|----------|----------|----------|
| Uncharacterized protein                                      | Q4Q0Z1_LEIMA  | 123 kDa | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Putative cation transporter                                  | Q4QD72_LEIMA  | 65 kDa  | 0   | 0   | 5.333333 | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4Q5N2_LEIMA  | 40 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 2.905933 |
| Putative ubiquitin-conjugating enzyme e2                     | Q9NED4_LEIMA  | 17 kDa  | 0   | 0   | 7.333333 | 0        | 0        | 0        |
| Nucleolar GTP-binding protein 1                              | Q4Q401_LEIMA  | 74 kDa  | 0   | 0   | 0        | 0        | 7.333333 | 0        |
| Uncharacterized protein                                      | Q4QHF3_LEIMA  | 82 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Serine/threonine-protein phosphatase                         | A4I9P2_LEIIN  | 34 kDa  | 0   | 0   | 4        | 2        | 4.666667 | 2.403701 |
| Protein kinase domain-containing protein                     | Q4QFM8_LEIMA  | 100 kDa | 0   | 0   | 3.333333 | 1.763834 | 6        | 0        |
| HSP70-like protein                                           | E9AC55_LEIMA  | 118 kDa | 0   | 0   | 4        | 2        | 0        | 0        |
| Vacuolar protein sorting-associated protein 4                | A4I4W4_LEIIN  | 50 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |
| Uncharacterized protein                                      | Q9N852_LEIMA  | 54 kDa  | 0   | 0   | 3.333333 | 1.763834 | 4        | 0        |
| Uncharacterized protein                                      | Q4Q9Z4_LEIMA  | 84 kDa  | 5.5 | 2.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4Q4E3_LEIMA  | 34 kDa  | 10  | 6   | 0        | 0        | 0        | 0        |
| 60S acidic ribosomal protein P2                              | A4H884_LEIBR  | 11 kDa  | 4.5 | 1.5 | 0        | 0        | 0        | 0        |
| Fe2OG dioxygenase domain-containing protein                  | Q4Q9Z3_LEIMA  | 48 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| GAF domain-containing protein                                | Q4QAZ2_LEIMA  | 58 kDa  | 6.5 | 3.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4Q875_LEIMA  | 73 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 2.905933 |
| Uncharacterized protein                                      | Q4Q0Y7_LEIMA  | 170 kDa | 0   | 0   | 4        | 0        | 0        | 0        |
| Transportin2-like protein                                    | Q4Q1E8_LEIMA  | 102 kDa | 0   | 0   | 4.666667 | 2.403701 | 0        | 0        |
| MRH domain-containing protein                                | Q4Q994_LEIMA  | 38 kDa  | 0   | 0   | 6.666667 | 3.527668 | 0        | 0        |
| Uncharacterized protein                                      | Q4QGGZ0_LEIMA | 107 kDa | 8.5 | 3.5 | 0        | 0        | 0        | 0        |
| Guanine nucleotide-binding protein subunit beta-like protein | Q4Q7R7_LEIMA  | 52 kDa  | 0   | 0   | 5.333333 | 0.666667 | 0        | 0        |
| Elongator complex protein 3                                  | Q4QB17_LEIMA  | 79 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Putative methyltransferase                                   | Q4QDT1_LEIMA  | 93 kDa  | 0   | 0   | 2.666667 | 1.333333 | 4.666667 | 0        |
| Dynein light chain                                           | A4HK04_LEIBR  | 10 kDa  | 0   | 0   | 7.333333 | 1.763834 | 0        | 0        |
| Putative p-nitrophenylphosphatase                            | Q4Q627_LEIMA  | 47 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Uncharacterized protein                                      | Q4QBV7_LEIMA  | 138 kDa | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Uncharacterized protein                                      | E9AX98_LEIMU  | 79 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Uncharacterized protein                                      | A4HTX5_LEIIN  | 93 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 0.666667 |
| Calponin-homology (CH) domain-containing protein             | Q4Q0S0_LEIMA  | 78 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Putative U5 snRNP-specific 40 kDa protein                    | Q4Q7W6_LEIMA  | 48 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| NAD-dependent protein deacetylase SIR2rp1                    | SIR2_LEIMA    | 41 kDa  | 0   | 0   | 0        | 0        | 6        | 2        |
| U5 snRNA-associated splicing factor                          | E9AFM0_LEIMA  | 277 kDa | 0   | 0   | 0        | 0        | 4        | 2        |
| Mitochondrial guide RNA binding complex subunit 1            | Q4QBT2_LEIMA  | 55 kDa  | 8   | 5   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                      | Q4Q172_LEIMA  | 23 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Glyceraldehyde-3-phosphate dehydrogenase-like protein        | E9AFV0_LEIMA  | 36 kDa  | 0   | 0   | 4.666667 | 0        | 4        | 2        |
| Alanine--glyoxylate transaminase                             | E9ACF2_LEIMA  | 48 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Putative quinone oxidoreductase                              | E9ACK2_LEIMA  | 34 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 0        |

|                                                             |              |         |     |     |          |          |          |          |
|-------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| FAA_hydrolase domain-containing protein                     | E9AF68_LEIMA | 30 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Uncharacterized protein                                     | E9AF03_LEIMA | 32 kDa  | 5   | 2   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4Q6A0_LEIMA | 19 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |
| Putative nuclear pore complex protein (NUP155)              | Q4Q065_LEIMA | 144 kDa | 0   | 0   | 3.333333 | 1.763834 | 4        | 2        |
| Uncharacterized protein                                     | A4I6U2_LEIIN | 54 kDa  | 0   | 0   | 4        | 0        | 4.666667 | 2.403701 |
| Putative co-chaperone, GrpE                                 | Q4Q7N3_LEIMA | 28 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Putative 60S acidic ribosomal protein P2                    | E9ACI8_LEIMA | 11 kDa  | 6   | 3   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | A4HUJ1_LEIIN | 26 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Signal recognition particle subunit SRP72                   | Q4QDE2_LEIMA | 81 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Putative peroxisomal enoyl-coa hydratase                    | Q4QDY4_LEIMA | 34 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Putative endosomal trafficking protein RME-8                | Q4Q772_LEIMA | 274 kDa | 0   | 0   | 2.666667 | 1.333333 | 2.666667 | 1.333333 |
| 2Fe-2S ferredoxin-type domain-containing protein            | Q4Q989_LEIMA | 18 kDa  | 0   | 0   | 4        | 2        | 0        | 0        |
| Protein phosphatase                                         | Q4Q9N3_LEIMA | 41 kDa  | 0   | 0   | 5.333333 | 1.333333 | 0        | 0        |
| Putative beta propeller protein                             | Q4QBH3_LEIMA | 60 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Putative GPI transamidase                                   | Q4Q2S2_LEIMA | 81 kDa  | 0   | 0   | 3.333333 | 1.763834 | 4.666667 | 2.403701 |
| JmjC domain-containing protein                              | Q4Q965_LEIMA | 65 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |
| Putative serine palmitoyltransferase                        | E9AEK9_LEIMA | 59 kDa  | 0   | 0   | 3.333333 | 1.763834 | 4        | 0        |
| Uncharacterized protein                                     | Q4QFA5_LEIMA | 226 kDa | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| GCV_T domain-containing protein                             | Q4QAF7_LEIMA | 41 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Putative ras-like small GTPases                             | E9ADL1_LEIMA | 21 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Cytochrome b5 heme-binding domain-containing protein        | Q4Q2N9_LEIMA | 20 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Putative glycosyl hydrolase                                 | Q4Q0N8_LEIMA | 129 kDa | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Methylthioribose-1-phosphate isomerase                      | MTNA_LEIMA   | 40 kDa  | 4.5 | 0.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4QEB5_LEIMA | 89 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |
| Putative eukaryotic translation initiation factor 4 gamma   | A4HX58_LEIIN | 71 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Putative ATP-binding cassette protein subfamily D, member 1 | E9AD24_LEIMA | 77 kDa  | 0   | 0   | 6        | 3.05505  | 0        | 0        |
| Uncharacterized protein                                     | E9ACP6_LEIMA | 17 kDa  | 4.5 | 1.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4Q5F6_LEIMA | 54 kDa  | 0   | 0   | 5.333333 | 2.666667 | 0        | 0        |
| Putative carboxypeptidase                                   | Q4QFW7_LEIMA | 57 kDa  | 10  | 4   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4QBW5_LEIMA | 61 kDa  | 5   | 1   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4Q0A5_LEIMA | 70 kDa  | 0   | 0   | 0        | 0        | 4        | 0        |
| Uncharacterized protein                                     | E9AFR2_LEIMA | 146 kDa | 0   | 0   | 0        | 0        | 5.333333 | 1.333333 |
| Putative serine peptidase family S51, peptidase E           | Q4QHW4_LEIMA | 36 kDa  | 4.5 | 1.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4Q9V9_LEIMA | 112 kDa | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Uncharacterized protein                                     | Q9XZY9_LEIMA | 50 kDa  | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| HEAT repeat-containing protein 1                            | Q4Q913_LEIMA | 296 kDa | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Uncharacterized protein                                     | Q4Q4Z7_LEIMA | 116 kDa | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |

|                                                             |              |         |     |     |          |          |          |          |
|-------------------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Uncharacterized protein                                     | A4I599_LEIIN | 29 kDa  | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Putative thymine-7-hydroxylase                              | E9ADM7_LEIMA | 36 kDa  | 5   | 1   | 0        | 0        | 0        | 0        |
| Na/H antiporter-like protein                                | Q4QB80_LEIMA | 165 kDa | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Cilia- and flagella-associated protein 300                  | Q4Q6F3_LEIMA | 42 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Uncharacterized protein                                     | Q4Q0F0_LEIMA | 62 kDa  | 5.5 | 2.5 | 0        | 0        | 0        | 0        |
| Putative ATP-binding cassette protein subfamily F, member 3 | Q4Q4I8_LEIMA | 75 kDa  | 3   | 0   | 0        | 0        | 0        | 0        |
| CCR4-NOT transcription complex subunit 11                   | Q4QBH5_LEIMA | 43 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| RNA helicase                                                | Q4QJI9_LEIMA | 73 kDa  | 6.5 | 3.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | E9AE47_LEIMA | 61 kDa  | 0   | 0   | 4        | 2        | 0        | 0        |
| Uncharacterized protein                                     | A4I1L3_LEIIN | 104 kDa | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Putative ras-related protein rab-2a                         | A4I825_LEIIN | 23 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Phosphoribosylpyrophosphate synthetase                      | A4H5F1_LEIBR | 41 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Aspartate carbamoyltransferase                              | Q4QEW6_LEIMA | 35 kDa  | 7   | 3   | 0        | 0        | 0        | 0        |
| Putative 60S ribosomal protein L22                          | A4IDM1_LEIIN | 16 kDa  | 4.5 | 0.5 | 0        | 0        | 0        | 0        |
| IU_nuc_hydro domain-containing protein                      | Q4Q9F7_LEIMA | 38 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Histone H2A                                                 | A4H8X1_LEIBR | 19 kDa  | 0   | 0   | 5.333333 | 2.666667 | 0        | 0        |
| Uncharacterized protein                                     | Q4Q717_LEIMA | 12 kDa  | 4.5 | 1.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | A4HA74_LEIBR | 31 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4QDA9_LEIMA | 131 kDa | 6   | 3   | 0        | 0        | 0        | 0        |
| Pescadillo homolog                                          | PESC_LEIMA   | 77 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Uncharacterized protein                                     | Q4Q164_LEIMA | 41 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Uncharacterized protein                                     | Q4QGH7_LEIMA | 144 kDa | 0   | 0   | 0        | 0        | 4        | 0        |
| Putative GPI-anchor transamidase subunit 8 (GPI8)           | A4HXR1_LEIIN | 40 kDa  | 0   | 0   | 0        | 0        | 4.666667 | 2.403701 |
| Uncharacterized protein                                     | A4HRF5_LEIIN | 24 kDa  | 0   | 0   | 4        | 0        | 0        | 0        |
| Uncharacterized protein                                     | E9ACZ3_LEIMA | 30 kDa  | 0   | 0   | 4        | 2        | 0        | 0        |
| Transmembrane 9 superfamily member                          | Q4Q7S3_LEIMA | 69 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Uncharacterized protein                                     | Q4QJG8_LEIMA | 41 kDa  | 0   | 0   | 4.666667 | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4Q464_LEIMA | 36 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Mitochondrial guide RNA binding complex subunit 2           | B6SBL6_LEIMA | 61 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| RRM domain-containing protein                               | E9AFE4_LEIMA | 74 kDa  | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Putative dihydrolipoamide dehydrogenase                     | E9AE44_LEIMA | 56 kDa  | 5.5 | 0.5 | 0        | 0        | 0        | 0        |
| DUF2779 domain-containing protein                           | Q4QH42_LEIMA | 125 kDa | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Protein kinase domain-containing protein                    | Q4Q3K6_LEIMA | 45 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Uncharacterized protein                                     | E9AEH0_LEIMA | 45 kDa  | 9   | 4   | 0        | 0        | 0        | 0        |
| Uncharacterized protein                                     | Q4Q0G6_LEIMA | 68 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| VHS domain-containing protein                               | Q4Q0P8_LEIMA | 56 kDa  | 6   | 3   | 0        | 0        | 0        | 0        |
| Protein kinase domain-containing protein                    | E9AET0_LEIMA | 54 kDa  | 0   | 0   | 4.666667 | 2.403701 | 0        | 0        |
| Uncharacterized protein                                     | Q4Q8Y8_LEIMA | 38 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |

|                                                     |              |         |     |     |          |          |          |          |
|-----------------------------------------------------|--------------|---------|-----|-----|----------|----------|----------|----------|
| Putative leucine-rich repeat protein                | A4I9M5_LEIIN | 66 kDa  | 0   | 0   | 0        | 0        | 4        | 2        |
| Putative dynein                                     | Q4Q5H6_LEIMA | 68 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Putative cytochrome b-domain protein                | E9AC79_LEIMA | 24 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Uncharacterized protein                             | Q4Q4A1_LEIMA | 13 kDa  | 6.5 | 3.5 | 0        | 0        | 0        | 0        |
| Actin-interacting protein 1                         | Q4QAA4_LEIMA | 59 kDa  | 0   | 0   | 0        | 0        | 5.333333 | 0        |
| Putative peptidase t                                | Q4QEH4_LEIMA | 47 kDa  | 7.5 | 3.5 | 0        | 0        | 0        | 0        |
| Ecotin-like protein 3                               | ECOT3_LEIMA  | 42 kDa  | 2.5 | 0.5 | 0        | 0        | 0        | 0        |
| Putative small GTP-binding protein                  | E9AKA7_LEIMU | 22 kDa  | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Putative PUF1                                       | Q4Q274_LEIMA | 61 kDa  | 0   | 0   | 2.666667 | 1.333333 | 0        | 0        |
| Putative ras-like small GTPases                     | A4HVT5_LEIIN | 41 kDa  | 0   | 0   | 0        | 0        | 3.333333 | 1.763834 |
| Uncharacterized protein                             | Q4QH90_LEIMA | 33 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Ysine decarboxylase-like protein                    | Q4QEB2_LEIMA | 35 kDa  | 4.5 | 2.5 | 0        | 0        | 0        | 0        |
| D-3-phosphoglycerate dehydrogenase-like protein     | E9ACF1_LEIMA | 56 kDa  | 3.5 | 0.5 | 0        | 0        | 0        | 0        |
| Putative ras-like small GTPases                     | Q4Q0H2_LEIMA | 41 kDa  | 0   | 0   | 0        | 0        | 2.666667 | 1.333333 |
| Putative 3-oxoacyl-(Acyl-carrier protein) reductase | Q4QAC7_LEIMA | 27 kDa  | 4.5 | 1.5 | 0        | 0        | 0        | 0        |
| Putative RNA guanylyltransferase                    | A4I7V3_LEIIN | 118 kDa | 3   | 0   | 0        | 0        | 0        | 0        |
| Putative surface protein amastin                    | Q4Q7L7_LEIMA | 21 kDa  | 0   | 0   | 3.333333 | 1.763834 | 0        | 0        |
| Ubiquitin carboxyl-terminal hydrolase               | Q4QA77_LEIMA | 25 kDa  | 6   | 4   | 0        | 0        | 0        | 0        |
| Putative acidocalcisomal exopolyphosphatase         | E9AC20_LEIMA | 43 kDa  | 2.5 | 0.5 | 0        | 0        | 0        | 0        |
| Coatomer subunit epsilon                            | Q4Q5A7_LEIMA | 35 kDa  | 3.5 | 0.5 | 0        | 0        | 0        | 0        |
| Uncharacterized protein                             | Q4Q9M6_LEIMA | 24 kDa  | 4.5 | 0.5 | 0        | 0        | 0        | 0        |
| Cytoplasmic dynein 2 light intermediate chain 1     | Q4Q4U9_LEIMA | 47 kDa  | 4   | 2   | 0        | 0        | 0        | 0        |
| Putative chaperonin HSP60/CNP60                     | Q4Q711_LEIMA | 58 kDa  | 3.5 | 1.5 | 0        | 0        | 0        | 0        |

*Supplemental Table 2. L. major WP protein content comparing WT L. major, Vps36<sup>(-/-)</sup> L. major, and Vps36 L. major parasites. Protein quantity is represented by total spectral count averaged between each replicate with accompanying SEM.*

## Chapter 4. General discussion and conclusion

This thesis represents a body of work that iterates upon previous findings and attempts to apply our larger understanding of EVs in higher eukaryotes to the biology of the ancient yet immensely complex parasite *Leishmania*. It is our hope that this work has deepened our understanding of *Leishmania* and the process of parasitic infection by further exploring the role of EVs in host-parasite interaction. In this section, the results from the above manuscripts will be briefly summarized and expanded upon, giving context to how they contribute to the field of EVs and parasitology as well as addressing the challenges and limitations that were encountered.

### 4.1 EVs isolated from unique *L. mexicana* clones are outwardly indistinguishable

In order to investigate EV heterogeneity, we compared the populations of EVs released by different clones of *L. mexicana*. Clones were generated by plating a heterogeneous (Ht) liquid culture of *L. mexicana* on solid Schneider's drosophila medium (SDM) mixed with agar. After allowing the parasites to grow, tiny colonies form on the surface of the plate which allows for clonal colony selection. 8 clones were picked from the plate and re-cultured in liquid SDM and expanded in order to isolate EVs. These clonal *L. mexicana* cultures still released EVs that were themselves Ht, as is apparent based on their particle size distribution from NTA. With NTA performed by NanoSight, we found that EVs isolated from all *L. mexicana* clones by ultracentrifugation are larger than 100 nm in diameter. Comparatively, other studies typically report EVs starting from around 50 nm in diameter, consistent with the optimal NanoSight range of 50-500 nm (Dragovic et al., 2011; Filipe et al., 2010; Nogueira et al., 2020). Regardless, we found EVs secreted from most *L. mexicana* clones exhibited multiple peak diameters, indicating the presence of multiple sub-populations of EVs (Liangsupree et al., 2021; van de Wakker et al., 2024). Even in NanoSight analyses where only a single peak is present, the wideness of the peak itself indicates there is likely heterogeneity in these preparations as well. Comparing particle size distribution between the EVs isolated from different *L. mexicana* clones, as well as Ht *L. mexicana*, shows that there is no

statistical distinction. Visualizing the clonal EVs by TEM also reveals the inherent heterogeneity of each clonal EV population but does not serve to differentiate the *L. mexicana* clonal EVs. To further characterize and quantify EV subpopulations in each preparation, further differentiation methods can be used like density gradients (Brennan et al., 2020; Willms et al., 2018). Additionally, while TEM did not have the resolution needed to confidently distinguish EV subpopulations, extensive cryo-TEM can be used to clearly differentiate EV subpopulations and quantitatively compare between *L. mexicana* clones.

#### 4.2 *L. mexicana* clonal EVs contain unique protein profiles but are functionally similar

Despite their apparent similarity, proteomic analysis reveals the unique nature of each population of *L. mexicana* clonal EVs. Overall EV protein abundance varies significantly between each clone of *L. mexicana*, even after normalizing EV samples based on measured protein quantity for liquid chromatography with tandem mass spectrometry (LCMSMS). For example, the total protein quantity of CL7 EVs was amongst the lowest, with many proteins being absent compared to EVs isolated from other *L. mexicana* clonal EVs. Of these proteins, 3 were uniquely absent from CL7 EVs, meaning they were present in every other preparation of EVs except CL7. Differentially abundant proteins have often been implicated in observed heterogeneity between *Leishmania* species, with regards to both the parasites themselves and disease outcome (Krobthong et al., 2022; Lima et al., 2020; Lynn et al., 2013). Thus, the varying protein content of the clonal EVs indicates that isolating EVs from different clonal colonies of *L. mexicana* can demonstrate observable heterogeneity between their EV populations. However, upon closer functional analysis of the EV protein profiles through gene ontology, the functionality of EV proteins remained consistent between *L. mexicana* clonal EVs, with no significant variation in functional categorization relating to molecular and biological functions (Mi et al., 2019). Most importantly, we found only minute variations in the quantity of common *Leishmania* virulence factors between *L. mexicana* clonal EVs. With STRING analysis, we also found that all clonal *L. mexicana* EVs carried proteins related to ribonucleotide triphosphate metabolism, GTP-binding, one-carbon metabolism, and stress response (Szkłarczyk et al., 2019). HSP70, HSP83, and GP63 were found amongst these

shared proteins, corroborating previous studies of the *Leishmania* exoproteome/secretome (da Silva Lira Filho et al., 2024; Douanne et al., 2020; Peysselon et al., 2013; Silverman et al., 2008). While *L. mexicana* clonal EV populations are variable, the phenotypic implications of this are unclear.

Further complicating this matter, we found that CL3 EVs exhibited abnormally low protein abundance and total proteins identified, most likely due to low yield during protein precipitation. Additionally, while it is logical that most of the high abundance proteins are shared between all clonal *L. mexicana* EVs, the presence of these high-abundance proteins interferes with the detection of lower-abundance proteins, an issue further exacerbated in EVs due to their small size (Bellei et al., 2011; Echan et al., 2005). Furthermore, the identification and categorization of *L. mexicana* proteins using existing tools like Panther and STRING proved challenging due to inadequate annotation of *Leishmania* proteomes, indicative of a wider issue of integration with regards to *Leishmania* proteomic databases (Sundar & Singh, 2018). Thankfully, this seems to be a problem that is quickly being addressed as the utility of proteomics for the of *Leishmania* is increasingly apparent, especially with regards to *Leishmania* development, differentiation, and drug resistance (Drummelsmith et al., 2003; Krobthong et al., 2022; Lynn et al., 2013; Nugent et al., 2004; Sundar & Singh, 2018; Veras & Bezerra de Menezes, 2016). As a result, there is continuous improvement of the related techniques, databases, and overall integration, even with regards to the *Leishmania* exoproteome (Braga et al., 2014; Douanne et al., 2020; Lima et al., 2017; Pawar et al., 2012; Tusnady et al., 2023).

### 4.3 *L. mexicana* clonal EVs differentially augment footpad infection and disease progression

Based on our early proteomic analysis of the *L. mexicana* clonal EVs, we selected CL1, CL7, and CL8 for further mouse footpad infection studies. When co-inoculating C57BL6 footpads with Ht *L. mexicana* and HT, CL1, CL7, and CL8 EVs, we found that CL7 EVs failed to augment infection compared to other *L. mexicana* EVs (Atayde et al., 2015; Silverman & Reiner, 2011). One potential explanation for this is the absence of two amastin-related proteins from CL7

EVs compared to the other CL and Ht EVs (Jackson, 2010). However, as previously noted, there was no overall variation in protein function between *L. mexicana* clonal EV populations. Furthermore, while CL8 EVs lacked cytochrome C, a *Leishmania* virulence factor, CL8 EVs augmented *L. mexicana* infection as expected, causing similar levels of lesion swelling compared to CL1 and HT EVs (Dey et al., 2010; Gerbaba & Gedamu, 2013; Lambertz et al., 2012). In comparison, the clonal and Ht *L. mexicana* parasites themselves exhibited similar infectivity when infecting C57BL6 footpads without EV co-inoculation, suggesting that CL7 EVs alone exhibit reduced infectivity. Without clear evidence emerging from our EV proteomic analysis, further functional assays will need to be done to identify the cause for reduced infectivity in CL7 EVs. Regarding the proteomic analysis itself, further refinement of the sample purification including the depletion of high-abundance proteins can sufficiently increase detection sensitivity to comprehensively compare low abundance proteins between *L. mexicana* clonal EV populations (Askeland et al., 2020; Reymond et al., 2023).

Although we observed that CL7 EVs exhibited reduced infectious capability, the cause of this is not clear. From our proteomic analysis, we noted that CL7 EVs possessed the lowest amount of GP63. It has been clearly demonstrated that EV-delivered GP63 is not only responsible for the deleterious effects of leishmaniasis but also that reduced EV GP63 content can impair *Leishmania* infection and disease severity (da Silva Lira Filho et al., 2021; Ghosh et al., 2013). However, the actual variation in GP63 between clonal EVs is so small that CL7 EVs cannot be considered to possess significantly less GP63. Furthermore, while CL7 EVs lack amastin-related proteins, these appear as low abundance proteins and thus it cannot be confidently stated that CL7 EVs entirely lack amastin. While not included in the chapter 2 manuscript, we have performed additional immunogenic assays on Ht, CL1, 7, and 8 EVs, specifically murine macrophage stimulation with the clonal EVs. However, the resulting ELISA showed that most cytokines and chemokines were not significantly induced by clonal *L. mexicana* EVs alone. Of the cytokines/chemokines induced by clonal EVs, only 3 showed differential induction by the clonal EVs with CL7 EVs inducing reduced expression compared to the other *L. mexicana* EVs. Altogether, the results of this experiment were

inconclusive, so a different experimental approach should be considered. It has been demonstrated that *Leishmania* EVs can induce cytokine expression by B-cells to a much greater degree than by macrophages, suggesting that using a different cell type may help better differentiate cytokine induction between clonal *L. mexicana* EVs (Barbosa et al., 2018).

#### 4.4 CRISPR/Cas9-mediated disruption of the *Vps36* gene in *L. major* reduces sEV release but does not prevent EV secretion

The *L. major* *Vps36* analogous gene, a component of the ESCRTII complex, was chosen as a target for CRISPR/Cas9-mediated gene disruption due to its importance to eukaryotic EV production (Im & Hurley, 2008; Teo et al., 2004). However, *L. major* parasites with successfully disrupted *Vps36* expression, termed *Vps36*<sup>(-/-)</sup> *L. major*, only secrete EVs at a lower level compared to WT *L. major* parasites, though not significantly so, both during growth in culture as well as after 37°C TS. Even within trypanosomes, *Vps36* disruption has been suggested to completely or near-completely abrogate EV secretion (Eliaz et al., 2017). Despite this, SEM images of the parasite surface did demonstrate a lack of augmented EV release after TS by *Vps36*<sup>(-/-)</sup> *L. major*. Indeed, upon closer analysis, *Vps36*<sup>(-/-)</sup> *L. major* parasites secrete significantly fewer sEVs, EVs of 140nm or smaller in diameter, compared to WT *L. major*. This suggests that *Vps36* disruption has negatively impacted the secretion of exosomes specifically, which are often defined by their smaller size (Raposo & Stoorvogel, 2013; van Niel et al., 2018). This is supported by the fact that exosome production and release is largely ESCRT-dependent, though ESCRT independent pathways exist (Cheng & Hill, 2022; Han et al., 2022). This inhibition of sEV release is also specific to *Vps36* disruption, as *Vps36* *L. major* parasites exhibit similar levels of sEV and EV release compared to WT *L. major*.

The failure to entirely inhibit EV or even sEV release in *L. major* may be due to the method chosen to disrupt *Vps36*, namely the insertion of a puromycin resistance gene into the *Vps36* exon rather than the excision of the entire gene, as this may have been insufficient in completely abrogating *Vps36* function. Additionally, the inherent genomic plasticity of

*Leishmania* parasites has always proven a challenge to traditional genetic manipulation, though CRISPR/Cas9 has been reported to have an improved success rate (Alpizar-Sosa et al., 2022; Duncan et al., 2017; Roberts, 2011). It should be noted that in the study targeting Vps36 in *Trypanosoma brucei*, Vps36 mRNAs were silenced using RNA interference (RNAi) rather than targeting the Vps36 gene on a DNA level (Eliaz et al., 2017). While this is an effective way to eliminate Vps36 expression, *L. major* does not possess an RNAi pathway as it is of the *Leishmania* subgenus as opposed to *Viannia*, making RNAi-mediated gene silencing impossible (Lye et al., 2010). It will be worthwhile to assess the effect of complete Vps36 deletion on EV release, but it should be noted that though *L. major* was chosen in this study, the same level of Vps36 disruption was attempted with *L. mexicana* without success. This may be a result of the higher level of genomic plasticity observed in *L. mexicana* as well as the potential essentiality of Vps36 causing chromosome duplication upon disruption (Ishemgulova et al., 2018; Rogers et al., 2011; Sterkers et al., 2012).

However, it should be noted that the necessity of ESCRTII for EV biogenesis is controversial. In fact, it was first hypothesized that protozoa only possessed ESCRTIII due to comparative genomic analysis performed across eukaryotic lineages (Leung et al., 2008). This and other studies contributed to the prevailing opinion that only ESCRTIII is necessary for EV biogenesis, as eukaryotic organisms could lack upstream elements of MVB biogenesis but never ESCRTIII (Williams & Urbe, 2007). In mammalian cells, siRNA-mediated depletion of ESCRTII component Vps25 failed to prevent EV-mediated HIV1 release, suggesting that HIV1 exploits an ESCRTII-independent pathway for cell egress (Langelier et al., 2006). Additionally, Vps25 depletion failed to completely abrogate the endosomal degradation of EGFR, suggesting continued endosome/MVB function even with ESCRTII depletion, albeit reduced. This is in contrast to the previously mentioned study using siRNA to deplete Vps36 in *T. brucei* (Eliaz et al., 2017). Another similar study demonstrated that the endosomal degradation of EGF and MHC class I molecules is entirely ESCRTII-independent, suggesting that ESCRTII is not required for MVB protein sorting (Bowers et al., 2006). These two studies call into question the necessity of ESCRTII, with ESCRTII depletion not even completely preventing EGFR degradation, which is ESCRTII-dependent. In the absence of ESCRTII, it has

been suggested that ALIX serves as an alternative link between ESCRTI and ESCRTIII due to observed two-hybrid interactions in yeast (Williams & Urbe, 2007).

Additionally, the potential for the existence of additional compensatory EV secretion mechanisms in *Leishmania* should not be discounted, as it has been demonstrated that *Leishmania* secretory pathways can be unconventionally regulated and utilized despite the conserved nature of eukaryotic EV secretion (Ansari et al., 2024; Corrales et al., 2010). If anything, the lack of tetraspanin analogs in *Leishmania* necessitates alternative regulatory mechanisms for EV release and targeting (Huang et al., 2005; Larios et al., 2020; Rana et al., 2012). For example, linoleic acid has been found to induce the release of angiogenic EVs from breast cancer cells, contradicting linoleic-acid-mediated inhibition of the release of *L. donovani* EVs (Garcia-Hernandez et al., 2021; Saini & Rai, 2020). An alternative cargo sorting mechanism may be necessitated by the unique structure of PPGs in *Leishmania*, especially considering that a large proportion of secreted and surface molecules are classified as PPGs, like LPG (Rogers, 2012; Sacks et al., 2000). It should also be considered that other classes of EVs may play more important roles in *Leishmania* infection and survival. In fact, one of the first studies of the *Leishmania* secretome hypothesized that infective *Leishmania* parasites make use of apoptotic bodies to enhance infection (Silverman et al., 2008). Compared to exosomes and MVs, apoptotic bodies garner much less attention due to their limited roll in cell-cell communication, and it is only recently that this perception is changing with regards to the study of other eukaryotes (Battistelli & Falcieri, 2020; Doyle & Wang, 2019).

#### 4.5 Vps36 disruption significantly alters EV protein, but not RNA, content

From our proteomic analysis, it was immediately apparent that Vps36<sup>(-/-)</sup> *L. major* EV protein content greatly differs from WT *L. major* EVs, with hundreds of proteins present only in WT *L. major* EVs. Although Vps36<sup>(-/-)</sup> *L. major* parasites retained the ability to release EVs, their remarkably altered protein profile proved promising. Vps36, as a member of the ESCRTII complex, targets ubiquitin, implying a role in cargo sorting (Babst, Katzmann, Snyder, et al., 2002; Teo et al., 2004). Although the most abundant *L. major* EV proteins were present in

both WT *L. major* and *Vps36*<sup>(-/-)</sup> *L. major* EVs, many of the proteins absent from *Vps36*<sup>(-/-)</sup> *L. major* EVs were sufficiently abundant in WT *L. major* EVs. In fact, the total protein abundance of *Vps36*<sup>(-/-)</sup> *L. major* EVs was reduced compared to WT *L. major* EVs despite normalization of samples based on protein quantity. This drop in abundance potentially results from defective ESCRT-mediated cargo sorting leading to non-protein cargos or many low-abundance proteins being sorted into *Vps36*<sup>(-/-)</sup> *L. major*, cargos which cannot be detected by traditional LCMSMS proteomic analysis (Anand et al., 2019; Lee et al., 2024; Millionni et al., 2011).

Amongst the proteins either absent or significantly reduced (greater than 2-fold reduction) in *Vps36*<sup>(-/-)</sup> *L. major* EVs were several ESCRT-related proteins. In particular, *Vps4* was found to be still present but significantly reduced in *Vps36*<sup>(-/-)</sup> *L. major* EVs. *Vps4* interacts with ESCRTIII in a process downstream of ESCRTII and has been implicated in membrane fission, ultimately remaining within excised EVs (Stuchell-Brereton et al., 2007). This reduction in *Vps4* abundance in *Vps36*<sup>(-/-)</sup> *L. major* EVs further suggests impaired ESCRT-mediated EV release (Han et al., 2022; Nickerson et al., 2010). Despite this significant drop in protein abundance, the overall functional profile of *Vps36*<sup>(-/-)</sup> *L. major* EVs remained similar to WT *L. major* EVs when assessing based on proportion. One standout category was metabolism, with *Vps36*<sup>(-/-)</sup> *L. major* EVs missing a significant number of proteins related to metabolic process, many of these being metabolite interconversion enzymes. Unsurprisingly, membrane traffic proteins were also depleted in *Vps36*<sup>(-/-)</sup> *L. major* EVs. Promisingly, protein content of the *Vps36*<sup>(-/-)</sup> *L. major* whole parasite (WP) remained largely unchanged compared to WT *L. major*, suggesting the effect of *Vps36* disruption is limited to EV cargo sorting.

While EV protein content was significantly altered by *Vps36* disruption, small RNA content was not significantly impacted in either *Vps36*<sup>(-/-)</sup> *L. major* EVs or WP compared to WT *L. major*. This was somewhat surprising as ESCRTII has been reported to be responsible for the trafficking of viral RNA (Ghoujal et al., 2012; Liu et al., 2022). However, it should be noted that the involvement of ESCRTII in viral replication and budding is somewhat controversial as early work indicated ESCRTII is dispensable in this process (Langelier et al., 2006).

Furthermore, these studies assessed cellular viral RNA load and localization but did not assess EV RNA content. Thus, these studies do not definitively implicate ESCRTII in EV RNA cargo sorting, with the overall involvement and regulation of ESCRT in RNA sorting unclear. It has been demonstrated that Vps25 is the specific ESCRTII component that targets RNA, so perhaps *Vps36* disruption failed to cause Vps25 degradation (Emerman & Blower, 2018). Of course, the possibility that EV RNA sorting in *Leishmania* proceeds through a different process due to the unique genomic organization and RNA regulation, or lack thereof in this case, of *Leishmania* (Charest et al., 1996; Clayton & Shapira, 2007; Iantorno et al., 2017; Lahav et al., 2011; Leifso et al., 2007). We did note that although total small RNA content was unchanged, many small RNA sequences were differentially enriched in the EVs from the WP when comparing between *Vps36*<sup>(-/-)</sup> and WT *L. major*. More detailed analysis of this enrichment will be necessary.

#### 4.6 *Vps36* disruption has catastrophic results for *L. major* infectivity *in vitro* and *in vivo*

With an observable phenotypic effect, we had confidence that *Vps36* disruption would impact *L. major* infectivity, and this was clearly demonstrated in our *in vivo* and *in vitro* infection experiments. Strikingly, *Vps36*<sup>(-/-)</sup> *L. major* parasites failed to cause significant footpad swelling or parasite burden in Balb/c mice, a typically susceptible mouse line (Sacks & Noben-Trauth, 2002). This loss of infectivity was further reflected in *in vitro* experiments, with *Vps36*<sup>(-/-)</sup> *L. major* parasites failing to successfully infect mouse bone-marrow-derived macrophages (BMDMs) and getting cleared within 24 hours post-infection. Comparatively, WT *L. major* and *Vps36* *L. major* parasites behaved as expected, causing significant footpad swelling in Balb/c mice and resisting clearance by BMDMs *in vitro*. This also served to prove the loss of infectivity is specifically caused by *Vps36* disruption as *Vps36* addback fully restored WT levels of infection for *L. major*. However, it is not immediately obvious what is causing this reduced virulence based on our proteomic analysis, as there was no significant alteration of WP protein content. Even with *Vps36*<sup>(-/-)</sup> *L. major* EVs, there was no significant reduction in virulence factor abundance. Additionally, WT *L. major* EVs did not enhance

Vps36<sup>(-/ -)</sup> *L. major* infection *in vivo* whereas WT *L. major* infection was augmented as expected. As observed in the case of GP63<sup>low</sup> *L. amazonensis*, WT EVs can rescue parasite infection to WT rates, demonstrating that *Leishmania* EVs are sufficient for the initial immunomodulation of host macrophages to establish successful infection even when the parasite itself has lower virulence factor abundance (da Silva Lira Filho et al., 2021).

While our intraperitoneal cavity infections exactly reflected our *in vivo* footpad infections, closer scrutiny of our BMDM infections gave further clues on the reduced virulence of Vps36<sup>(-/ -)</sup> *L. major*: although Vps36<sup>(-/ -)</sup> *L. major* parasites are almost entirely cleared by 24 hours post-infection, their BMDM infection rate is only slightly lower compared to WT *L. major* and Vps36<sup>(-/ -)</sup> *L. major* at 3 hours post-infection. This shows that during early infection, Vps36<sup>(-/ -)</sup> *L. major* parasites are still able to invade host macrophages and survive initial immune responses but cannot maintain infection. This seems counterintuitive as the popular hypothesized role of *Leishmania* EVs is that they are deliverers of a pre-emptive strike, predominantly impacting early infection (de Menezes et al., 2016; Liu & Uzonna, 2012; Silverman & Reiner, 2011). As such, it stands to reason that if defective sEV release is the cause of reduced infectivity in Vps36<sup>(-/ -)</sup> *L. major* parasites, their infection rate would be drastically lower during the first hours of infection. Furthermore, repeating our *in vivo* experiments but co-inoculating mice footpads with WT *L. major* parasites together with WT *L. major*, Vps36<sup>(-/ -)</sup> *L. major*, or Vps36<sup>(-/ -)</sup> *L. major* EVs showed that EVs from all 3 *L. major* strains augmented infection at similar levels. These results together suggest that Vps36 disruption has caused a fundamental defect in Vps36<sup>(-/ -)</sup> *L. major* parasites outside of its effect on sEV release and protein cargo sorting which is the real culprit for the catastrophic loss of virulence.

#### 4.7 Vps36 disruption has no obvious effect on *L. major* promastigotes but greatly inhibits their differentiation and life cycle

With Vps36<sup>(-/ -)</sup> *L. major* EVs possessing similar infectious capabilities to both WT *L. major* and Vps36<sup>(-/ -)</sup> *L. major*, the observed loss of infectivity caused by Vps36 disruption seems to originate from a cellular source rather than from the EVs themselves. However, Vps36<sup>(-/ -)</sup> *L. major* promastigotes grew at a comparable rate to WT *L. major* and Vps36<sup>(-/ -)</sup> *L. major* in

culture and were morphologically identical, meaning such a defect is not obvious or not affecting *L. major* promastigotes. Based on our EV proteomic analysis, we suspected that the metabolism of Vps36<sup>(-/-)</sup> *L. major* parasites may have been impacted by Vps36 disruption. Although there was no significant alteration of Vps36<sup>(-/-)</sup> *L. major* WP protein content, we did find that several nucleobase/nucleoside transporter proteins were missing. These transporters, especially purine transporters, are essential to parasite survival as *Leishmania* parasites are unable to synthesize purines (Carter et al., 2008; Ortiz et al., 2007). Additionally, a previous study found that mutating Vps4 made *L. major* less able to withstand starvation (Besteiro et al., 2006). Unfortunately, we found that Vps36<sup>(-/-)</sup> *L. major* promastigotes starved at a similar rate compared to WT *L. major* and Vps36 *L. major* in PBS. Furthermore, we found that our WT *L. major* was less resistant to starvation compared to the *L. major* used in the previous study.

Without strong leads, the utility of further experiments with promastigotes seemed limited, so we decided to assess Vps36<sup>(-/-)</sup> *L. major* amastigote EV release and metabolism. However, there are very few reports of successful *in vitro* generation of axenic *L. major* amastigotes, with only one described protocol and study (Clos et al., 2022; Dias-Lopes et al., 2021; Wenzel et al., 2012). Following this protocol, we unexpectedly found that Vps36<sup>(-/-)</sup> *L. major* promastigotes could not survive in amastigote differentiation conditions and failed to differentiate into amastigotes. Furthermore, Vps36 *L. major* expanded at a lower rate compared to WT *L. major* and less axenic amastigotes were collected after 14 days in differentiation conditions. For the first time, Vps36 addback was insufficient in restoring Vps36<sup>(-/-)</sup> *L. major* to WT levels. This result was unexpected despite Vps4 mutation similarly impacting *L. major* due to the fact that Vps36<sup>(-/-)</sup> *L. major* promastigotes possessed no visible defects and expanded in regular culture conditions at the same rate as WT *L. major*, whereas mutant Vps4 *L. major* (E235Q) prematurely exited the logarithmic growth phase, resulting in a lower stationary phase promastigote population and morphologically peculiar promastigotes (Besteiro et al., 2006). Further corroborating these results, we found that Vps36<sup>(-/-)</sup> *L. major* metacyclogenesis was completely impaired within the sandfly midgut, and this time Vps36 addback failed to restore metacyclogenesis at all: no metacyclic

promastigotes were found in the midguts of *Phlebotomus duboscqi* sandflies 21 days post-infection with Vps36<sup>(-/-)</sup> *L. major* and Vps36 *L. major*.

While Besteiro et al. attribute the effects of Vps4 mutation to autophagy dysfunction, our study did not include detailed investigation in this direction. It should be noted that the authors relate *L. major*<sup>Vps4E235Q</sup>'s increased susceptibility to starvation to autophagy dysfunction due. However, Vps36<sup>(-/-)</sup> *L. major* parasites did not exhibit significantly reduced starvation resistance compared to WT *L. major*. Furthermore, whereas Vps4 is a member of the ESCRTIII complex and is therefore present in the late endosome, Vps36 is a member of the ESCRTII complex which transiently binds the endosomal membrane (Babst, Katzmann, Snyder, et al., 2002). Therefore, despite similar phenotypic outcomes, it seems unlikely that Vps36 disruption-mediated attenuation of *L. major* occurs by the same mechanism as Vps4 mutation. Although our initial metabolic experiments failed to demonstrate significant differences between WT *L. major*, Vps36<sup>(-/-)</sup> *L. major*, and Vps36 *L. major* parasites, a metabolic defect still remains the most likely cause of Vps36<sup>(-/-)</sup> *L. major* reduced virulence. *Leishmania* differentiation from promastigote to amastigote is accompanied by important metabolic changes necessary for the parasite to adapt and survive within the macrophage phagolysosome (McConville et al., 2015; Saunders et al., 2014; Saunders et al., 2021). Although we failed to generate axenic Vps36<sup>(-/-)</sup> *L. major* amastigotes, we can still capture the moment promastigotes encounter the stressors that induce amastigote differentiation, the predominant of which being TS (Alcolea et al., 2010). To investigate Vps36<sup>(-/-)</sup> *L. major* metabolism further, metabolites have been isolated and purified from the 3 strains of *L. major* and sent for metabolomic analysis.

## 4.8 Conclusions and future directions

In conclusion, successful *Leishmania* infection and intramacrophage survival are inextricably linked with EV biogenesis and function. Even slight variations in secreted EV populations can result in varied parasite infectivity, as observed with EVs isolated from clonal *L. mexicana* cultures. While our proteomic analysis clearly demonstrates the varying protein cargo between clonal *L. mexicana* EVs, there is no clear link between these observed

variations and the resulting murine footpad infection. To further establish this link, the clonal EVs should be further characterized using additional analytical techniques like lipidomics and RNA sequencing. These two analyses will be especially useful to assess EV population heterogeneity in greater detail, giving a view beyond protein content that may shed more light on the observed differences in parasite infectivity. Furthermore, the clonal *L. mexicana* cultures themselves should be sequenced in full in order to establish the extent of genetic homogeneity achieved through clonal colony picking. This will also contribute a clearer understanding of potential causes for the variations in EV content across the *L. mexicana* clones.

Through CRISPR/Cas9-mediated *Vps36* disruption, we found that *Vps36<sup>(-/-)</sup> L. major* parasites secreted significantly less sEVs with altered protein content. However, total EV and WP small RNA content remained unchanged. *Vps36<sup>(-/-)</sup> L. major* parasites also exhibited severely impaired infectivity both *in vitro* and *in vivo*. However, it seems that this reduction in infectivity is unlinked to impaired sEV release but is rather caused by an impairment of parasite differentiation: *Vps36<sup>(-/-)</sup> L. major* has almost completely abrogated amastigote differentiation *in vitro* and metacyclogenesis *in vivo*. Furthermore, while *Vps36* addback fully restored sEV release and parasite infectivity, it did not restore parasite differentiation. Although our initial metabolic assays were inconclusive, we would like to further perform metabolomics in order to gain a more complete picture of *Vps36<sup>(-/-)</sup> L. major* metabolism compared to WT since metabolic adaptation is a necessary process for intramacrophage survival and our preliminary proteomic analysis shows a reduction in TCA-related proteins in *Vps36<sup>(-/-)</sup> L. major* EVs. It will also be interesting to further analyse the enrichment of small RNAs in *L. major* EVs from the WP in comparison to WT to determine if this is an indicator of *Vps36* involvement in RNA cargo sorting in *Leishmania*. It is imperative to complete our *in vivo* sandfly experiments and perform a sandfly-mediated murine infection to replicate a true *in vivo* infection as closely as possible.

## 4.9 Original contributions to science

First use of clonal colonies to study *Leishmania* EV heterogeneity

First observation of *Leishmania mexicana* clonal EV heterogeneity using proteomic analysis

First demonstration of disparate EV infectious capability due to EV heterogeneity resulting from clonal colonies

First confirmation of impaired sEV release caused by *Vps36* disruption in *Leishmania major*

First observation of altered EV protein content but not RNA content as a result of *Vps36* disruption in *Leishmania major*

First confirmation of impaired parasite infectivity and intramacrophage survival caused by *Vps36* disruption in *Leishmania major*

First observation of impaired amastigote transformation and metacyclogenesis caused by *Vps36* disruption in *Leishmania major*

First confirmation of *Vps36*<sup>(-/-)</sup> *L. major* infection of *Phlebotomus duboscqi* sandflies and resulting abrogation of metacyclogenesis

## Chapter 5. References

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