Characterization of glycosomal RING finger proteins of *Leishmania donovani*

By

Anand Vardhan

Institute of Parasitology, McGill University, Montreal

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Abstract

A really interesting new gene (RING) finger protein complex is part of an integral membrane structure that is suggested to play an essential role in the import of proteins into peroxisomes/glycosomes. This RING finger complex is comprised of three subunits, *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 that have not yet been characterized in *Leishmania donovani*. The aim of this study was to purify and characterize these proteins by incorporating them into liposomes. *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 were cloned into the pET30b(+) and pRP-H-BSD plasmids for their individual expression in *E. coli* C41(DE3) and *Leishmania donovani* expression system. Inclusion bodies containing the PEX proteins were isolated from *E. coli* cells and purified using Ni^{2+}-NTA affinity chromatography. In contrast, to *E. coli*, expression of *Ld*PEX2 and *Ld*PEX12 in *Leishmania donovani* appeared to be a lethal event in these parasites. Purified proteins were incorporated into proteoliposomes composed of a phospholipid mixture that mimics the composition of the glycosomal membrane. These proteoliposomes were generated using detergent dialysis, and isolated in the first fraction of floatation assay. *Ld*PEX10 remained intact within the lipid even after the harsh treatment with urea and alkaline carbonate, suggesting it exists as an integral membrane protein. Our proteoliposome model was successful in incorporating RING finger proteins into lipid bilayers as demonstrated using the sucrose density floatation and alkaline carbonate experiment. This model can be exploited to further characterize other genes in *Leishmania*.

Abstrait

Un nouveau complexe de protéines RING (Really Interesting New Gene) qui fait partie d'une structure membranaire intégrale est suggèré de jouer un rôle essentiel dans l'importation de protéines dans les peroxisomes / glycosomes. Ce complexe RING comprend trois sous-unités protéiques, *Ld*PEX2, *Ld*PEX10 et *Ld*PEX12, qui n'ont pas encore été caractérisées dans *Leishmania donovani*. Le but de cette étude était de purifier et de caractériser ces protéines en les incorporant dans des liposomes. *Ld*PEX2, *Ld*PEX10 et *Ld*PEX12 ont été clonés dans les plasmides pET30b (+) et pRP-H-BSD et les protéines recombinantes ont été exprimées en utilisant *E. coli* C41 (DE3) et le système d'expression *Leishmania donovani*. Les corps d'inclusion contenant les protéines PEX ont été isolés à partir de cellules de *E. coli* et purifiés par chromatographie d'affinité Ni2 + -NTA. Par contre, l'expression de *Ld*PEX2 et *Ld*PEX12 chez *Leishmania donovani* semblait être léthale pour le parasite. Des protéines purifiées ont été incorporées dans des protéoliposomes composés d'un mélange de phospholipides imitant la composition de la membrane glycosomale naturelle. Ces protéoliposomes ont été générés par dialyse au détergent et isolés dans la première fraction du test de flottation. *LdPEX10* est resté intact dans le lipide même après le traitement rigoureux avec de l'urée et du carbonate alcalin, suggérant qu'il existe en tant que protéine membranaire intégrale. Notre modèle de protéoliposomes a réussi à incorporer les protéines RING dans les bicouches lipidiques, comme l'a démontré l'expérience de flottation de la densité de saccharose et de carbonate alcalin. Ce modèle peut être exploité pour mieux caractériser d'autres gènes de *Leishmania.*

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Abbreviations

ATP - Adenosine-5'-TriPhosphate

ADP - Adenosine-5'-TriPhosphate

CL - Cutaneous Leishmaniasis

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeat

ddFKBP- Destabilization Domain of FKBP12 Protein

DNA – Deoxyribonucleic acid

DOPE - 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine

DOPC - 1,2-Dioleoyl-sn-glycero-3-phosphocholine

DOPG - 1,2-Dioleoyl-sn-glycero-3-phosphoglycerol

DAT - Direct Agglutination Test

DCL - Diffuse Cutaneous Leishmaniasis

ER - Endoplasmic Reticulum

FBS – Fetal Bovine Serum

HMG-CoA - Hydroxymethylglutaryl-CoA synthase

His-Tag - Histidine-Tag

HIV – Human Immunodeficiency Virus

HRP – Horse-Radish Peroxidase

IFAT - Indirect Fluorescent Antibody Test

IMD - Intensified Disease Management

IPTG - Isopropyl β-D-1-thiogalactopyranoside

LCL - Localized Cutaneous Leishmaniasis

***Ld*PEX –** *Leishmania donovani* Peroxin

MCL - Mucocutaneous Leishmaniasis

mRNA - Messenger Ribo nucleic Acid

NTD - Neglected Tropical Disease

NAD^+/NADH - Nicotinamide Adenine Dinucleotide

PKDL - Post-kala-azar dermal leishmaniasis

PCR - Polymerase Chain Reaction

PEP - PhosphoEnolPyruvate

PEX - PEroXin protein

PTS - Peroxisomal Targeting Signal

PBS – Phosphate Buffered Saline

PBST - Phosphate Buffered Saline + Triton X-100

PI - Phosphatidyl Inositol

RING - Really Interesting New Gene

RNAi – Ribonucleic Acid Interference

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TCA – Trichloroacetic Acid

TRPs - Tetratricopeptide Repeats

TAP - Tandem Affinity Purification

VL - Visceral Leishmaniasis

WD40 – 40 Amino Acid Tryptophan-Aspartic Domain

WHO - World Health Organization

Claim of originality

1. This is the first study that has reports to purify recombinant proteins *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 from the bacterial expression system.
2. Incorporation of proteins *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 in proteoliposome.
3. Characterization of *Ld*PEX10 to show that it is an integral membrane protein.

Contribution of authors

AV performed all the experiments in this study, including cloning, protein expression, protein purification, protein characterization and wrote the thesis. (Anwer Hasil Kottarampatel) AHK was available for constructive discussion, design of experiments and consultation for this document. (Phil Yates) PY provided pRP plasmid for the study. (Armando Jardim) AJ supervised the work, provided insight for designing the experiment and editing of this document.

General Introduction

Neglected tropical diseases (NTDs) have been classified by the World Health Organization (WHO) as a group of fungal, bacterial, viral and parasitic diseases affecting the poorest individuals across the globe [1]. As the name suggests, these diseases are mostly associated with the tropical and subtropical regions of the world. NTDs have received increased attention in the past few decades. Despite this progress, the poor sectors of society, mostly in developing countries, suffer from insufficient access to donated drugs from various pharmaceutical companies. The current 2020 roadmap target of the WHO to provide treatments, diagnostics and prevention strategies against NTDs suffers from limited funding, as the present budget of $300 million annually is almost half of the actual required annual budget [2]. One class of NTDs, categorized as innovative and intensified disease management (IMD), requires special attention as they lack cost effective control tools while many of the existing tools are also limited. This class include Chagas disease, human African trypanosomiasis, leishmaniasis and *Buruli* ulcer [3].

Among the IMD NTDs, leishmaniasis is found to be spread world wide and endemic in Asia, the Americas, the Mediterranean region and Africa [4]. This uneven spread of the disease throughout the globe is facilitated by the diverse distribution pattern of reservoir host species and, transmission vector of the parasite. Sandflies are the primary vectors responsible for parasite transmission, with various species of the genera *Phlebotomus* and *Lutzomyia* especially responsible for transmission of leishmaniasis to humans. Animal reservoirs like dogs, cats, foxes, jackals and rodents also play an important role in maintaining the prevalence in various areas [5]. The disease is transmitted to humans by blood feeding from infected sandfly. There are three different clinical forms of leishmaniasis: Visceral (VL), Cutaneous (CL) and Mucocutaneous (MCL) [6]. According to the WHO 2017 report, 97 countries and territories are still found to be

endemic for leishmaniasis, out of which VL and CL are endemic in 65 countries. Approximately 700,000 to 1 million new cases occur annually from which 20,000 to 30,000 lead to death [4].

Currently available first line and second line treatment drugs for leishmaniasis are facing problems of high toxicity, drug resistance, high cost and parenteral administration. There exist no vaccines, either prophylactic or preventive, that can induce long term protection. Therefore, there is an urgent need to search for novel effective drugs or vaccines to treat and control leishmania infections [7, 8].

Proteomic analysis, microarrays and several bioinformatics tools have exploited the genome of this parasite to find species-specific genes that may lead to the development of drugs targeting novel enzymes and receptors [9]. In leishmania, various biochemical pathways (glycosylphosphatidylinositol biosynthetic pathway, purine salvage and glycolytic pathways), proteinases, topoisomerases and hypusine pathways have been found to be potential drug targets [10]. The different metabolic pathways which are compartmentalized in the organelle called glycosome are quite distinct in leishmania as compared to its mammalian host, thereby making glycosome the potential drug target. Glycosomes, as compared to other organelles, are evolutionarily and structurally related to peroxisomes in higher eukaryotes [11, 12]. Compared to peroxisomes, glycosome requires the post-translational import of enzymes critical for functional metabolism compartmentalized into the lumen of the organelle [13]. As these necessary enzymes are encoded in the nucleus, the pre-requisite to import them into the glycosome makes this process a potential drug target.

The peroxin proteins such as PEX5, PEX7, PEX14, PEX13, PEX2, PEX10, PEX12, PEX6 and some others, have so far been recognized to play important roles in matrix protein import. The import cycle starts when PEX5 receptor protein interacts with a protein carrying type 1

peroxisomal-targeting signal (PTS1) and/or PEX7 receptor protein interacts with a protein carrying type 2 peroxisomal-targeting signal (PTS2). The two cytosolic receptors, PEX5 or PEX7 then transport the bound protein to the docking complex of the peroxisomal membrane [14]. This docking complex is composed of PEX13, PEX14 and PEX17 as revealed in yeast. Interactions between PEX5 and PEX14 is thought to be responsible for the formation of a pore, as paralleled in peroxisomal transport [15]. An additional protein complex composed of RING finger proteins (PEX2, PEX10, PEX12) and PEX4 (Ubc10) (in yeast) or UbcH5a/b/c (in mammalian cells) is responsible for ubiquitination of receptors, acting as a signal for its retrieval [16]. Receptors are then translocated back to the cytosol in an ATP-dependent manner by two predominantly cytosolic ATPases of the AAA+- protein family, PEX1 and PEX6 [17, 18].

The current study focuses on exploring how the import machinery in glycosome works, specifically, the role of RING finger proteins in export and import of necessary PTS-containing proteins into the glycosome. *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 constructs were inserted into the cloning site of pET30b(+) expression vector and pRP-H-BSD [19]. RING finger proteins were successfully expressed and purified using the pET30b(+) plasmid. These proteins were later inserted into liposomes. The liposomes were composed of phospholipids and fatty acids, mimicking the glycosomal membrane [20]. They were further characterized by using biochemical experiment like alkaline carbonate extraction. The result of this study will build on the existing knowledge of import machinery of glycosomes. Further experiments can be carried out to see the protein-protein interaction of RING finger proteins with other partner proteins.

Research Objectives and Project Goals

The aim of this study was to express, purify and characterize RING finger proteins as a step to further investigate their role in the import/export of receptor proteins. To this end, the following objectives were determined:

1. Purification of RING finger proteins *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12.
2. Incorporation of these proteins in liposomes to create proteoliposomes that mimic glycosomal membranes.
3. Characterization of RING finger proteins to determine how they behave in the proteoliposome.

Literature review

Leishmaniasis

Leishmaniasis is a disease caused by the protozoan parasite *Leishmania* which was named after William Boog Leishman, the Scottish pathologist who identified the parasite in a sample recovered from the spleen of a patient in India in the 1900s. A few months later, Charles Donovan another doctor in India observed similar parasites, hence, their names were used to designate the species associated with the visceral form of leishmaniasis, now known as *Leishmania donovani* [21].

Sandfly is the insect vector which includes more than 600 species responsible for the transmission of the parasite to the mammalian host. Five genera of sandflies have been characterized and these include: *Lutzomyia*, *Brumptomyia*, and *Warileya* in the New World and *Phlebotomus* and *Sergentomyia* in the Old World. Among these only the species and subspecies of the genus *Phlebotomus* and *Lutzomyia* are responsible for transmission of human leishmaniasis. The parasite is transmitted to a mammalian host when the sandfly takes a blood meal. The disease is diverse and 53 species of the *Leishmania* have been described globally, of these 20 species are pathogenic and cause infections in humans [22, 23]. The disease ranges from non-fatal but disfiguring causing cutaneous lesions to a potentially fatal visceral form of the infection if not treated [24, 25, 26]. Geographical distribution of pathogenic *Leishmania* species and their respective reservoir host is represented in Table 1.

Leishmaniasis is one of the most significant vector-borne human diseases that remains endemic to many areas of the Mediterranean basin, the tropics and subtropics regions of the globe [24]. Countries like Brazil, Ethiopia, India, Somalia, South Sudan and Sudan are the most impacted

	Species of *Leishmania*	Type of Leishmaniasis	Geographical Distribution	Vector
Old World Species	*Leishmania major*	CL	Central Asia, Middle East, North and Central Africa, Sahel belt	*Phlebotomus bergeroti, P. duboscqi, P. papatasi, P Salehi, P. mongolensis*
	L. aethiopica	LCD and DCL	East Africa (Ethiopia, Kenya)	*P. sergenti, P. pedifer, P. longipes*
	L. donovani	VL and PKDL	Ethiopia, Sudan, Kenya, India, China, Bangladesh, Burma, Central Africa, South Asia, Middle East	*P. alexandri, P. argentipes, P. martin, P. orientalis, P. sichuanensis, P.* celiae
	L. infantum	VL and CL	North, Central and South America, Southeast Europe, Middle East, Central Asia, North Africa, Mediterranean countries	*P. arias, P. pcrniciosufi, P. balcanicus, P. alexandri, P. brevis, P. chinensis, p. halepensis, P. kandelakii*
	L. tropica	CL and VL	Central and North Africa, Middle East, Central Asia, India	*P. aculeatus, P. saevus, P. rossi, P. arabicus, P. chabaudi, P. guggisbergi, P. sergenti*
New World Species	*L. mexicana*	CL and DCL	Ecuador, Peru, Venezuela and Central America	*Lutzomyia anthophor, L. christophei, L. columbiana*
	L. amazonensis	CL, DCL and MCL	South America (Bolivia, Brazil, Venezuela), north of the Amazon	*L. diabolica, L. olmeca reducta, L. flaviscutellata, L. longipalpis,*
	L. braziliensis	CL and MCL	Brazil, Bolivia, Western Amazon basin, South America, Peru, Guatemala, Venezuela,	*Psychodopygus, L. cruciate, L. anduzei, L. ayrozai, L. cruciate, L. fischeri, L. intermedia*
	L. peruviana	CL and LCD	Peru, Bolivia, Argentine highlands	*L. verrucarun, L. pvmenis, L. whitmani, L. noguchii, Lu. tejadai*
	L. guyanensis	CL and MCL	Northern South America, Bolivia, Brazil	*L. anduze, L. ayacuchensis, L. longiflocosa, L. umbratilis, L. whitmani*

Table 1: Most prevalent infectious leishmania species infecting humans in different parts of the world
Most prevalent *Leishmania* species of the New and Old World with their associated vectors and forms of leishmaniasis. Visceral leishmaniasis (VL) is the most dangerous form of leishmaniasis, during this infection the parasite can migrate to internal organs like spleen and liver. Post-kala-azar-dermal leishmaniasis (PKDL) is characterized by development of scars on skin or mucosal membranes in patients who recovered from VL. Cutaneous leishmaniasis (CL) causes ulcers on the skin. It can be either localized (LCD) or disseminate from the site of infection (DCL). Mucocutaneous leishmaniasis (MCL) is characterised by ulcers on both skin and mucosal membranes mainly in mouth and nose tissue. [22, 27, 28].

nations [29, 30]. Worldwide, there are ~70,000 deaths reported and around 1.5 to 2 million new cases each year [31].

Leishmaniasis is characterized as a "Neglected Tropical Disease" (NTD) by World Health Organization (WHO). This categorization includes the likes of Chagas disease, Schistosomiasis, African Sleeping Sickness, Onchocerciasis, soil-transmitted helminths which are all prevalent in tropical countries affecting mostly the poorest section of these population [32]. Despite this drastic socioeconomic impact of NTDs, pharmaceutical companies have devoted minimal research effort for the development of treatment or drugs against them [33, 34]. As a result of the increased morbidity and mortality due to NTDs WHO started a program (a roadmap for implementation [1]) in 2012 to eradicate many of these NTDs by 2020. This eradication program focused on the top ten NTDs, an initiative supported by donations from various agencies in the London Declaration on NTDs [35]. Although a significant effort has been made by the WHO and other funding agencies towards eradication and treatment of NTDs, more than one billion people remain affected in ~149 countries [1]. A research consortium from Médecins Sans Frontière argues, "tropical diseases have become progressively neglected because of the insufficient return on investment that such drug development and disease research financial efforts offer" [36].

Clinical signs of Leishmaniasis

Leishmaniasis can be present in three clinical forms, namely cutaneous, mucocutaneous, or visceral leishmaniasis. Cutaneous leishmaniasis (CL) involves only the skin and may be characterized by one or several lesions. Symptoms may manifest as ulcers, smooth nodules, flat plaques or hyperkeratotic wart-like lesions depending on the species of *Leishmania* involved. Although the pace of healing varies with the species, most skin lesions heal immediately. HIV-infected individuals may present extremely severe cases and the disease proves more difficult to

cure [24]. In the Old World (Africa, Asia, and Europe) CL is mainly associated with *L. major* and *L. tropica,* whereas, in the New World (North and South America) it is associated with *L. mexicana* with an estimated 1.5 million new cases every year (Table 1). The localized form (LCD) is most common, consisting of chronic self-healing ulcers on the exposed area of infection, localized in skin tissue [37]. In contrast, the diffuse form (DCL) is the less common manifestation of cutaneous leishmaniasis. It is characterized by non-ulcerative nodules that disseminate from the initial site of infection and can potentially cover large areas of the body. This has been seen especially with *L. amazonensis* in the Western Hemisphere, although other organisms may also be involved [38].

In mucocutaneous leishmaniasis (MCL), the parasite attacks the pharyngeal mucosal membranes to establish a chronic infection. A small percentage of these cutaneous infections metastasize to mucosal tissues by dissemination through the lymphatic or haematogenous system [38]. MCL usually results from *L. braziliensis* infections which are predominantly found in Latin America (Table1) [39]. During the infection, macrophages colonize the nasopharyngeal mucosa making these lesions non-self-healing, which leads to diminished healing capacity [40, 41]. Deaths associated with MCL are usually related to secondary bacterial infections that cause further tissue destruction [39].

Visceral leishmaniasis (VL) is the most severe form of the disease; and is responsible for most of the fatalities associated with leishmaniasis [42]. 500,000 new cases are reported each year and it is estimated that VL causes 50,000 deaths per year, a number that is likely a gross underestimate due to inaccurate reporting [43]. VL is predominantly found in India, Sudan, South Sudan, Kenya, Ethiopia, and Brazil [6, 23]. In India and Eastern Africa, *L. donovani* is the predominant causative agent of VL, while *L. infantum* and *L. chagasi* are more common in the Mediterranean region and

the New World [44]. The clinical symptoms presented by patients with VL include; fever, weakness, fatigue, weight loss, appetite suppression, and swelling of visceral organs such as the spleen (splenomegaly), lymph nodes, and liver (hepatomegaly) [23]. It is strongly associated with fatality without proper diagnosis and treatment; within two years after the onset, VL can be fatal in more than 95% of cases. In 2014, Brazil, Ethiopia, India, Somalia, South Sudan and Sudan had more than 90% of new cases as reported by the WHO.

Post-kala-azar dermal leishmaniasis (PKDL) is characterized by skin lesions, or macular, maculo-papular, or nodular rashes and is thus classified as a cutaneous manifestation of VL. This dermatological disorder is often observed after the treatment of visceral leishmaniasis in Sudan, East African countries, and in the Indian subcontinent [23, 45]. The time interval taken to detect PKDL after treatment of VL varies from 0-6 months as reported in Sudan, and 3-6 years in India [46, 47]. All three forms of leishmaniasis and their respective disease-causing species are represented in Figure 1.

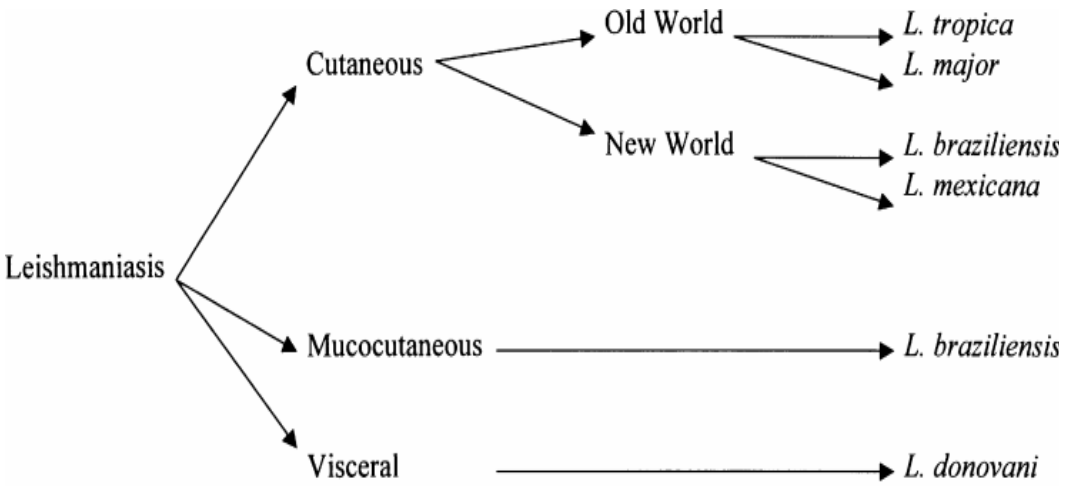

Figure 1. Different forms of leishmaniasis and associated *Leishmania* species [48].

Geographical Distribution

Leishmania spp. have been reported on every continent except in Australia and Antarctica [49]. In tropical and sub-tropical regions, these organisms are predominantly endemic. Humans are mainly affected by the organism in Africa, parts of Asia, the Middle East, Latin America and the Mediterranean region. In Europe, leishmaniasis seems to be spreading gradually northward from its traditional foci.

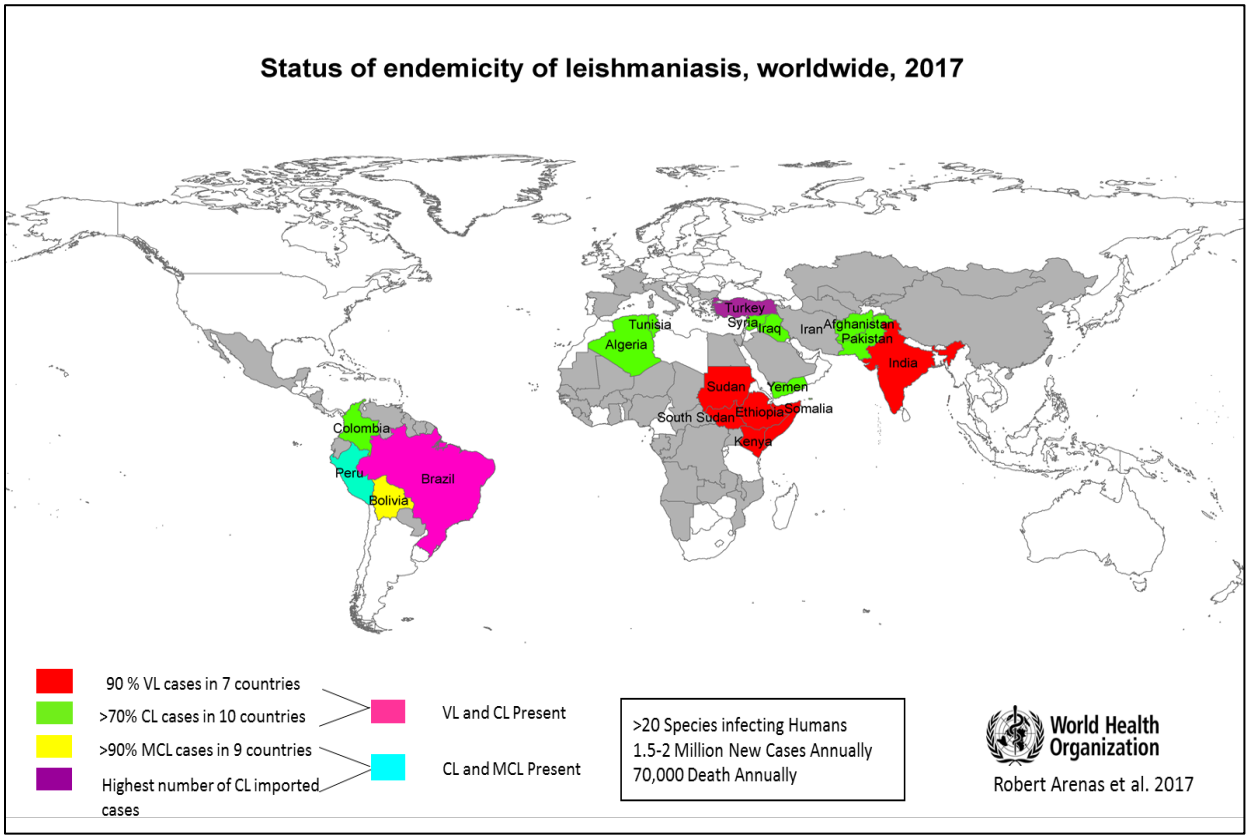

Figure 2. Geographical distribution of leishmaniasis worldwide World map highlighting areas with 90% visceral leishmaniasis concentrated in 7 countries (red color), more than 70% cases of cutaneous leishmaniasis concentrated in 10 countries (green color) and more than 90% cases of mucocutaneous leishmaniasis concentrated in 9 countries (yellow color). Other endemic countries are represented in grey color. Highest number of imported CL cases has been reported in Turkey as of 2016 (purple color) [6, 31]

Visceral leishmaniasis is majorly concentrated in southern Asia and Africa and spread by *L. donovani*, while in Mediterranean, the Middle East, Latin America and parts of Asia it is spread by *L. infantum.* [50]. Cutaneous leishmaniasis is caused by *L. major* in Africa, the Middle East and parts of Asia; by *L. tropica* in the Middle East, the Mediterranean and parts of Asia; and by *L. aethiopica* in parts of Africa. Many different species may be found in the Western Hemisphere, where CL can be found throughout South America and Mexico. In North America, limited foci of infection have been reported in Canada and the United States called canine leishmaniasis, caused by *L. infantum,* and is found primarily in foxhounds [24]. Leishmaniasis is present in over 88 countries, mainly in developing and underdeveloped nations. While new cases are reported annually, and the number of cases continually increases, originating in newer areas of the world. Countries like India, Nepal and Bangladesh harbor 67% of the global count of VL [51, 52]. Figure 2 shows current scenario of geographically spread leishmaniasis worldwide in 2017 as reported by the WHO.

Life-cycle of the *Leishmania* Parasite

The bite of an infected female phlebotomine sandfly (*Lutzomyia* or *Phlebotomus*) is responsible for the transmission of *Leishmania* parasites. Since the insect vector needs a blood meal to produce eggs, infection occurs when an uninfected sandfly feeds on an infected individual or a mammalian vertebrate reservoir host. Many mammalian species including rodents or dogs act as reservoir hosts [53, 54]. Host macrophages containing intracellular amastigotes are ingested by the sandfly vector during feeding [51]. Amastigotes are 3 – 7 μm in diameter, round and non-motile obligate intracellular form of the parasite found on the skin (32 ºC) or liver (37 ºC) of the host. In the posterior abdomen of the sandfly midgut amastigotes transform into promastigotes (22 ºC), thus starting their extracellular life cycle in the vector.

The promastigote stage of *Leishmania* is highly motile, 10 – 20 µm long, and flagellated (Figure 3). When an infected sandfly pierces the skin of the host with its proboscis during feeding, (which occurs commonly during the night while the host is asleep) promastigotes are released into the host together with sandfly saliva. The metacyclic stage of the promastigote are taken up by host macrophages, where they differentiate to amastigotes which multiply within the phagolysosome. Amastigotes released from macrophage rapidly undergo a receptor mediated

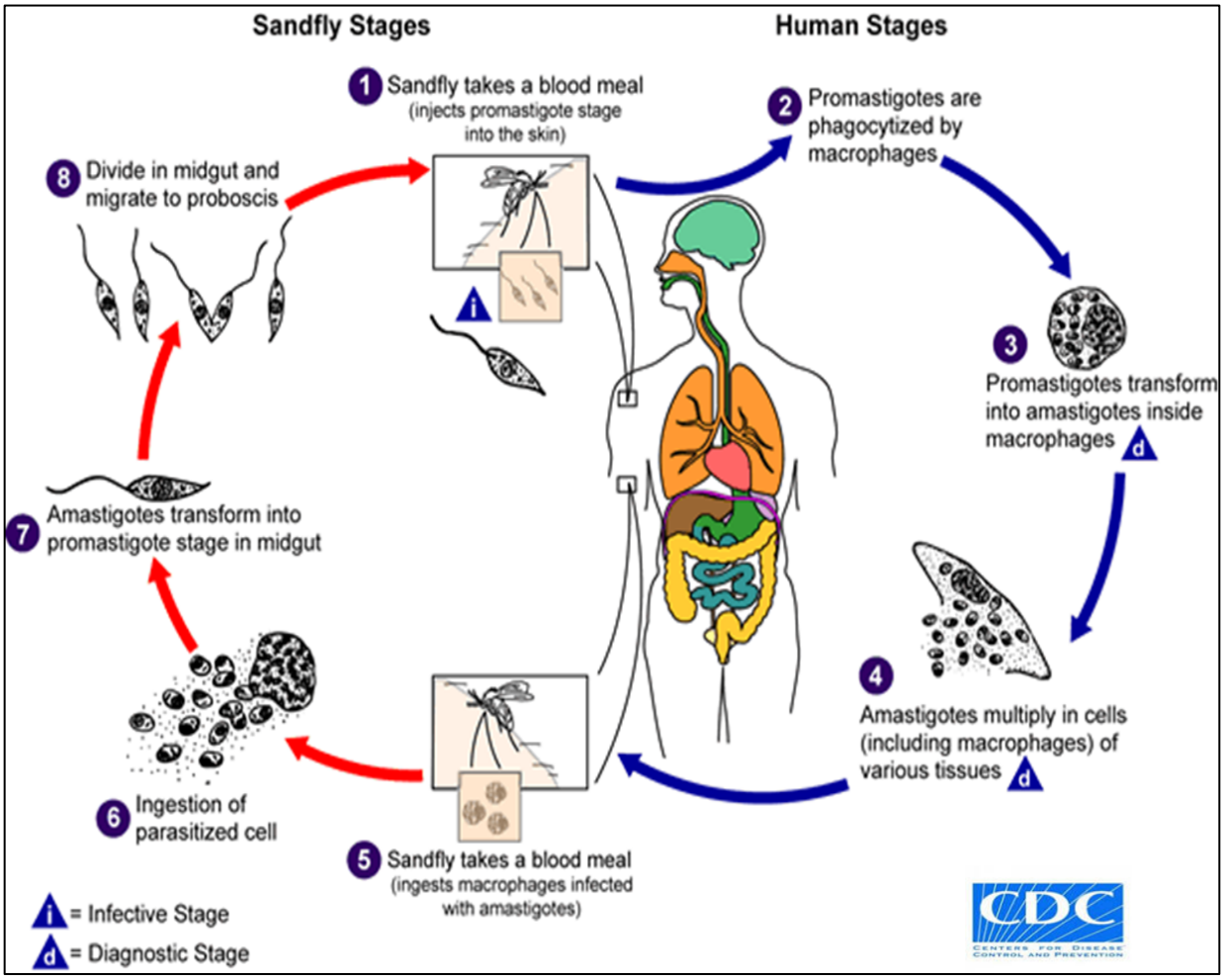

Figure 3: Life cycle of *Leishmania*. Promastigote are injected into the human host by sandflies (1), promastigotes are then phagocytized by macrophages and other mononuclear phagocytic cells (2), then it is transformed into amastigotes inside macrophages, where they multiply and start to infect other phagocytic cells (3, 4). When sandfly take blood meal from infected host, it ingests the amastigote (5, 6) which then gets transformed into promastigote in the gut (7) and migrates to the proboscis (8) ready to infect another human host [51]

infection of neighboring macrophages and thus continues the cycle and the infectious stage of leishmaniasis. In the case of visceral leishmaniasis, the parasites residing in macrophages and phagocytes are colonized in organs such as lymph nodes, spleen, liver and bone marrow [55, 56, 57].

Diagnosis of Visceral Leishmaniasis

The first-line approach in the diagnosis of visceral leishmaniasis is by detection of amastigotes in the splenic aspirates, bone marrow, and lymph nodes by microscopy of biopsies. These tests are highly specific and confirmatory but due to their invasive nature they may cause severe haemorrhaging in patients and therefore require high degree of technical expertise for sample collection [58]. In the past, tests like aldehyde test (also known as Formol-Gel test, is the interaction of serum globulins with formaldehyde resulting in jellification caused by their binding) were used but they have been abandoned because of very low specificity and sensitivity [59, 60].

Several less invasive serological tests like the Direct Agglutination Test (DAT), rK39 ELISA, and other molecular tests, for example, PCR, are becoming more popular but presently these tests are limited to laboratories with skilled personnel [61, 62]. The Indirect Fluorescent Antibody Test (IFAT) is based on detecting antibodies produced in the initial stages of infection and are generally undetectable six to nine months after cure. ELISA is used as a serodiagnostic test for VL, whereas DAT employs Coomassie Brilliant Blue to stain whole promastigotes incubated with sera of the patients and agglutination is observed after overnight incubation [62, 63, 64]. Recently, immuno-chromatographic strips using K39 antigen have become popular diagnostic tools [65].

Visceral Leishmaniasis treatment strategies

Drugs necessary for the management of anemia, malnutrition, bacterial or parasitic infection and some specific anti-leishmanial drugs are generally used for the treatment of visceral leishmaniasis [66]. The first line of treatment for VL has been the pentavalent antimonial, sodium stibogluconate and meglumine antimoniate in many areas of the world for over 70 years [67, 68]. Conventional treatment with amphotericin B has replaced antimonials as the first-line treatment for VL in some areas of the state of Bihar in India where the failure rates for antimonials has reached >60% [69]. Liposomal amphotericin B is used as the first-line treatment in Europe and the United States. Until recently, the high cost (USD $2,800 per treatment) of the drug hindered its use in developing countries [70, 71]. Miltefosine (an anticancer drug) is the first effective oral drug for VL, but as a side effect it causes severe gastric toxicity. When compared to other anti-leishmanial drug, the associated risk of toxicity with miltefosine is high due to its long half life (~150 hours) [72]. Paromomycin (formerly aminosidine), is an aminoglycoside antibiotic with good anti-leishmanial activity, but is related to renal and ototoxicity [73]. Sitamaquine is an aminoquinoline drug which is taken orally and showed evidence of efficacy against VL more than 20 years ago. But it causes nephrotoxicity and has been abandoned as it has low efficacy at tolerated doses [74]. The association of sodium stibogluconate and paromomycin was found to be safe and effective in early trials conducted in India and East Africa [75, 76, 77]. Table 2 summarizes current drugs and issues related to treatment of visceral leishmaniasis.

On the other hand, the progress in developing a protective vaccine against the different human leishmaniasis has been limited. The process of ‘leishmanization’, live infection is simulated by using live *L. major* as a vaccine for protection against CL. Leishmanization has been carried out

Drug	Trade Name	Known Issue
Pentavalent antimonials	Pentostam, Glucantime	Resistance, toxicity, quality of generics
Amphotericin B	Fungizone	Intravenous infusion, toxicity
Lipid-associated amphotericin B	Ambisome	Less toxic, cost prohibitive
Pentamidine	Pentacrinate	Second line treatment, toxicity, intravenous infusion.
Miltefosine	Impavido, Miltex	Teratogenicity, resistance
Paromomycin	Humatin	Resistance
Sitamaquine	(WR-6026)	May cause methemoglobinemia

Table 2 Current VL treatments and their main characteristics [23, 44, 78, 79, 80].

in Uzbekistan, Iran and Israel [78, 81]. However, since this technique is not suitable for large-scale use or for use in HIV endemic areas, it has been discontinued. Preparations of killed parasites are crude and difficult to define and standardization with or without adjuvant have not shown any significant prophylactic efficacy [82].

Although all treatment options were adapted to control visceral leishmaniasis, none of them have been successful in eliminating the disease from endemic regions (Table 2). There is an urgent need for more intensive research and development in the area to discover new antileishmanial drug targets. This could be achieved by understanding and exploration of novel processes of the parasite biology [82].

Biology of the Parasite

Kinetoplastida are protist organisms that include the genus *Leishmania*. Thought to have diverged early from the eukaryotic lineage, they received their name from the presence of a kinetoplast organelle [83]. The kinetoplast is a network of circular DNA (called kDNA) found in a large single mitochondrion that contains many copies of the mitochondrial genome [83, 84]. The class of Kinetoplastida contains the order of Trypanosomatida, which are characterized by the presence of a single flagellum and a smaller kinetoplast [83, 85]. Additionally, they possess unique molecular biological traits such as atypical RNA editing and transcription of long multi-cistronic mRNA molecules. This unusual RNA editing, which is characteristic of kinetoplastids, involves the insertion, or deletion of uridine residues at particular sites of coding regions. These create new initiation codons, new open reading frames, stop codons, and even correct frame shifts from long multi-cistronic RNA molecules [85, 86, 87]. The other characteristic feature of kinetoplastids is the compartmentalization of several metabolic processes into the organelles called glycosomes, which are similar to peroxisomes in eukaryotic organisms [88, 89, 90].

Role of glycosomes in trypanosomes

In 1977, Opperdoes and Borst, [91] discovered that trypanosomes compartmentalize several glycolytic enzymes in highly specialised microbodies, named glycosomes. These organelles, present in all kinetoplastids, are evolutionarily related to the peroxisomes of fungi and mammalian cells, and to the glyoxysomes of plants [92]. These microbodies are spherical organelles with a diameter of 0.2 to 0.3 μm and are bound by a single phospholipid bilayer. They are devoid of DNA and have a proteinaceous electron-dense matrix [93]. Glycosomes compartmentalize several essential metabolic functions required for: glycolysis [94, 95], pentose-phosphate pathway [91,

96], purine salvage [97, 98], pyrimidine biosynthesis [97], β-oxidation of fatty acids [98], ether-lipid biosynthesis [99, 100], and oxidant defence mechanisms [101]. Therefore, glycosomes represent an attractive chemotherapeutic target as mistargeting of glycosomal enzyme trafficking or disruption of glycosome biogenesis leads to a lethal phenotype [102, 103, 104].

The glycosomal membrane is impermeable to most glycolytic metabolites, thus a mechanism is required to balance the ratio of ADP/ATP and NAD+/NADH [103]. Glycolysis is the metabolic pathway that keeps a check on ADP/ATP and NAD+/NADH ratios in the glycosome. In the bloodstream form of *T. brucei,* the first seven enzymes involved in the conversion of glucose to 3-phosphoglycerate or 1, 3-bisphosphoglycerate, are localized to the matrix of the glycosome. On the other hand, downstream enzymes required for glycolysis, including phosphoglycerate mutase and pyruvate kinase, are present in the cytosol [94, 105, 106, 107]. These enzymes further metabolize 3-phosphoglycerate to pyruvate (producing ATP), which is then secreted from the cell (Figure 4) [94, 105].

The overall ratio of ATP/ADP following glycolysis does not have any net change inside the glycosome. This is because the two ATP molecules are consumed during the first part of glycolysis are restored by the action of downstream kinases involved in substrate level phosphorylation inside the glycosome (Figure 4) [107]. There is net gain of two ATP molecule which is produced when phosphoenolpyruvate is converted to pyruvate in the cytosol. Similarly, the ratio of NAD+/NADH remains unaltered by the end of glycolysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is responsible for the production of NADH. In the subsequent step, NADH is oxidized to NAD^+ by the actions of glycerol-3-phosphate dehydrogenase (G3PDH). As the NADH molecule is reduced, the electrons produced are transferred to the mitochondrion with the help of a shuttle pathway that includes the transport of glycerol-3-phosphate to the outer face of the inner membrane

of the mitochondrion where glycerol-phosphate oxidase system converts it to dihydroxyacetone phosphate (DHAP). A putative G3P/DHAP antiporter system helps facilitate the exchange of

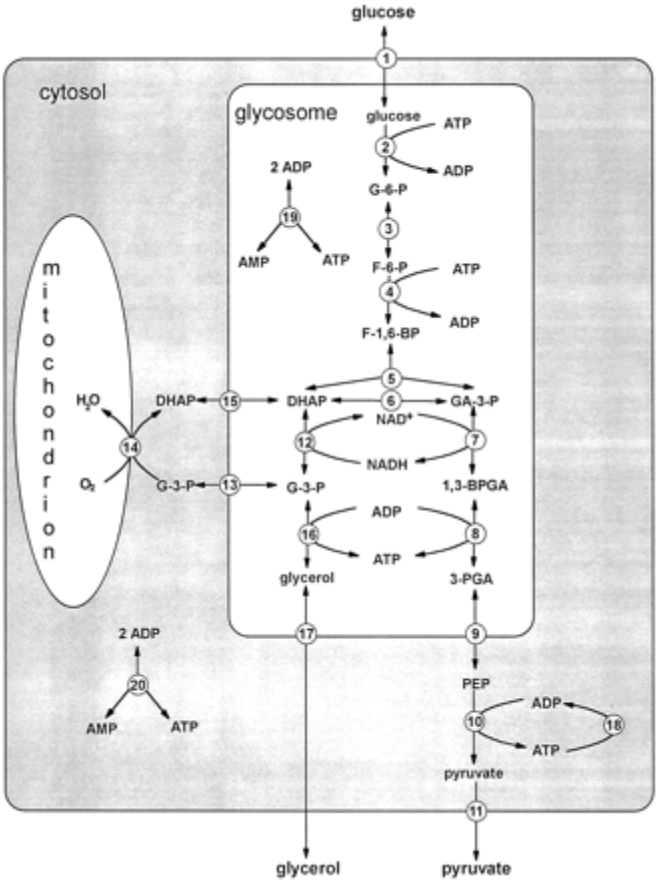

Figure 4. The stoichiometric scheme of the model of glycolysis in the bloodstream form of *T. brucei.* Chemical reactions 3, 6, 9, 13, 15 and 17, 19 and 20 were treated as equilibrium reactions. *1*, transport of glucose across the plasma membrane and the glycosomal membrane; *2*, HK; *3*, PGI; *4*, PFK; *5*, ALD; *6*, TIM; *7*, GAPDH; *8*, PGK; *9*, transport of 3-PGA across the glycosomal membrane, PGM, and ENO; *10*, PYK; *11*, pyruvate transport across the plasma membrane; *12*, GDH; *13*, transport of Gly-3-P (*G-3-P*) across the glycosomal membrane; *14*, GPO; *15*, transport of DHAP across the glycosomal membrane; *16*, GK; *17*, transport of glycerol across the glycosomal membrane and the plasma membrane; *18*, ATP utilization; *19*, glycosomal AK; *20*, cytosolic AK. G-6-P, Glc-6-P; F-6-P, Fru-6-P; F-1, 6-BP, Fru-1, 6-BP [108]

DHAP with another glycerol-3-phosphate (G3P). Hence, NADH is once again oxidized to NAD^+ causing no net gain of NADH after completion of glycolysis [94, 107].

The partial reduction of glycolytic enzyme levels leads to decreased glycolytic flux, of which a 50% reduction results in parasite death [109, 110]. Interestingly, high levels of glycolytic flux are only associated with trypanosomatids that live on high glucose medium [102, 111]. The catalytic activity of a given enzyme controls the rate determining step of glycolytic flux in *T. brucei* [85, 112]. These facts are contradictory to the postulation of a relationship between compartmentalization of glycolysis and glycolytic flux. Mistargeting of enzymes like hexokinase and phosphofructokinase results in cytosolic toxicity in the bloodstream form of *T. brucei* indicating the importance of compartmentalization in overcoming metabolic interference (Figure 4). Compartmentalization also helps in recovering from starvation, as the closed system in these parasites prevents loss of ATP and restarts the glycolysis process when substrates are available once again. Enzymes hexokinase and phosphofructokinase have another interesting role to play in catalysing the rate limiting step of the first stage of glycolysis.

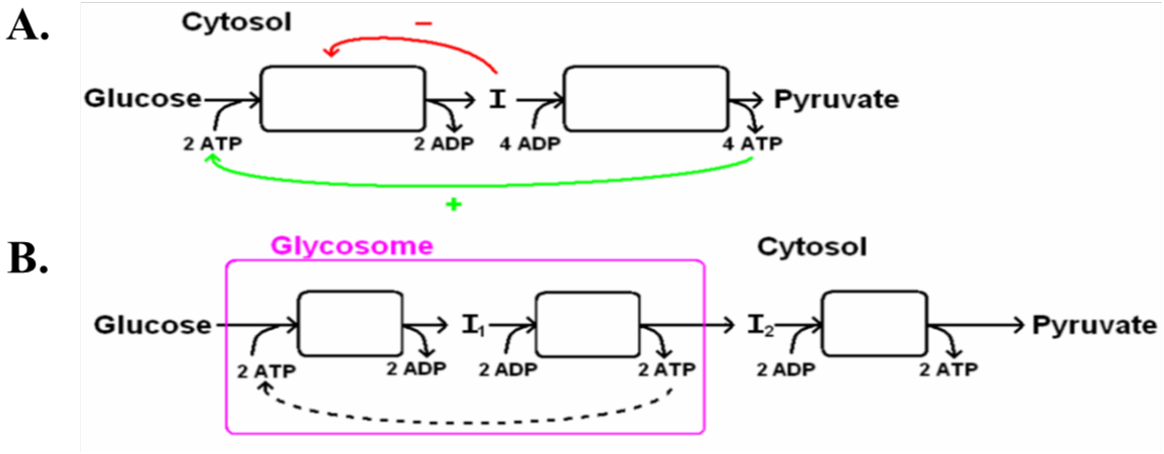

Figure 5: Necessity of compartmentalization for keeping the enzymes under control without product or feedback inhibition. Product or feedback inhibition. **(A)** Hexokinase and phosphofructokinase are involved in first few steps of the glycolytic pathway resulting in constant investment of ATP, while accumulating intermediates which are not under control of a negative feedback mechanism (P: product, I: metabolic intermediate, S: substrate). **(B)** Low ATP/ADP ratio (no net ATP production in the glycosome) is maintained through compartmentalization of first few steps of glycolysis which reduces the turnover rate of the enzymes and avoids accumulation of intermediates (P: product, I: metabolic intermediate, S: substrate) [85].

Surprisingly, they are not regulated by feedback inhibition through allosteric effectors or reaction products [113, 114, 115]. The low ratio of ATP to ADP also controls the activity of phosphofructokinase and hexokinase enzymes (Figure 5). Decreased levels of ATP causes a reduction in the turnover rate of the afore mentioned enzymes that maintain glucose-6-phosphate levels below the toxic range [85, 115, 116, 117] (Figure 5). This process is like how a turbo engine works, as engine exhaust is used to boost its performance. This property was termed the "turbo-design" of glycolysis [13]. Hence, compartmentalization of glycolysis has been shown to be extremely significant for critical regulation of the glycolysis pathway.

Transport of protein across glycosomal membrane

Peroxisomal targeting signals, the C-terminal PTS1 or N-terminal PTS2 topogenic signal sequence, are responsible for translocation of select cytosolic proteins across the glycosomal membrane [118, 119]. Proteins with a PTS1 receptor signal are bound by peroxin 5 (PEX5), located in the cytosolic compartment. Similarly, PTS2 signal sequences are bound by peroxin 7 (PEX7) [14, 120]. Another type of protein targeting involving a polypeptide internal sequence (iPTS) has also been established for the glycosomal transport of triosephosphate isomerase in *T. brucei*. Surprisingly, this enzyme does not contain any PTS1 and PTS2 motifs. A 22-amino acid sequence containing residues such as K155, D158, W159, A160 and K161 on the surface of a glycolytic enzyme called phosphoglycerate kinase isoenzyme (PGK-A), was found to play a role in this transport [121]. Moreover, there are some glycosomal proteins that do not have any recognized import sequence. It is assumed that the transport of such proteins into the glycosomal matrix occurs with the help of a piggy-back transport mechanism in complex with a PTS1 or PTS2 signal-carrying protein [122, 123, 124]. While some of these proteins lack a PTS signal, they are still transported by PTS1 receptor protein, PEX5. Glycosomes are

evolutionarily related to peroxisomes and share similar protein import and biogenesis mechanisms. They have many common homologous protein and share similar structure [125]. Other than the mechanism of protein transport discussed above, a few peculiar protein import mechanisms have been observed in peroxisomal protein import. For example, in a study conducted with the *S. cerevisiae* Pex5p, it was shown that the presence of a N-terminal half of Pex5p alone was sufficient for the import of Fox1p into peroxisomes [126]. The study also showed that when Pex18p was fused to the C-terminus of Pex5p it was able to import Fox1p into peroxisomes. This shows that accessory proteins like Pex18p, which are required for the

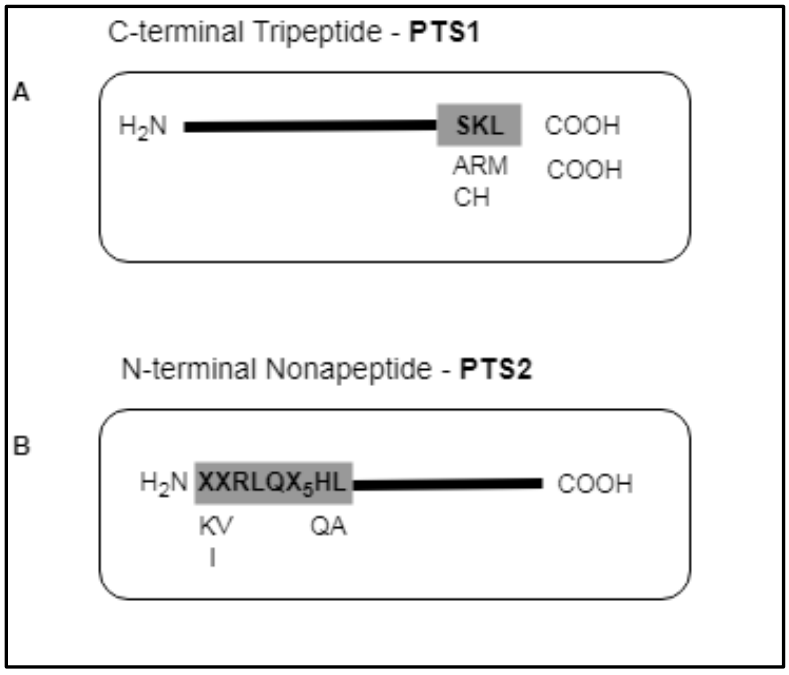

Figure 6: PTS1 and PTS2 signaling pathway. A. The PTS1 signal is recognized by the C-terminally located tripeptide serine lysine leucine represented in grey box (SKL). **B**. PTS2 signal is recognized by the N-terminally located degenerative nonapeptide arginine, leucine, X_5, histidine, leucine (RLX_5HL) represented in a grey box. Degenerate sequence is represented below the respective box of both PTS1 and PTS2 signals [119].

transport of cargo proteins via PTS2 receptor proteins, in conjunction with Pex7p shows similarity to N-terminus region of Pex5p. [126] The details of PTS1 and PTS2 mediated transport is discussed in detail in the following section.

PTS1-Signaling Pathway

Our understanding of peroxisomal protein import was pioneered when a group of researchers working with recombinant luciferase found that it co-localized with catalase inside peroxisomes [127]. Later, the C-terminal region of luciferase was defined as the first peroxisome targeting signal (PTS1), a sequence necessary for targeting protein to peroxisomes. The C-terminus of the PTS1 signal sequence contains a degenerative tripeptide (S/A/C) (K/R/H) (L/M) [127]. Further studies found that for some proteins, a consensus tripeptide signal alone was insufficient for peroxisomal targeting. For example, addition of the PTS1-SKL to the C-terminus of a mouse dihydrofolate reductase or PTS1-AKL to the C-terminus of a chimeric chloramphenicol-acetyl-transferase (CAT) failed to target these proteins to the peroxisome. In contrast, when PTS1-SKL was attached to C-terminus of CAT it was sufficient for import into the peroxisomal matrix [127]. It is possible that the accessibility of the C-terminal signal sequence and interaction with the PEX5 receptor may account for these observations. Presently it is well known that a majority of peroxisomal matrix proteins carry a PTS1 signal, in contrast to a N-terminal PTS2 signal [127]. In trypanosomatids, enzymes important for metabolism of glucose inside the glycosome such as glucose-6-phosphate isomerase (PGI), phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), glycerol-3-phosphate dehydrogenase and glycerol kinase all carry a PTS1 signal. The translocation of these proteins happens when they are recognized by

the PTS1 binding domain (P1BD) of PEX5, which is composed of seven tetratricopeptide repeats [128].

PTS2-Signaling Pathway

PTS2 was first identified near the N-terminus of rat liver thiolase. In contrast to the PTS1 signal sequence, the PTS2 signal was found to be cleaved from the thiolase as it enters into peroxisomes. Enzymes such as watermelon malate dehydrogenase, *H. polymorpha* amine oxidase and aldolase from *T. brucei* were found to have similarly conserved sequences characterized by an N-terminal nonapeptide with a PTS2 consensus R/K-L/V/I-X_5-H/Q-L/A where X is any residue (Figure 6) [129]. PTS2 was also shown to target passenger proteins into the peroxisomes and in many cases the PTS2 sequence remains attached, unlike rat liver thiolase. Site-directed mutagenesis of the PTS2 sequence showed that first two and last two amino acids of the PTS2 consensus play an essential role in this targeting. It is interesting to see how some changes in the amino acid region near the PTS2 nonapeptide in the N-terminal region of peroxin proteins can affect the targeting. Mutations to the rat thiolase, 5 amino acids downstream of PTS2, from a glutamtic acid to a basic or hydrophobic amino acid shuttled the protein to the mitochondria as it was targeted to peroxisomes [129].

Peroxin 5 is the PTS1 receptor

More than 70% of glycosomal proteins bearing peroxisomal targeting sequence type 1 (PTS1) are trafficked to the glycosome through interaction with PEX5. *L. donovani* proteins bearing a PTS1 signal interact with a highly conserved seven tetratricopeptide repeat (TRPs) on the C-terminal region of PEX5, localized within residues 324 and 588 **(**Figure 7**)**. The TRPs

comprise of a helix-turn-helix motif with interacting anti-parallel alpha helices and are composed of 34 degenerate amino acids. They are arranged in particular manner; TRP1-3 are present together in a fold, TRP 5-7 are arranged in 3 domain fold and TRP 4 act as a hinge between both of them forming a continuous α helix.

*Ld*PEX5 is also a bidomain protein like other PEX5 proteins; the N-terminal region of the protein has three conserved WXXXY/F motifs, and a LVAEF pentapeptide sequence which was recently identified on the N-terminus of PEX5. The NMR structure of Pex5(57-71) and the Pex14-N-terminal complex showed that the novel sequence LVAEF interacts in a similar α-helical orientation as the WXXXF/Y motif interacted with the Pex14-N-terminal domain i.e. mainly through electrostatic interactions. The difference lies in the interaction between the first residues of the LVAEF (Leu_{62}) and WAQEF (W_{118}) motifs with K_{56} of PEX14. When the WXXXF/Y motif was replaced with the novel motif, protein import into the peroxisomes was impaired. Therefore, it was suggested that cargo protein may initially bind to this novel site of Pex5 but later is processed in sequential manner [130] **(Figure 7)**.

Protein sequence analysis of *Ld*PEX14 revealed that only the N-terminal region of this protein shares maximum sequence homology when compared to other PEX14 [120]. Studies show that *Ld*PEX14 is a soluble peripheral membrane-associated protein, oriented facing the cytosolic face of the glycosomal membrane and hence, associates with PTS1-loaded *Ld*PEX5 to form a docking complex in the presence of other proteins. It was also confirmed that the N-terminal signature motif AX2FLX7SPX6FLKGKGL/V between residues 23-63 of *Ld*PEX14 is critically important for its binding to *Ld*PEX5. As demonstrated in *L. donovani*, there is decrease in *Ld*PEX5 affinity for PTS1 cargo as it interacts with *Ld*PEX14, suggesting the

release of cargo protein [131, 132, 133, 134, 135]. Cargo import is discussed further in the upcoming section.

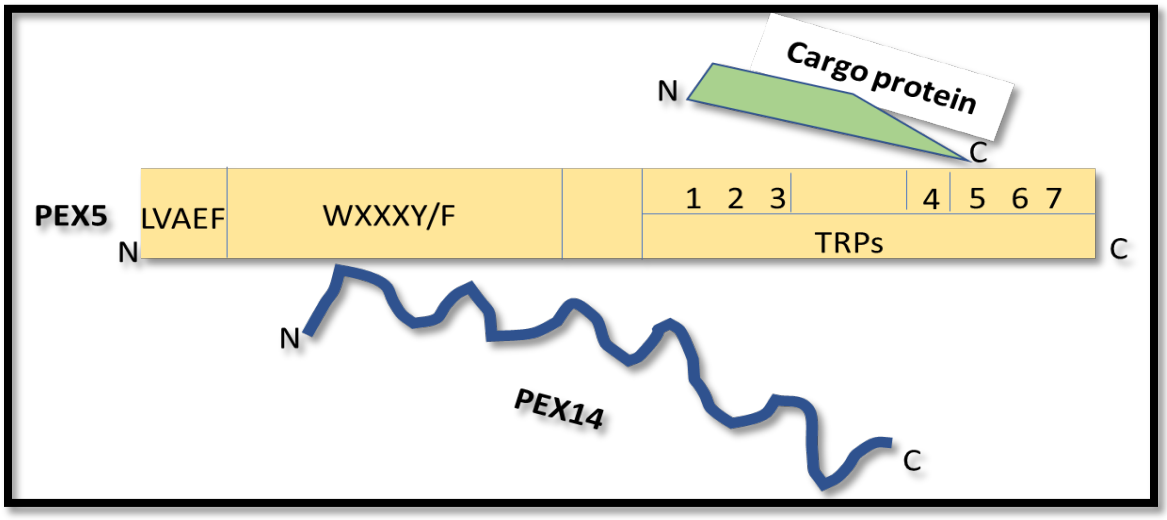

Figure 7: PEX5 representing the PTS1 and PEX14 binding sites. The cargo protein containing the C-terminal PTS1 signal binds to the seven tetratricopeptide repeat (TRPs) motif of PEX5. PEX14 N-terminal signature motif between the residues 23-63 bind to the novel N-terminus conserved LVAEF and pentapeptide WXXXF/Y of PEX5 protein [130, 135].

Peroxin 7 is the PTS2 receptor

PEX7 is a member of the WD-40 protein super family which is characterized by the presence of ~40-60 amino acids that contain conserved tryptophan-aspartate residues. Six WD40 repeats have been predicted in *L. donovani* to form a β-propeller structure. The β-propeller structure forms in a way to produce interlocking structure hence it provides increased stability to the protein. Each repeat in β-propeller structure consists of 4 β-stands contributing to propeller blades in a 3+1 manner i.e. each blade is built of 3 stands of one WD40 repeat plus one strand is from previous repeat [136]. Approximately 20% of all glycosomal proteins bearing PTS2 signal, are imported into the glycosome utilize the PEX7 receptor.

PEX7 has a proline rich region near its C-terminus which forms a helical structure that interacts with the SH3 class 11 domain found in the proteins Src and Grb2 [137]. The N-terminus of PEX7 was found to interact with PEX5, acting as a co-receptor in some trypanosomes, plants and mammals [138]. It was observed that the PTS2-PEX7 interaction was weak and unstable, but upon binding of PEX5 a stable tri-component complex was formed, implying that PEX5 acts as a locking device for PTS2 and PEX7 interaction facilitating the trafficking of the PTS2 protein to the glycosome/peroxisome. Similarly, it was identified that import-competent complexes in yeast requires several PEX7 co-receptors including PEX18, PEX20 and PEX21 [14, 137, 139, 140, 141]. *Ld*PEX14 and *Ld*PEX5 were also found to interact with *Leishmania major* PEX7 (LPEX7). The first 52 amino acids of the LPEX7 N-terminus is critical for binding to *Ld*PEX14 [142]. LPEX7 was found to interact with liposomes; the presence of a hydrophobic domain suggested that it could possibly play a role in transporting PTS2 proteins across the glycosomal membrane [143].

In higher eukaryotes, PEX7 interacts with PEX5-carrying the cargo proteins and enters the peroxisomes, where it exposes its N-terminus to the matrix, but this complex is resistant to proteases [144]. Therefore, it was not known whether the mammalian PEX7 is fully transported into the peroxisome along with its cargo protein or if the complex is retained at the docking/translocation machinery (DTM) and only the PTS2-protein is transported inside the peroxisome. More recently, an *in vitro* co-import and export assay was conducted with a pre-assembled PEX5-PEX7-PTS2 complex, showing that PEX5 is necessary for the cargo export competency of PEX7 (Figure 8). The conformational alteration in PEX5 destabilizes the trimeric complex PEX5-PEX7-PTS2 and is responsible for the release of PTS2 proteins into the peroxisomal matrix. Hence, cargo proteins are released into the peroxisomal matrix, while

the PEX7 is embedded at the DTM. This suggests that the release step does not require proteases for PTS2 cleavage [144].

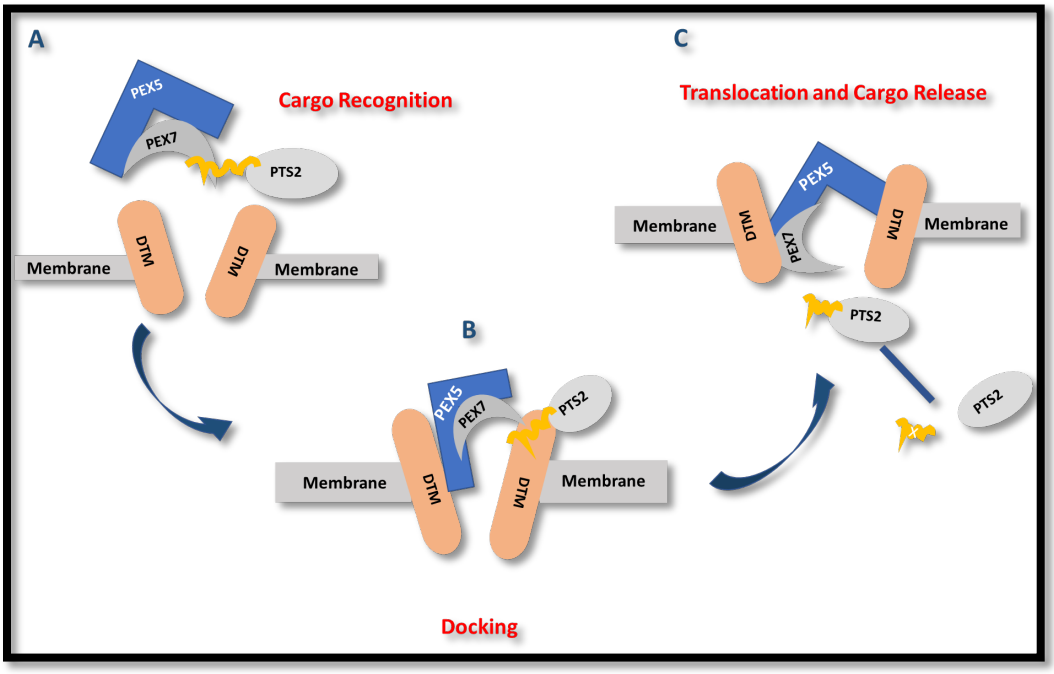

Figure 8: Import model of protein bearing PTS2 signal. A. Cargo protein bearing the PTS2 signal interacts with PEX5 and PEX7 cytosolic receptors forming a trimeric complex. **B.** A PEX5-PEX7-PTS2 tricomplex docks at the docking/translocation machinery (DTM) on the membrane of the peroxisome. **C.** Conformational changes occur when the tricomplex is inserted into the membrane, resulting in dissociation of PEX5 from the dyad of PEX7-PTS2. This causes the PTS2 to be released into the matrix and later is cleaved off from the cargo protein by the peroxisomal protease TYSND1 [144].

In another study conducted on a mammalian cell line, a Pex7p binding protein named P7BP2 was identified, which contains an N-terminal PTS2 region that binds to Pex7. P7BP2 was identified as a novel dynein type AAA+ protein. P7BP2 was transported into peroxisomes by binding to Pex5pL and Pex7p. This transport was confirmed by peroxisomal localization of P7BP2 using its cleavable N-terminal region. Peroxisomal localization and binding to Pex7p suggested that it is a new PTS2 protein [145]. Hence, suggesting that PTS2 proteins are

similarly released into the peroxisomal matrix as PTS1 proteins from DTM-embedded receptors.

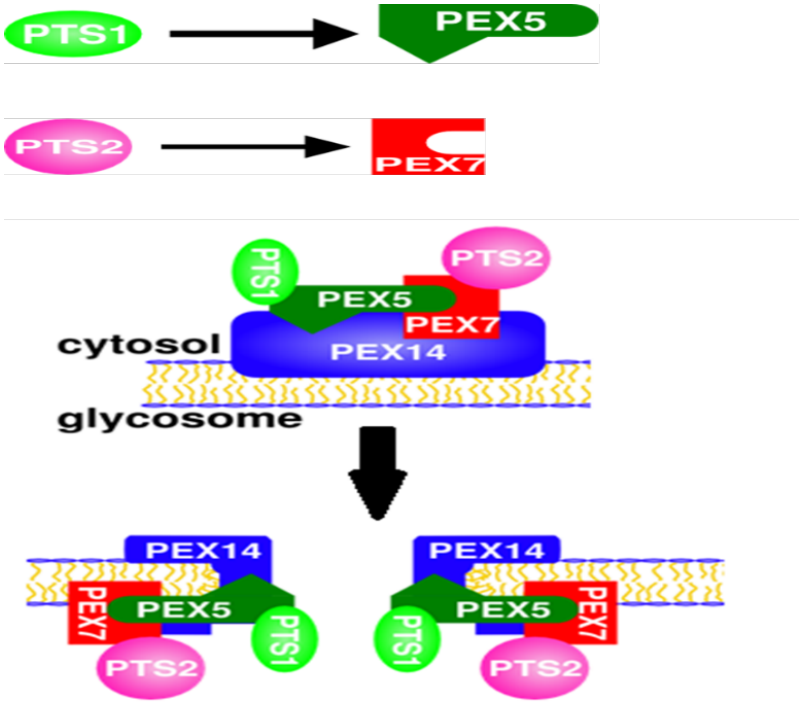

Figure 9: Working model of glycosomal protein import. *Leishmania* glycosomal proteins are synthesized in the cytosol and post-translationally imported into the glycosome via a C-terminal PTS1 or N-terminal PTS2 signal sequence. These are bound by the cytosolic receptor proteins *Ld*PEX5 and *Ld*PEX7, respectively, which then dock to the glycosomal membrane associated protein *Ld*PEX14. The cargo proteins are translocated to the lumen of glycosome followed by possible recycling receptors back to the cytosol (thesis Normand Cyr) [131, 142].

Furthermore, considering the mechanism of protein import, the whole process can be divided into four different steps which include; i) cargo recognition in the cytoplasm, ii) membrane docking of the cargo-receptor complex, iii) cargo release, and iv) recycling of the receptors back into the cytosol. Proteins are imported into peroxisomes or glycosomes as folded proteins or oligomeric complexes [14, 146, 147]. Various peroxin proteins have been identified in humans, yeast/fungi and trypanosomatids, that play important roles in protein import mechanism. A list of

identified peroxins in these organisms is presented in Table 3. The present model of *Leishmania* glycosomal protein import suggests that PTS1 proteins first bind to *Ld*PEX5 and this complex docks to *Ld*PEX14. Comparatively, PTS2 proteins bind to *Ld*PEX7, then this (PTS2-*Ld*PEX7) complex, with or without the help of *Ld*PEX5 (under investigation), docks to *Ld*PEX14. *Ld*PEX5 and *Ld*PEX7, after unloading their cargo protein are suggested to be recycled back to the cytosol (under investigation) (Figure 9).

In *Saccharomyces cerevisiae* the docking complex usually contain membrane proteins PEX13, PEX14 and PEX17. The presence of PEX13 in the docking complex suggests that it may play a role in the mechanism of protein transport [148, 149]. This was confirmed in a study which found that *T. brucei* TbPEX13 plays an important role in the formation of the docking complex for the PTS1 receptor protein TbPEX5, by interacting with TbPEX14 at the glycosomal membrane. A SH3 domain of TbPEX13 was shown to interact with the third pentapeptide motif of TbPEX5. The involvement of this motif in the interaction of TbPEX14 and TbPEX13 possibly suggests that TbPEX13 may play an important role in orienting TbPEX14 at the glycosomal membrane [18]. In *T. brucei* two isoforms of TbPEX13, TbPEX13.1 and TbPEX13.2, have been identified and confirmed to play role in biogenesis of glycosomes. Notably, TbPEX13.2 does not have PTS1-like motif and lacks the SH3 domain. However, the absence of TbPEX13.2 in cells disrupts the import of PTS2 proteins, suggesting it to be a supplementary factor for PTS2 import [150]. Furthermore, RING finger proteins (PEX2, PEX10, PEX12) are also found to participate in the import and export of glycosomal proteins [121, 151, 152]; their role is discussed in the following section.

Peroxin	Leishmania species	Trypanosoma Species	Yeast/fungi	Human
PEX1	✓	✓	✓	✓
PEX2	✓	✓	✓	✓
PEX3			✓	✓
PEX4			✓	
PEX5	✓	✓	✓	✓
PEX6	✓	✓	✓	✓
PEX7	✓	✓	✓	✓
PEX8			✓	
PEX9			✓	
PEX10	✓	✓	✓	✓
PEX11	✓	✓	✓	✓
PEX12	✓	✓	✓	✓
PEX13	✓	✓	✓	✓
PEX14	✓	✓	✓	✓
PEX15			✓	
PEX16	✓	✓	✓	✓
PEX17			✓	
PEX18			✓	
PEX19	✓	✓	✓	✓
PEX20			✓	
PEX21			✓	
PEX22			✓	
PEX23			✓	
PEX24			✓	
PEX25			✓	
PEX26			✓	✓
PEX27			✓	
PEX28			✓	
PEX29			✓	
PEX30			✓	
PEX31			✓	
PEX32			✓	

Table 3. Peroxin proteins across different organisms

Symbol (✓) represents the presence of homologous peroxin proteins present in various *Leishmania species*, *Trypanosoma species*, humans and yeast or fungi, Experimental confirmation of expression has not been confirmed in all the species [106, 119, 153, 154, 155].

Significance of the RING finger proteins (PEX2, PEX10 and PEX12)

The acronym RING stands for "Really Interesting New Gene". RING finger proteins share greater than fifty percent sequence identity among the trypanosomatids and roughly twenty percent when compared to humans [156]. Studies show that the RING finger proteins (RNF) play different roles such as signaling [157], DNA repair during transcription and apoptosis [158], and are also involved in cell cycle regulation [159]. In *T. brucei*, RING finger proteins (PEX10 and PEX12) were found to localize in the glycosome and behave as integral membrane proteins using a Green Fluorescent Protein (GFP) fusion of the coding regions in its procyclic form [121]. RING finger proteins play a significant role in the compartmentalization of important glycosomal functions and hence are vital for cell growth. They are believed to be integral membrane proteins embedded as a complex of three proteins *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 in the glycosomal membrane, playing crucial role in import and export machinery (Figure 10). It has been demonstrated that RNAi-dependent degradation of these proteins affects the growth of both bloodstream and procyclic forms of trypanosomes. However, the bloodstream form trypanosomes are more susceptible, as they rely on glycolysis for ATP production[156].

RING domains in these proteins behave as zinc binding regions and have characteristics similar to many E3 enzymes, which play a role in ubiquitin ligase activity. For instance, RING domain are suggested to have E3 ligase activity in trypanosomatids [17]. This property of RING finger protein present in various organelles of mammalian and yeast cells, have already been studied and are discussed here to better understand their importance and function.

RING finger known as E3s has been found to influence the balance between proliferation and apoptosis. As an example, the Mdm2 protein, which has a heterologous RING finger, ubiquitinates

p53 protein, as well as itself by binding p53 through its N terminal end and targets both to proteasomes. Similar to p53, a p73 protein is also bound by Mdm2 protein and hence ubiquitinated, but in this case p73 is not targeted for proteasomal degradation. This shows how RING finger protein Mdm2 can perform two different functions [160].

RING finger E3 proteins play a vital role in the secretory pathway. Hrd1p is an endoplasmic reticulum (ER) membrane RING finger protein found in yeast that regulates quality control in the ER via proteasomal degradation of abnormal proteins. Hrd1p also mediates the ubiquitination of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) a resident ER membrane protein, known as a rate controlling enzyme of the pathways that produce cholesterol and other isopreniopoids. Similarly, other RING finger E3 proteins facilitate the disposal of membrane proteins from the ER [161]. Another RING finger protein BRAC1 mediates its own *in vitro* ubiquitination. If the ubiquitination function is lost, it leads to the malignancy and dysregulated cell growth. N-terminal mutations of RING finger proteins are associated with familial breast and ovarian cancer [162, 163]. However, *in vivo* activity is yet to be demonstrated in this regard. The above-mentioned examples accentuate the critical role that RING finger proteins play in the functioning of different metabolic pathways.

PEX2, PEX10 and PEX12 comprise a predicted network of integral membrane proteins present in the glycosomal or peroxisomal membrane (Figure 10) [17]. In yeast, these proteins act downstream of the docking complex, forming a translocation subcomplex with other peroxisome proteins including PEX22, PEX4, PEX1 and PEX6, to translocate the PEX5 receptor (Figure 10) [18, 164]. As such, RING peroxins are suggested to be important for the matrix protein import/export cycle [148, 165]. Previous studies have demonstrated that knockdown of PEX2 in

yeast resulted in rapid degradation and functional disability of PEX12 and PEX10 (like mono- and polyubiquitination of PEX5) [16]. Hydrophobicity plots of predicted sequences putatively identified as PEX12 proteins from *T. cruzi* and *L. major,* predicted two transmembrane domains. In comparison, the hydrophobicity plot for TbPEX12 predicts one or possibly two weak transmembrane domains, while two transmembrane domains were predicted for all putative Tritryp PEX10 predicted proteins [121, 149, 166, 167]. This suggests that RING finger protein behaves as integral membrane protein in trypanosomatids.

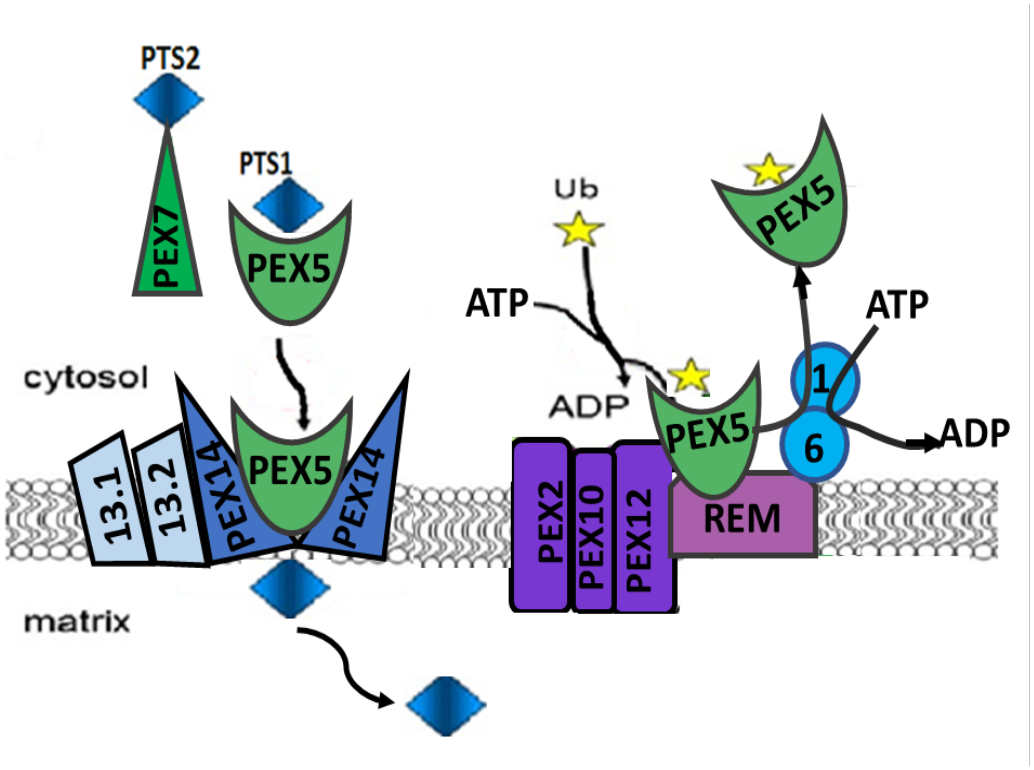

Figure 10: Glycosomal protein import/export machinery. Role of RING finger proteins in export of PEX5. PEX5 carrying the PTS1 protein docks at the docking complex composed of PEX14 and PEX13. Once the cargo is released into the glycosome/peroxisome, PEX5 is either signaled for degradation or recycling back to the cytosol. RING finger proteins PEX2, PEX10 and PEX12 act as a E3 ligase in the ubiquitination pathway, hence, the substrate (PEX5) is either monoubiquitinated and recycled back to the cytosol with the help of translocation subcomplex located downstream to docking complex or PEX5 is polyubiquitinated and signalled for degradation [18, 168].

The glycosomal and peroxisomal RING finger proteins contain a C-terminal RING or RING-like motif that is predicted to extend into the cytoplasm. It has been observed in yeast and mammals

that PEX2 and PEX10 contain a C_3HC_4 type cysteine rich RING domain, which binds two zinc ions. On the other hand, PEX12 in all species that have been studied, retained only five of these cysteine residues [17, 169]. For the binding of the first zinc ion: C1, C2, C5, and C6 cysteine residues are conserved, while only the C7 cysteine residue is found to be conserved for the binding of the second zinc ion (Figure 11). RING fingers are further classified into subcategories, namely RING-HC and RING-H2 depending on whether the fifth coordination site is occupied by a Cysteine (Cys) or Histidine (His), respectively. Structures of RING-HC fingers show two interleaved zinc binding sites which is in contrast to the tandem arrangement of zinc binding sites found in zinc fingers [162]. Irrespective of whether the RING domain is coordinated by either one or two zinc ions, they are essential for the interaction within themselves and with other proteins like PEX5 in case of glycosomal RING finger protein [17, 151, 164, 170, 171, 172, 173, 174, 175].

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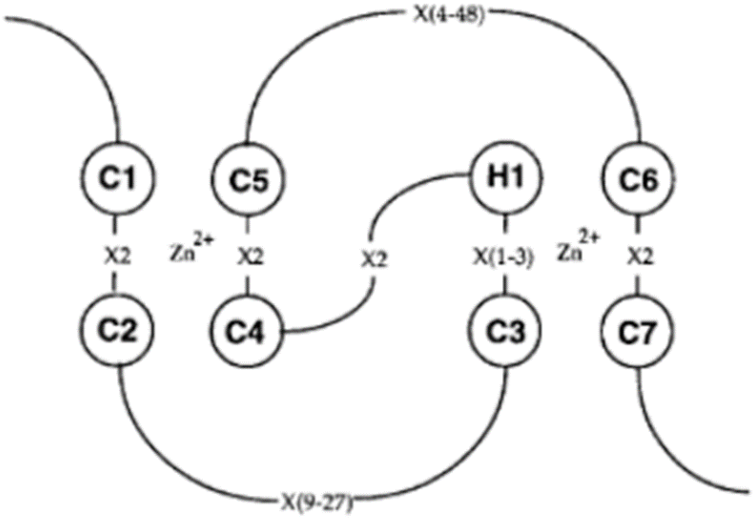

Figure 11: Zinc binding motifs of RING finger domain. Cysteine (C) and histidine (H) in circles represent conserved zinc-binding ligands. There are four ligands that tetrahedrally coordinates each of the zinc atom [167].

The presence of the RING domain in PEX10 and PEX2 (and possibly PEX12), suggest a possible role in ubiquitin ligase activity (as E3 ligases) [162]. Recent studies have notably

delineated the specific role(s) that these proteins have in ubiquitylation and hence recycling back PEX5. Many E1 (ubiquitin-activating), E2 (ubiquitin conjugating) and E3 (ubiquitin-ligating) genes are present in yeast, mammals and trypanosomatids (*T. brucei*), as seen by from GeneDB [121].

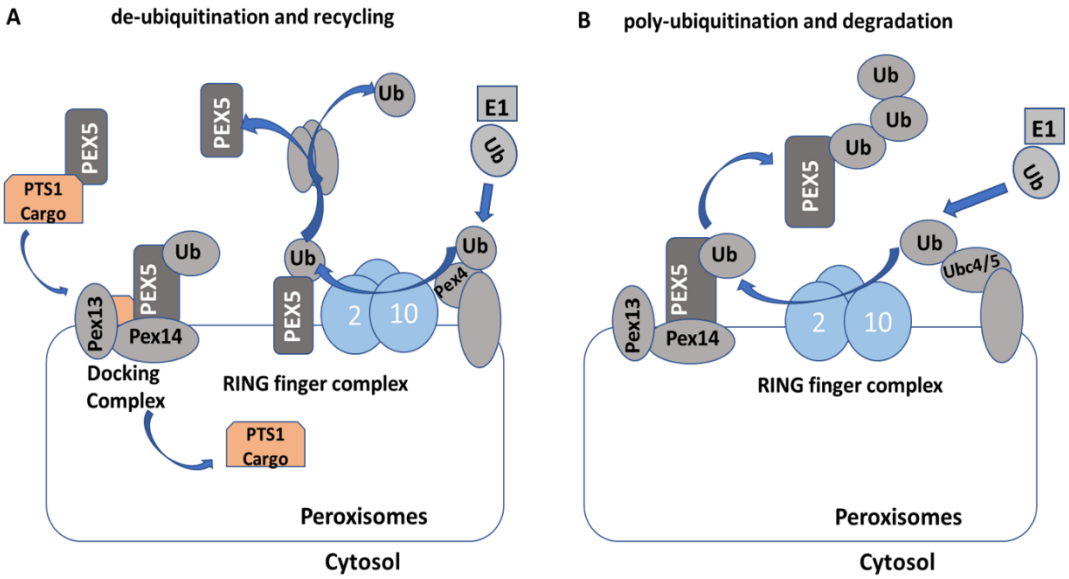

Figure 12: Signalling of PEX5 for recycling or degradation through ubiquitination or polyubiquitination. RING finger proteins have been shown to ubiquitinate Pex5 in yeast. **A.** Peroxisomal E2 enzyme Pex4 and Pex12 as protein-ub ligase (E3) mediated monoubiquitination of Pex5, signalling for the recycling of Pex5 from membrane to the cytosol, and making it available for subsequent import. **B.** Ub conjugating enzyme Ubc4 and Pex2 as protein-Ub ligase was found to mediate polyubiquitination of Pex5, hence signal for its degradation [16].

Protein ubiquitination is initiated with the formation of a thiol-ester linkage between the cysteine (Cys) at the active site of Ub activating enzyme (E1) and C terminus of Ub. Again, through a thiol-ester linkage, Ub is transferred to a Ub conjugating enzyme E2 (Ubc or Pex4) (Figure 12B). E3 proteins are primarily responsible for providing specificity to Ub conjugation. They interact with E2 and its substrate, thus facilitating the formation of isopeptide bonds between the C terminal end of Ub and lysines (Lys) either on a target protein or on the last Ub of a protein-bound multi-Ub chain (Figure 12B). Ubc4 and PEX4 (Ubc10) in yeast have been identified as the

ubiquitin conjugating enzymes (E2 enzymes) (Figure 12B) [16], while Pex2, Pex10 and Pex12 behave as E3 ligases that bind both E2 and substrate, facilitating the conjugation of Ub on to the substrate (Pex5) (Figure 12) [162, 176]. On the other hand, initial models for ubiquitination suggested that E3 proteins facilitate the direct transfer of Ub from E2 to substrate. Zinc ions present on the C-terminus of RING finger protein are necessary for this ubiquitination. It was observed that Pex4 or Ubc4-dependent ubiquitination was exhibited by RING finger proteins (Pex2, Pex10 and Pex12) and replacing Pex4 or Ubc4 with any other protein (like Ubc7) hampered the ubiquitination process. This showed that glycosomal RING finger protein (E3 ligases) exhibit selectivity for these two E2 enzymes [16]. To prove the E3 ligase activity of RING finger proteins (which were considered to be present on the peroxisomal membrane during experimentation), mono- and polyubiquitination of Pex5 was monitored in the presence and absence of TPEN (*N*,*N*,*N*',*N*' -tetrakis-(2-pyrididylmethyl)ethyl-enediamine), to determine specifically which class of E3s enzyme are involved [16]. TPEN was chosen as it chelates zinc ions and inhibits the activity of RING finger-containing protein (Ub E3 ligases), while not affecting other E3s enzyme such as U box or HECT (homologous to E6-assciated protein C terminus). *S. cerevisiae* UTL-7A cells were grown in the presence of NEM (*N*-ethylmaleinide) to prevent deubiquitination of Pex5. Pex5 was isolated via immunoprecipitation and it was observed that ubiquitinated Pex5 was present; when only NEM was used. In contrast, when TPEN was applied to the sample prior to the addition of NEM it prevented the formation of monoubiquitinated Pex5 [16]. As TPEN prevented Pex5 monoubiquitination, this could not be changed later, even after the addition of NEM. Similar experiments to determine the effect on polyubiquitination of Pex5 were also performed. MG132 was used for the inhibition of proteasomes which led to the accumulation of polyubiquitinated Pex5. When TPEN was added to the samples prior the addition of MG132, it prevented the

formation of polyubiquitinated Pex5 [16]. This suggested that RING finger protein (E3 ligases) are necessary for ubiquitination of Pex5, while their activity is inhibited when TPEN is used. Later experiment suggested that Pex12 specifically plays a role in Pex4-dependent monoubiquitination of Pex5 (Figure 12A) and Pex2 facilitates Ubc4-dependent polyubiquitination of Pex5 in yeast (Figure 12B) [16].

Database mining using *T. brucei* sequences helped to identifying the homologous proteins in *L. donovani*. The roles of *Leishmania* PEX2, PEX10 and PEX12 proteins are poorly understood and biochemical characterizations has been previously hampered due to their hydrophobicity, complex folding patterns and difficulties expressing *in vitro*. To express these proteins, we employed strategies involving; induction in presence of chemical chaperon 'benzyl alcohol' for high yield and proper protein folding. Expression for less time to reduce the overexpression and hence misfolding of proteins. Denaturants like urea were used, due to hydrophobic nature of these proteins, preventing them to be expressed in soluble fraction.

Materials and Methods

Expression of recombinant RING finger proteins in *E. coli*

Protein expression in *E. coli* - *L. donovani* RING finger proteins (*Ld*PEX10 and *Ld*PEX12) were cloned into the pET30b(+) vector (New England Biolabs). A codon optimized *Ld*PEX2 pET30b(+) construct was purchased from GenScript USA inc. (Figure 13C). *Ld*PEX10 and *Ld*PEX12 were amplified by PCR and cloned into the NdeI/HindIII sites of the pET30b(+) (Figure 13A, 13B). Open reading frames for all the *Ld*PEX constructs contained a hexahistidine tag at the C-terminus. *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 bearing plasmids were used to transform *Escherichia coli* C41(DE3) (New England Biolabs) and were grown with vigorous shaking (200 rpm) in Terrific Broth containing 50 µg/ml kanamycin until an optical density of 0.6 at 37 ºC. For protein induction, two different methods were attempted; (i) induction in presence of 10 mM of benzyl alcohol and Zn^{2+} ions; four different cultures of *Ld*PEX12 were supplemented with different concentration (µm) of ZnCl (0, 5, 10 and 100 µm) and the induction samples were collected at different time point for each (0, 40, 80 and 130 min), (ii) The cultures for each of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 were supplemented only with 10 mM of benzyl alcohol and incubated for 20 minutes at 16 º C. Induction of the RING finger proteins was carried out by the addition of 0.5 mM isopropyl β-D-1-thiogalactoside (IPTG) for 2 h at 37 ºC.

Isolation of inclusion body - Cells were harvested by centrifugation (6,000 rpm for 20 min at 4 ºC) and the pellet was washed once with PBS (phosphate buffer saline). The cell pellet was resuspended in 10 ml of solution A (PBS pH 7.0 containing 0.75 M sucrose, 0.2 mg/ml lysozyme)

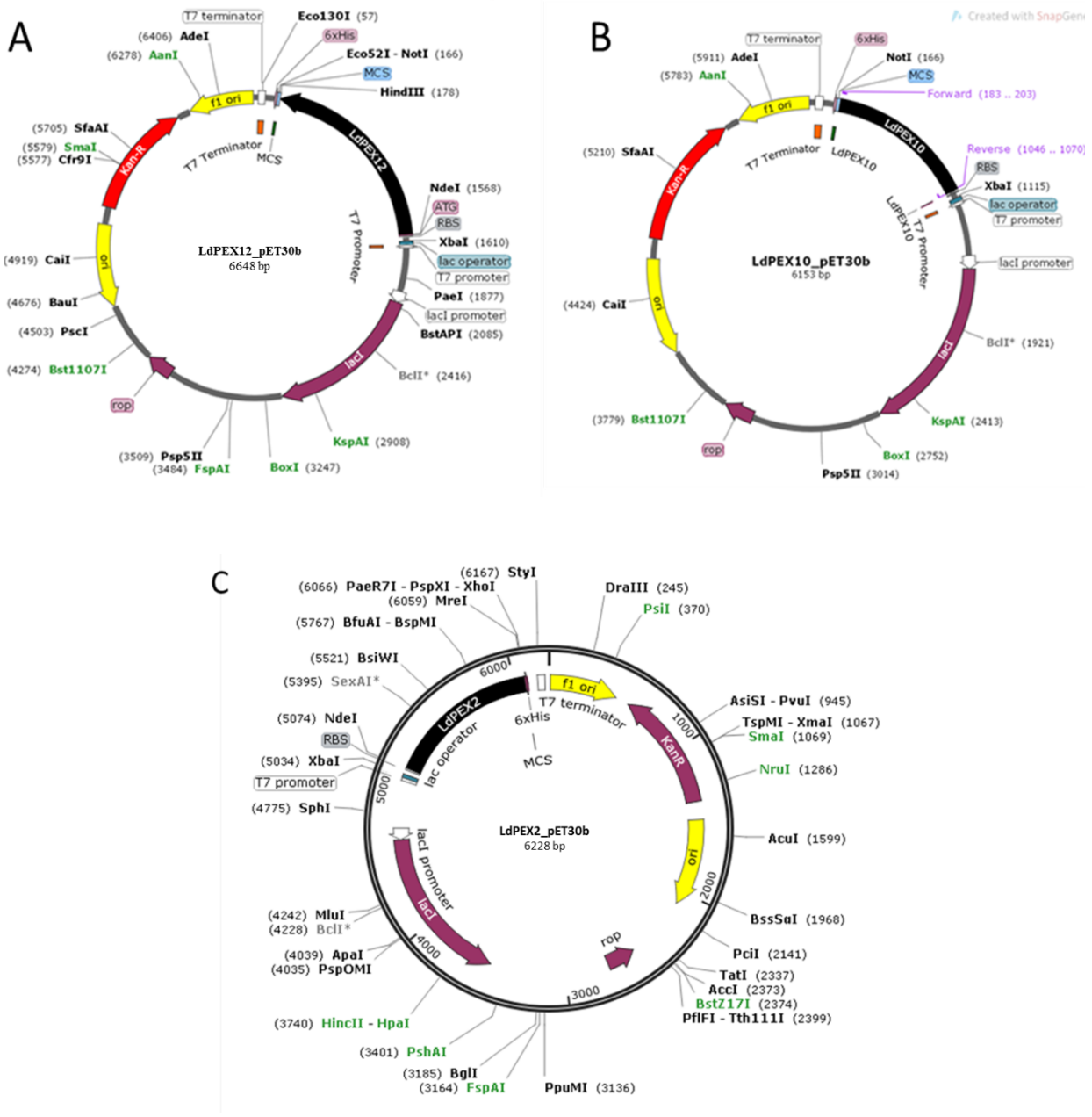

Figure 13. Maps of pET30b(+) plasmid encoding open reading frames (ORF) for *Leishmania* Peroxins
A map of pET30b(+) with the (**A.**) *Ld*PEX10 and (**B.**) *Ld*PEX12 ORFs with hexahistidine tag fused on the C-terminus, kanamycin resistance gene (Kan-R) is for drug selection in *E. coli.* (made with SnapGene 4.3.10 [https://www.snapgene.com/snapgene-viewer/]) **C.** Codon optimized *Ld*PEX2 gene cloned in pET30b has C-terminus 6X-HisTag and kanamycin gene (Kan-R) for drug selection in *E. coli.*

and incubated at room temperature for 10 min. Inclusion of lysozyme helps to break down bacterial cell wall, hence, improve purity of inclusion body. The digest was then supplemented with 3 mM

EDTA and incubated on ice for 5 min. Cells were then lysed and subjected to centrifugation (14,000 x*g* for 30 min at 4 ºC). The pellet was resuspended in 12 ml of solution B (PBS pH 7.0 containing 0.25 M sucrose, 1 mM EDTA, 0.1 % sodium azide) and then layered over a 40-53-67% (w/w) sucrose step gradient made in PBS (pH 7.0). The gradient was then subjected to ultracentrifugation at 108,000 x*g* for 90 min at 4 ºC. Six fractions were collected and the protein concentration was determined [177].

Protein purification - The induced *E. coli* cells were harvested by centrifugation (6,000 x*g* for 15 min) and resuspended in 25 ml of 0.2 % Triton X-100 (SigmaAldrich) in PBS pH 6.8 then lysed by sonication, the clarified supernatant was removed, and the pellet was resuspended overnight in 25 ml 8.0 M urea, 0.2 % Triton X-100 and 20 mM ammonium acetate in PBS (phosphate buffer saline) to extract protein from inclusion bodies.

The suspension was centrifuged (13,000 x*g* for 20 min) and supernatant was then affinity purified using Ni^{2+}-NTA resin (Qiagen), equilibrated with PBS pH 6.8, 8.0 M urea, 0.2 % Triton X-100. The column was washed sequentially with 150 ml of buffer A (PBS pH 6.8, 8.0 M urea, 0.2% Triton X-100, 10 mM β-mercaptoethanol), 100 ml of buffer B (PBS pH 6.8, 6.0 M urea, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 10 mM imidazole), 50 ml of buffer C (PBS pH 6.8, 4.0 M urea, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 20 mM imidazole) and 25 ml of buffer D (PBS pH 6.8, 4.0 M urea, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 40 mM imidazole) to remove any nonspecific protein binding. The *Leishmania* peroxin were eluted with 15 ml of elution buffer (PBS pH 6.8, 4.0 M urea, 0.2 % Triton X-100, 500 mM imidazole, 10 mM β-mercaptoethanol). Elution fractions containing peroxin proteins were concentrated using an Amicon Ultra filter 3 kDa (Millipore).

Protein concentration determination - Concentrated protein samples were dialyzed against PBS containing 4.0 M urea and aliquots of 200 µl were stored at -80 ºC. The protein sample final concentration was made to 8.0 M urea and stored at room temperature for 30 min to ensure complete unfolding. The protein sample (100 µl) was then aliquoted into 100 µl quartz cuvettes for measuring the absorbance at 280 nm using visible/UV spectrophotometer (Beckman-Coulter). Protein concentration of each sample was calculated using Beer's law $A_\lambda = \epsilon\ c\ l$ where A_λ is the absorbance at 280 nm, ϵ is the molar extinction coefficient of the protein, c is the concentration of the sample and l is the path length (1 cm) [178].

SDS-PAGE and Western blot analysis - Recombinant peroxin proteins were resolved on 8% SDS-PAGE and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) using a semi-dry transfer unit. 2 % skim milk powder in Tris-buffered saline [40 mM Tris-HCL pH 8.0, 150 mM NaCl, 0.1 % Tween-20] (TBST) was used to block the PVDF membrane for 1 h. Anti-His tag antibody (Abcam Rabbit monoclonal 6X His-tag) in 2 % milk TBST (dilution 1: 10,000) was used as a primary antibody to probe for recombinant proteins overnight at 4 ºC. PVDF membranes were washed three times with TBST for 15 min intervals, followed by incubation of the membrane with a secondary antibody (Bio-Rad, mouse anti-rabbit conjugated to horse radish peroxidase (HRP)) (dilution 1: 20,000) at 20 ^{0}C for 1 h. PVDF membranes were washed three times with TBST for 15 min intervals then developed using Western Lightning chemiluminescence reagent (Perkins-Elmer).

Transmembrane domain prediction - The transmembrane prediction program 'Phobius' (http://phobius.sbc.su.se/) was used to predict the number of transmembrane domains and the orientation (C- and N- terminus) of each of the Peroxin proteins (*Ld*PEX2, *Ld*PEX10 and *Ld*PEX12) in the glycosomal membrane. Amino acid sequences of *Ld*PEX2 (accession

XP_003861563.1), *Ld*PEX10 (accession XP_003861570.1) and *Ld*PEX12 (accession XP_003860299.1) were used as input on software tool Phobius. The output generated by the program included posterior probability of transmembrane, cytoplasmic and non-cytoplasmic regions of the protein.

Preparation of proteoliposomes - Liposomes entrapping fluorescein-isothiocyanate (FITC) conjugated-dextran and *Leishmania* peroxin proteins (*Ld*PEX2, *Ld*PEX10 and *Ld*PEX12) were prepared using a mixture of dioleoyl phosphatidylethanolamine (DOPE), dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylglycerol (DOPG), bovine liver phosphatidylinositol (PI) and cholesterol in a 55:25:15:2.5:2.5 molar ratio [20]. A thin layer of lipid film was made by dissolving lipids in chloroform in a glass tube by evaporating the solvent under a nitrogen steam. Residual chloroform was removed under vacuum for 16 h. Liposomes containing entrapped FITC conjugated-dextran were prepared by dissolving the lipid thin film (10 mg) in 1 ml PBS at pH 7.0 using 50 mM octylglucoside in dissolved FITC-dextran [20 k MWCO] (3 mg/ml).

Proteoliposomes were made by dissolving the lipid film in 1 ml PBS pH 7.0 to maintain a concentration of 10 mg/ml, in the presence of 50 mM octylglucoside, 10 mM β-mercaptoethanol, 0.2 % sodium azide and FITC-conjugated dextran (3 mg/ml), along with individual *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 (1.0 mg each). Above mentioned mixture was dialyzed [SnakeSkin Dialysis Tube] (3.5 K MWCO) for 4 h at 4 ºC against the PBS at pH 7.0 containing 10 mM β-mercaptoethanol, with continuous stirring. The dialysis buffer was changed after 4 h and dialysis continued for another 12 h to form proteoliposomes (small unilamellar vesicles) of sizes ~150 to ~250 nm which are comparable to the size of *Leishmanial* glycosomes [179].

Dye leakage experiment - Proteoliposomes were subjected to centrifugation at 100,000 x*g* at 4 ºC for 60 min in a Beckman-Coulter tabletop centrifuge using a TLA100.3 rotor to separate proteoliposomes from nonencapsulated FITC-dextran. The proteoliposome pellet was washed by resuspension in 2 ml of PBS pH 7.0 and centrifuged again to remove any residual FITC-dextran. Total FITC-dextran content was determined by addition Triton X-100 (0.2 % v/v) to the liposomes by measuring the dye release using an excitation and emission wavelength of 511 nm and 548 nm at 25 ºC (Figure 14). The following equation was used to calculate percentage dye leakage:

$$\% \text{ leakage} = F_P/F_{\text{Triton X-100}} \text{ x } 100.$$

Where, Fp is fluorescence intensity in presence of protein and $F_{\text{Triton X-100}}$ is the fluorescence intensity in presence of Triton X-100

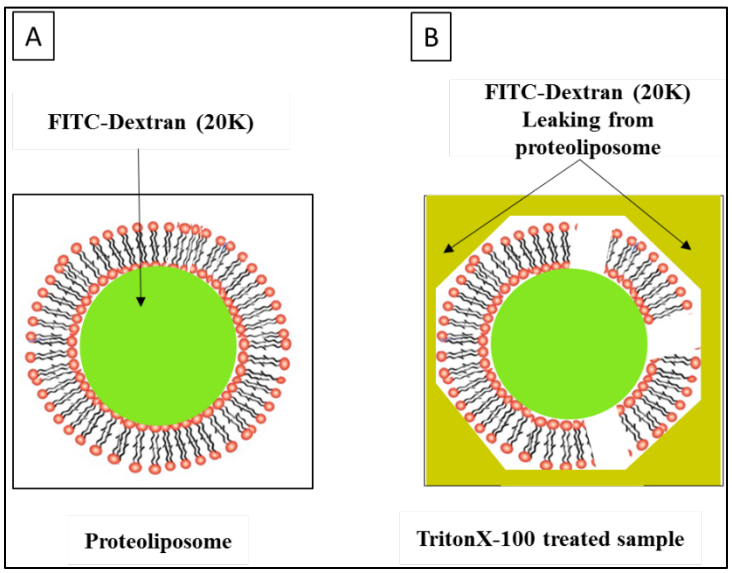

Figure 14. FITC-dextran leakage assay. A. FITC-Dextran (20 kDa) was entrapped in liposome using a detergent dialysis method [179]. **B.** Triton X-100 (0.2 % v/v) was used to dissolve the proteoliposome membrane to measure the fluorescence intensity using excitation and emission wavelength of 511 nm and 548 nm.

Sucrose density gradient - Sucrose gradient intervals (55 %, 40 % and 0 %) were prepared in PBS at pH 7.0 containing 10 mM β-mercaptoethanol. An 18-gauge syringe was used to make the

sucrose gradient in a 5.2 ml polypropylene open top ultracentrifuge tube using a SW 55 Ti Rotor (Beckman Coulter) in following manner: the last layer comprising of proteoliposomes was made to 55 % sucrose in a 1.6 ml volume, the second layer was 40 % sucrose in 2.6 ml volume and first layer was made of PBS pH 7.0 in 1 ml volume. This gradient was centrifuged at 74,000 x*g* for 16 h at 4 ºC (SW 55 Ti Rotor). Gradients were fractionated into 740 µl fractions from the top and proteins were precipitated using 12 % TCA and resolved using SDS-PAGE and finally analyzed by Western blot using anti-His tag antibody.

Alkaline carbonate extraction - Proteoliposomes isolated from the sucrose density gradient were sequentially treated with 500 mM NaCl, 100 mM sodium carbonate pH 11.5, and 4 M urea in 100 mM sodium carbonate pH 11.5 at 0 ºC for 30 min. Samples were separated into supernatant and pellet fractions by centrifugation (Beckman-Coulter table top ultracentrifugation) at 100,000 x*g* for 30 min at 4 ºC using a TLA 100.3 rotor. Supernatant and pellet samples collected after each treatment were precipitated by 12% TCA and visualized using Western blot analysis.

Expression of *Ld*PEX2 and *Ld*PEX12 in *Leishmania donovani*

***Leishmania donovani* culture -** *Leishmania donovani* promastigotes were cultured and grown to late logarithmic stage in M199 media (SigmaAldrich) supplemented with penicillin/streptomycin, and 10 % dialyzed heat-inactivated FBS at 26 ºC in 5 % CO_2 incubators.

Construction of *Ld*PEX2 and *Ld*PEX12 RING finger proteins in pRP plasmids - ORFs for *Ld*PEX2 and *Ld*PEX12 were cloned into the SFiI site of pRP high expression plasmid bearing a C-terminal blasticidin (BSD^R) drug resistance cassette and histag on C-terminus (*Ld*PEX2-pRP-H-BSD-His and *Ld*PEX12- pRP-H-BSD-His) (Figure 15) [180]. FLAG tagged-*Ld*PEX14 was cloned in the pRP high expression plasmid with a BSD drug resistance cassette (pRP-H-BSD-flag-

*Ld*PEX14) was under the *L. donovani* rRNA promoter (rRNA) and it was used as protein expression control.

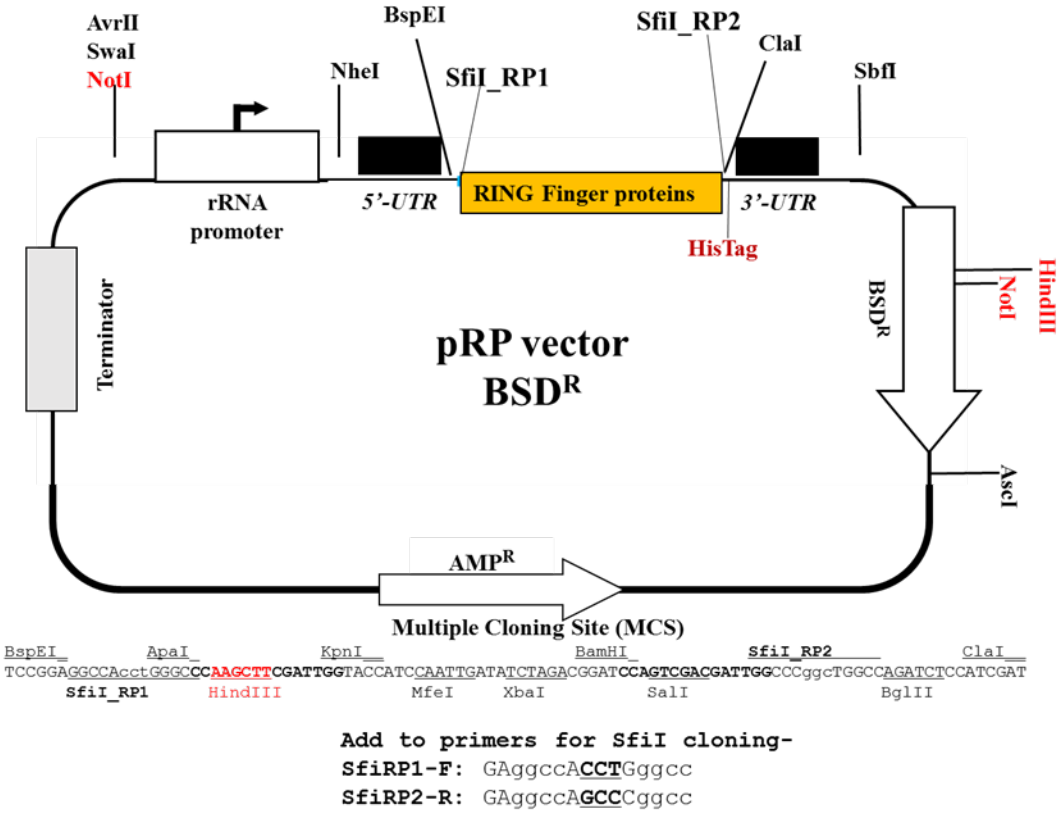

Figure 15. Map of pRP plasmid with Histag RING finger protein. Schematic of pRP high expression plasmid having multiple cloning site (*Ld*PEX2 and *Ld*PEX12 cloned in MCS) SfiI sites were used for cloning with Histag fused on the C-terminus. Ampicillin resistance gene (Amp^R) is for drug resistance in *E. coli* and Blasticidin resistance gene (Bsd^R) is for drug selection in eukaryotic cells [19].

Transfection and cloning - Plasmids containing *Ld*PEX2 and *Ld*PEX12 ORFs were linearized with the AvrII restriction endonuclease and used to transfect *L. donovani* 1S2D promastigotes, using Bio-Rad gene pulser II electroporator at following condition 25 µF, 1500 V (3.75 kV/cm) pausing 10s pulses [181]. Clones that contained integrated construct were selected in bulk culture using 30 µg/ml of blasticidin, 36 h after transfection. Clones were further isolated by performing a limiting dilution in 96 well plate as follows [182]. The first column was plated with 1 X 10^6 cells

per well and a step-wise 10-fold dilution was performed across the plate to acquire single clones from the last wells. Cells were grown for one to two weeks and then expanded using a 24 well plate before transferring clones to T-25 culture flasks.

Western blot analysis - Culture densities were determined using a hemocytometer and 1 X 10^7 cells were collected and lysed (100 µl) using 8 % SDS-PAGE to resolve on the polyacrylamide gel. Proteins were transferred to a PVDF membrane and the membrane was blocked with 2 % w/v skim milk powder in TBST (0.1 % Tween 20) and then incubated with diluted primary antibodies diluted anti-His (1: 10,000), anti-Flag (1: 10,000) overnight at 4 ºC. It was then probed with secondary antibody (Bio-Rad, mouse Anti-rabbit) conjugated to horse radish peroxidase (HRP) (1: 20,000) for 1 h at room temperature and visualized using Western Lightning chemiluminescence reagent (Perkins-Elmer).

Results

Expression and purification of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 in *E. coli*

To further understand the role of RING finger proteins in *Leishmania* glycosomal import/export mechanisms, it is essential to purify *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12, to allow for downstream *in vitro* assays.

Transmembrane domain prediction - Protein sequences for *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 were submitted to the Phobius web server (http://phobius.sbc.su.se/) which uses a Hidden Markov model (HMM) for *in silico* analysis. It models the query sequence in a series of interconnected manner, while simultaneously differentiating regions of signal peptide and transmembrane topology of a protein to avoid cross-reaction between these two regions, to give optimal output. Hence, it allows more constrained and homology-enriched prediction compared to conventional tools like TMHMM and SignalP, that overlaps transmembrane topology and signal peptide predictions [183]. *Ld*PEX2 protein sequence was predicted with non-cytoplasmic N-terminus and cytoplasmic C-terminus, along with three weak transmembrane domains spanning regions; 142-158, 178-204, and 247-262 (Figure 16A). The *Ld*PEX10 protein sequence was predicted with high posterior probability having three transmembrane domains spanning regions; 91-109, 129-147, and 167-186. Cytoplasmic region predicted ranged between (110-128 and 187-296) amino acids, non-cytoplasmic region predicted ranged between (148-166), whereas N-terminus was predicted to be non-cytoplasmic and C-terminus to be cytoplasmic (Figure 16B). *Ld*PEX12 protein sequence was predicted with two transmembrane domains, one with high posterior probability between (343-361) amino acid residues, compared to another weakly predicted transmembrane domain.

The N and C-terminus of *Ld*PEX12 was predicted with high posterior probability towards the cytoplasm (Figure 16C).

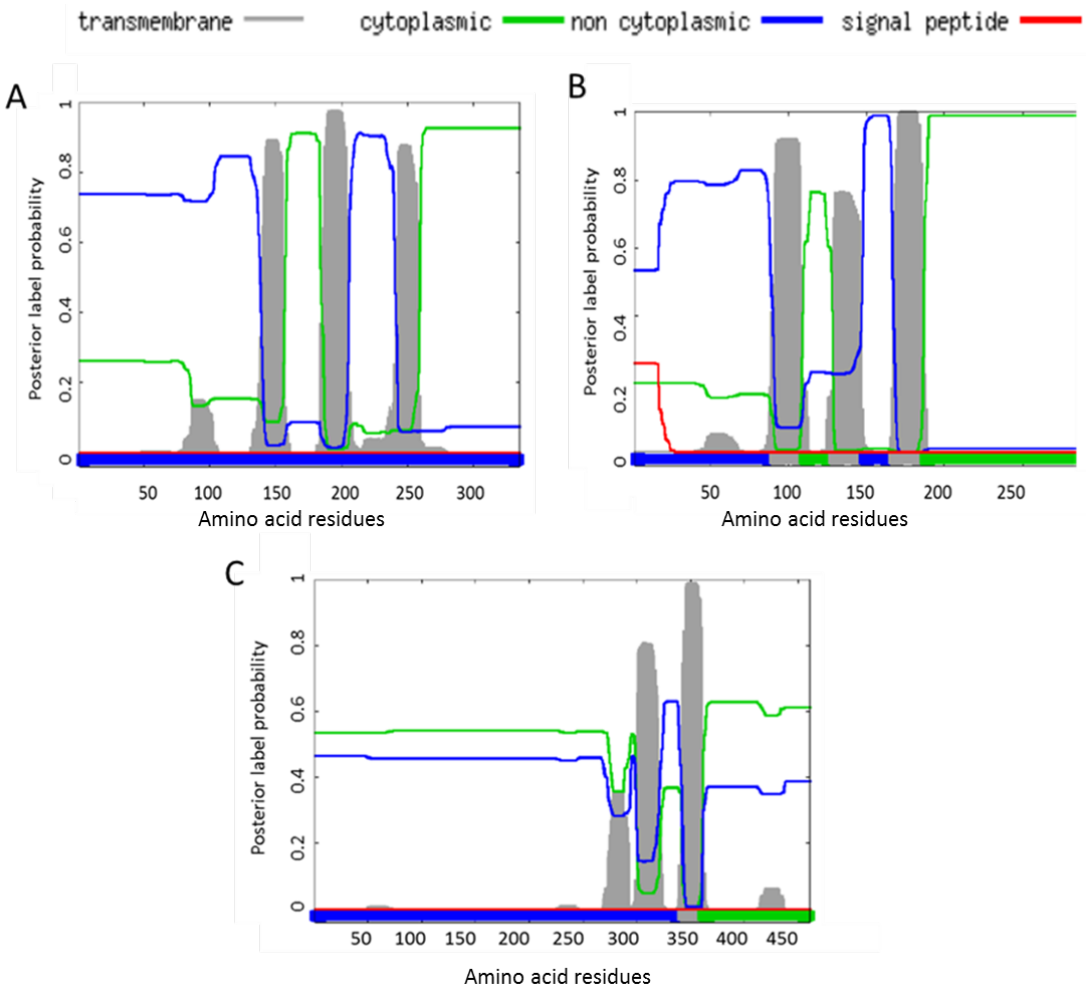

Figure 16. Transmembrane prediction for RING finger proteins.
The transmembrane prediction software Phobius was used to predict transmembrane domains of RING finger protein. The regions with high posterior probability were considered for each of the protein predictions. **A**. *Ld*PEX2 was predicted to have three weak transmembrane domains with cytosolic C-terminus and non-cytosolic N-terminus. **B**. *Ld*PEX10 was predicted to have three transmembrane domains with high posterior probability. The software predicts that the C-terminus is towards the cytosol and N-terminus is towards the lumen of the glycosome. In *T. brucei* the hydrophobicity plot using TMpred suggested at least two transmembrane domains for *Tb*PEX2 and *Tb*PEX10 [17]. **C**. *Ld*PEX12 was predicted to have two transmembrane domains, one with high posterior probability and one transmembrane domain with low probability, both of its N and C-terminus facing towards the cytosol. These results comply with the hydrophobicity plot prediction for *Tb*PEX12 that showed two weak transmembrane domains [17].

Isolation of inclusion bodies - Inclusion bodies containing recombinant *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 (RING finger) proteins were isolated from *E. coli* using sonication and centrifugation

as described in the methods section (Isolation of inclusion bodies). Cells were lysed and then fractionated to separate inclusion bodies from outer membranes, inner membranes and other cell debris on the basis of size and density. The density of inclusion bodies is comparable to protein (1.3-1.4 g/ml), ribosome (1.5 g/ml) and outer membrane vesicle is (1.22 g/ml) [177]. Cell lysates containing *Ld*PEX12 were subjected to sucrose density ultracentrifugation. Fractions were collected, and aliquots analyzed by Western blot analysis. *Ld*PEX12 was predicted to migrated as a 51 kDa protein based on the primary amino acid sequence. Figure 17A (lane 2) shows SDS-PAGE result of induced protein in the cell lysate upon induction by IPTG in *E. coli* cells and is used as a control (Western blot (Figure 17B)). According to the density of the inclusion body,

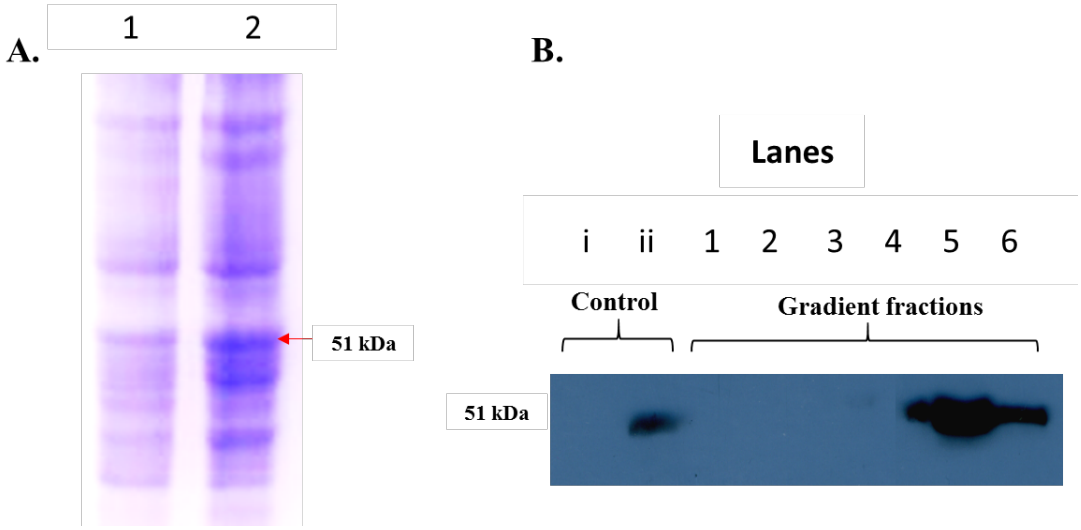

Figure 17. SDS-PAGE and Western blot of pure inclusion bodies for *Ld*PEX12.
A. SDS-PAGE visualized using Coomassie blue showing *Ld*PEX12 present in inclusion body, lane 1 shows uninduced cell lysate and lane 2 shows induced cell lysate. **B.** Inclusion bodies isolated after sucrose density gradient ultracentrifugation were analyzed using Western blot analysis (anti-HisTag antibody). (i) un-induced cell lysate (ii) induced cell lysate (c) Lane 1-6, represents fractions from top to bottom of sucrose density gradient. *Ld*PEX12 was detected in fifth fraction while partially the protein was present in sixth fraction

protein was expected to be present at the interface of the (53 % - 67 %) sucrose layer, which corresponds to the fifth fraction where antibody detection was observed (Figure 17B).

Expression and Purification of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 - Structure analysis of RING finger complexes of trypanosomatid orthologues show that they have at least one transmembrane domain. The hydrophobic nature of transmembrane domains cause aggregation of these protein [17]. For this reason, it is highly likely that partially denatured and misfolded *Ld*PEX12 accumulated in inclusion bodies. Our initial attempt to produce large amount of recombinant protein (*Ld*PEX2 and *Ld*PEX12) in *E. coli* were confounded with either negligible or very low level of expression (Figure 19E). These proteins contain a RING domain that coordinates the binding of Zn^{2+}, and the induction media was supplemented with different concentration of Zn^{2+} ions. It is known that Zn^{2+} plays a critical role in protein stabilization and even folding of protein subunits [184]. To our surprise the aliquots collected from different induction setup (with varying Zn^{2+} ion concentration), when analyzed by Western blot analysis showed negligible differences in induction compared to the control that was supplemented only with benzyl alcohol and no zinc (Figure 18). Therefore, further inductions were carried out only in presence of benzyl alcohol (a chemical chaperon) that alone, was effective in improving the expression (Figure 17A). Our next goal was to extract these proteins from inclusion bodies (Figure 17A) for purification using denaturing agents like urea in addition to a detergent. Figure 17A shows the induced protein, while, Figure 19A shows proteins bound in inclusion bodies, proteins present in urea pellet after extraction by 8.0 M urea, and some still bound to Ni^{2+}-NTA beads after elution. *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 were solubilized from inclusion bodies using 8.0 M Urea in PBS pH 6.8 (Triton X-100, 20 mM ammonium acetate) and bound to Ni^{2+}-NTA columns. A gradient of PBS wash buffer, starting from 8.0 M urea to 4.0 M urea was passed through the proteins bound to Ni^{2+}-

NTA column. Proteins were eluted in PBS pH 6.8, 4.0 M urea, 10 mM β-mercaptoethanol and 500 mM imidazole.

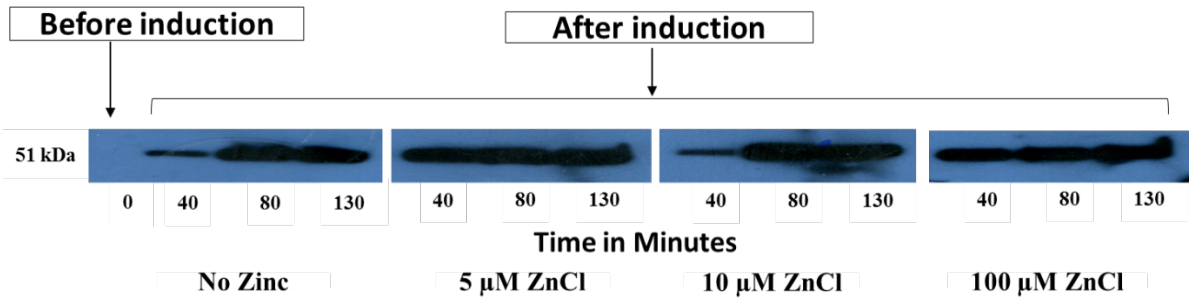

Figure 18. Western blot analysis of *Ld*PEX12 induction in presence of zinc.
*Ld*PEX12 was induced in presence of different concentration of zinc (5 µM, 10 µM and 100 µM), one induction was carried out without zinc, as a control. In comparison to non-induced cell lysate sample the induction with and without zinc showed negligible difference in the protein induced.

*Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteins were resolved on an 8% SDS-PAGE and transferred to a PVDF membrane for Western blot analysis (Figure 19B, 19C, 19D). When the proteins were resolved directly from cell lysate, a monomeric signal was observed for each of the *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteins at their expected molecular weight. In contrast, the purified protein resolved on SDS-PAGE gel showed oligomeric units along with the monomer band. We speculated that boiling (100 ºC) the protein in SDS-PAGE sampling buffer produced heat-induced micelle aggregates which shows higher number of oligomeric units when resolved on a gel. Therefore, we prepared the SDS-PAGE sample at a low temperature (70 ºC) to avoid the formation of micelleßs, but oligomeric bands were still detected. The oligomerization observed could be explained by aggregation of the hydrophobic regions of monomeric unit, by strong hydrophobic interactions.

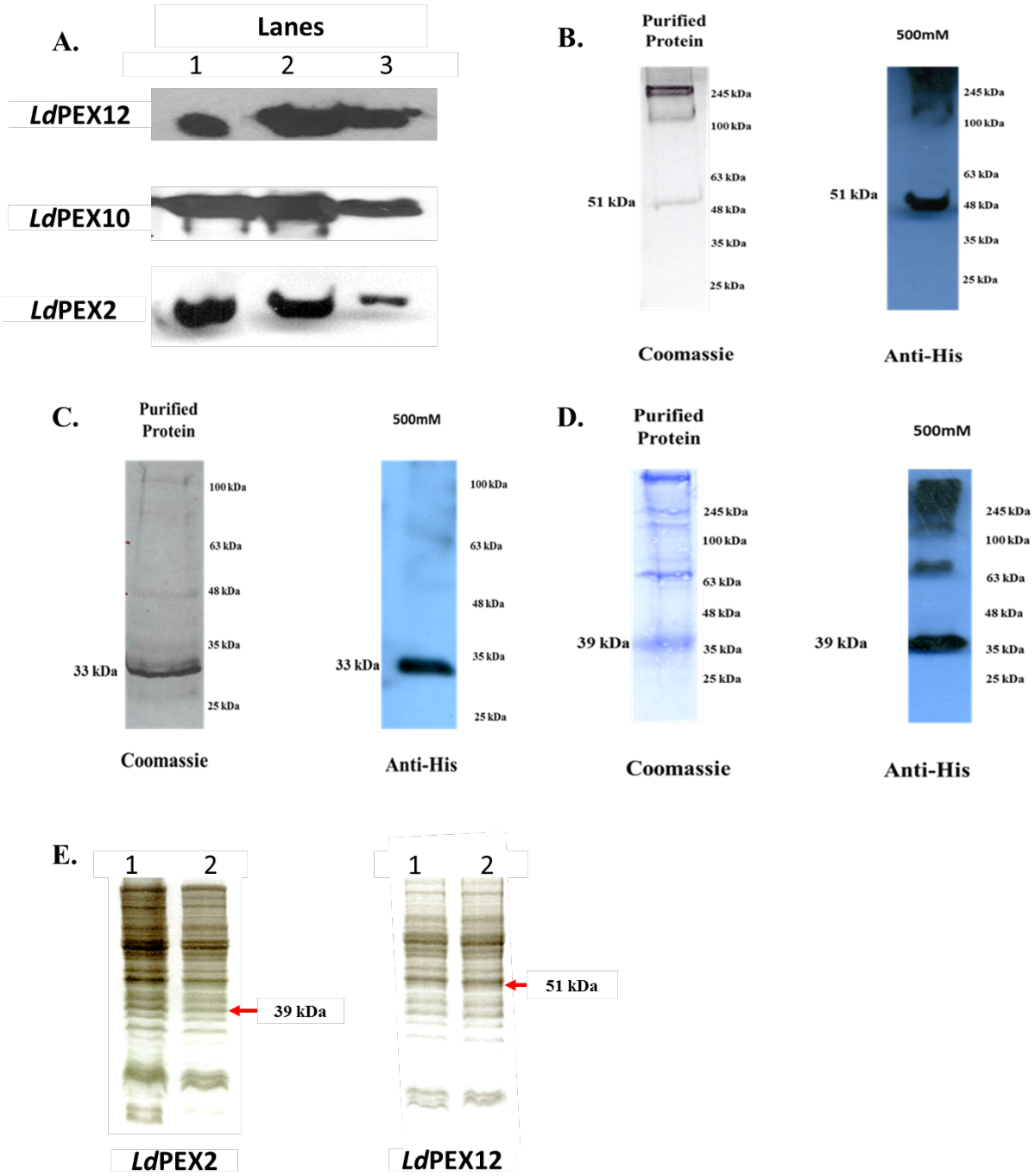

Figure 19. Selection of elution buffer and purification of RING finger protein by Ni^{2+}-NTA column.
A. Western blot of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 present in inclusion body (lane 1), urea pellet (lane 2) and bound to Ni^{2+}-NTA beads (lane 3). Elution buffer included PBS pH 6.8, 4 M urea, 10 mM β-mercaptoethanol, 500 mM imidazole. Samples were prepared using 500 ml bacterial culture, expressing *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteins in the pET30b(+) bacterial expression vector during purification. **B.** (Lane 1) *Ld*PEX12 was resolved on SDS-PAGE and stained with Coomassie blue for visualization and, purified *Ld*PEX12 migrated to 51kDA. (Lane 2) Western blot analysis was performed using 1: 10,000 rabbit anti-his antibody showing the protein migrating to 51 kDa. Interestingly both gels showed bands at higher molecular weight. **C.** (Lane 1) *Ld*PEX10 was resolved on SDS-PAGE and stained with Coomassie blue for visualization, purified *Ld*PEX10 migrated to 33kDa. (Lane 2) Western

blot analysis was performed using 1: 10,000 rabbit anti-his antibody showing the protein migrating to 33 kDa. Compared to *Ld*PEX12 very light oligomeric bands were observed for *Ld*PEX10. **D.** (Lane 1) *Ld*PEX2 was resolved on SDS-PAGE and stained with Coomassie blue for visualization and, purified *Ld*PEX2 migrated to 39 kDa. (Lane 2) Western analysis was performed using 1: 10,000 rabbit anti-his antibody showing the protein migrating to 39 kDa. Similarly, to *Ld*PEX12 and *Ld*PEX10, *Ld*PEX2 also showed intense, putative oligomeric bands. **E. (1)** induced and **(2)** uninduced cell lysate of *Ld*PEX2 and *Ld*PEX12 showing very negligible expression of proteins.

Characterization of RING finger proteins

***Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteoliposomes -** To confirm the formation of liposomes a FITC-dextran encapsulation experiment was performed. FITC-dextran (20 kDa) was incorporated into the liposomes, as it would not dialyze out due to its large size compared to pore diameter of the dialysis bag (3 kDa), secondly the encapsulation of dye inside the lipid will prove the formation of liposome. Encapsulation of FITC-dextran dye in liposomes was successful and was later detected by fluorescence spectrophotometer and UV visualization [179]. Proteoliposome entrapped FITC-dextran floated in a sucrose gradient and was visualized using a low intensity UV lamp (365 nm) in a dark room (Figure 20). Treatment of liposome collected from the top fraction of sucrose gradients with Triton X-100 showed 100 % dye leakage when compared to 64.5 % fluorescence observed in non-Triton X-100 treated sample as the encapsulated dye quenches its own fluorescence. In the PBS sample, negligible fluorescence was observed, which shows that the dextran associated FITC was not dialyzed out of the dialysis bag (Figure 21).

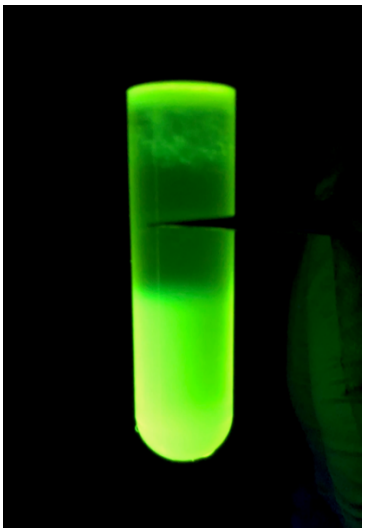

Figure 20. Sucrose density gradient for FITC-dextran encapsulated proteoliposomes
Nonencapsulated FITC-dextran at the bottom of centrifuge tube, 55% sucrose density. No FITC-dextran in the middle layer, 40% sucrose density. FITC-dextran at the top with cloudy appearance (possibly due to FITC-dextran encapsulated in proteoliposome) in PBS.

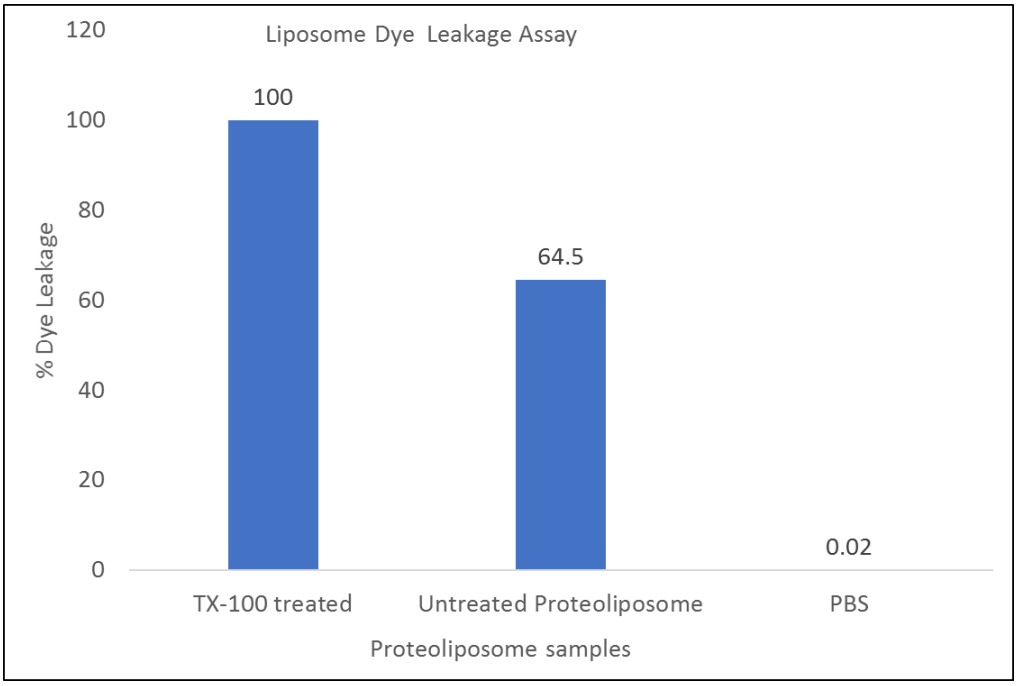

Figure 21. Triton X-100 induced dye leakage from the liposome entrapping FITC-dextran.
Bar graph for liposome samples collected from dialysis bag treated with Triton X-100 showed 100 % leakage. Non-Triton treated liposome samples showed only 64.5 % fluorescence, due to self-quenching of FITC. While the PBS samples that were collected from the buffer, showed negligible fluorescence, this ensures that FITC-dextran was not dialyzed out of dialysis bag.

*Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteins as predicted by Phobius, are integral membrane proteins. To study these proteins, detergent mediated reconstitution into proteoliposome was used [17]. To solubilize both the recombinant peroxin proteins and lipids, octylglucoside was used as a detergent because of its small micelle size and high critical micelle concentration (CMC) [185], which permits the removal of this detergent by dialysis. Individual proteoliposomes containing *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 were formed by dialyzing a mixture of detergent, phospholipids, respective proteins, FITC-dextran and β-mercaptoethanol against the PBS buffer. Proteoliposomes are formed spontaneously as detergent is removed during dialysis. The proteoliposomes and unbound protein mixture obtained from dialysis bag was subjected to sucrose density gradient. Fractions were later collected from sucrose density gradient and 200µl aliquots of each fraction were precipitated using 12% TCA and analyzed by Western blot. Figure 22 shows that the buoyant proteoliposomes were formed on top of the sucrose density gradient with signal detected in the first fraction by Western blot analysis for each of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12. Heavier free *Ld*PEX2 and *Ld*PEX10 that were not incorporated into liposomes were detected in the fifth and sixth fractions at the bottom of the tube (Figure 22A, 22B). In contrast, *Ld*PEX12 was observed only in the first fraction (Figure 22C). Individual proteins alone were used as a control for the sucrose density gradient, and all localized to the last fraction collected corresponding to the bottom of the tube (Figure 22A, 22B, 22C).

Alkaline carbonate extraction - To further characterize proteoliposomes and to confirm if *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 were incorporated into the lipid bilayer as integral membrane proteins, proteoliposomes underwent serial membrane extraction. Proteoliposomes containing *Ld*PEX10 were treated with 500 mM NaCl, which removes proteins bound to lipid bilayers via

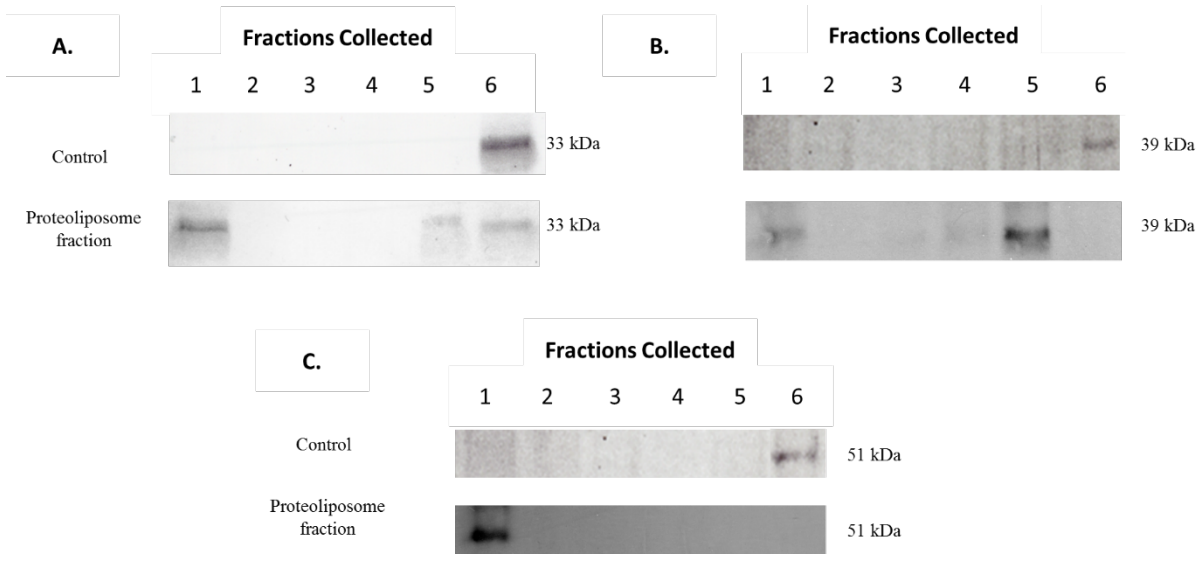

Figure 22. Floatation assay for individual *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteoliposomes and their control. **A**. Fractions 1-6 collected from floatation of sucrose gradient for *Ld*PEX10 proteoliposomes. Fraction 1 shows protein contained within proteoliposomes, while signals from fractions 5 and 6 indicate free protein. Control represents the protein that was floated in the absence of lipid, therefore it remained in the bottom most fraction. **B.** Fractions 1-6 collected from floatation of sucrose gradient for *Ld*PEX2 proteoliposomes. Fraction 1 shows protein from proteoliposomes, while signal from fractions 5 indicates free protein. *Ld*PEX2 alone was used as a control. **C.** Fractions 1-6 collected from floatation of sucrose gradient for *Ld*PEX12 proteoliposomes. Fraction 1 shows protein from proteoliposomes, while no free protein was detected. *Ld*PEX12 alone was used as a control.

electrostatic interaction. In the subsequent treatment, the membrane pellet was extracted with 100 mM alkaline carbonate which converts vesicles into planar monolayers a structural change that is predicted to release proteins entrapped in the lumen of the proteoliposome (these proteoliposome did not contain FITC-dextran) [186]. The membrane pellet was finally extracted with 4 M urea and alkaline carbonate to remove any protein bound to the membrane loosely via hydrophobic interactions or proteins that were not inserted into the phospholipid bilayer but formed aggregates [186]. Following treatment,100 µl aliquots of the supernatant and pellet were collected for Western blot analysis. For *Ld*PEX10 proteoliposomes, after each treatment the recombinant proteins remained associated with membrane-containing pellets and no protein was detected in the

supernatant fraction (Figure 23). This result indicates that *Ld*PEX10 is an integral membrane protein.

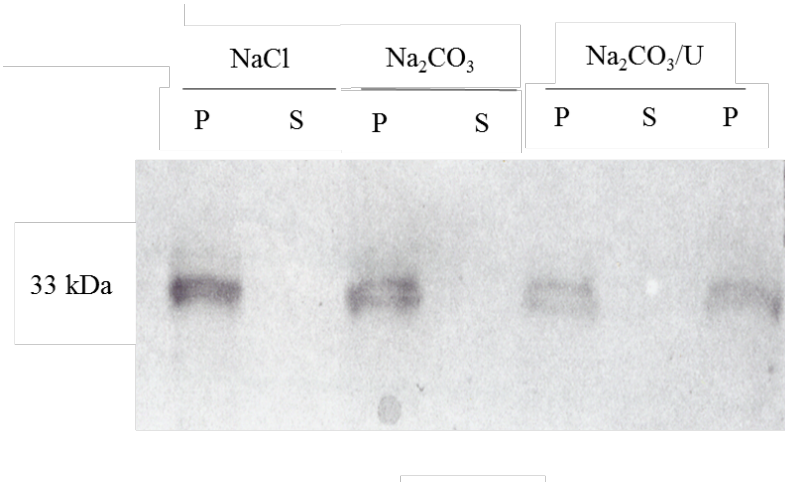

Figure 23. Alkaline carbonate extraction of *Ld*PEX10 proteoliposome.
Protein was retained in the pellet after the treatment with 500 mM NaCl and Na_2CO_3 of *Ld*PEX10 proteoliposome and nothing was detected in the supernatant fraction. Similar results were observed after the treatment of 4 M urea and Na_2CO_3. This result suggest that *Ld*PEX10 tends to remain integrated into the liposome.

RING finger protein expression and purification in *Leishmania donovani*

Expression of RING finger protein in *Leishmania donovani* - *Leishmania donovani* 1S2D cells were used to express RING finger protein to provide proper post translational modification, and to prevent misfolding of proteins thus preventing formation of inclusion bodies, which were the main problems encountered using *E. coli* cells. *Ld*PEX2 and *Ld*PEX12 were cloned into pRP for stable expression in the absence of drug selection as they can be integrated into the ribosomal RNA (rRNA) locus. Another advantage of using the pRP vector was that it provided a wide range of transgene expression options from very high to very low levels [180]. Proteins were cloned and expressed in the pRP-H-BSD plasmid, with FLAG tagged *Ld*PEX14 used as a control. *Leishmania* cells were grown to log phase before collecting the sample for Western blot analysis. No protein expression was observed in the clones of *Ld*PEX12 (clones $P12_5$, $P12_1$ and $P12_{10}$) and *Ld*PEX2

(clones $P2_3$, $P2_8$ and $P2_6$) (Figure 24A, 11B). Whereas, FLAG tagged *Ld*PEX14 clones $P14_4$, $P14_7$ and $P14_8$ were successfully expressed by *Leishmania* parasites as verified by western blot analysis using anti-flag antibody (Figure 24C)

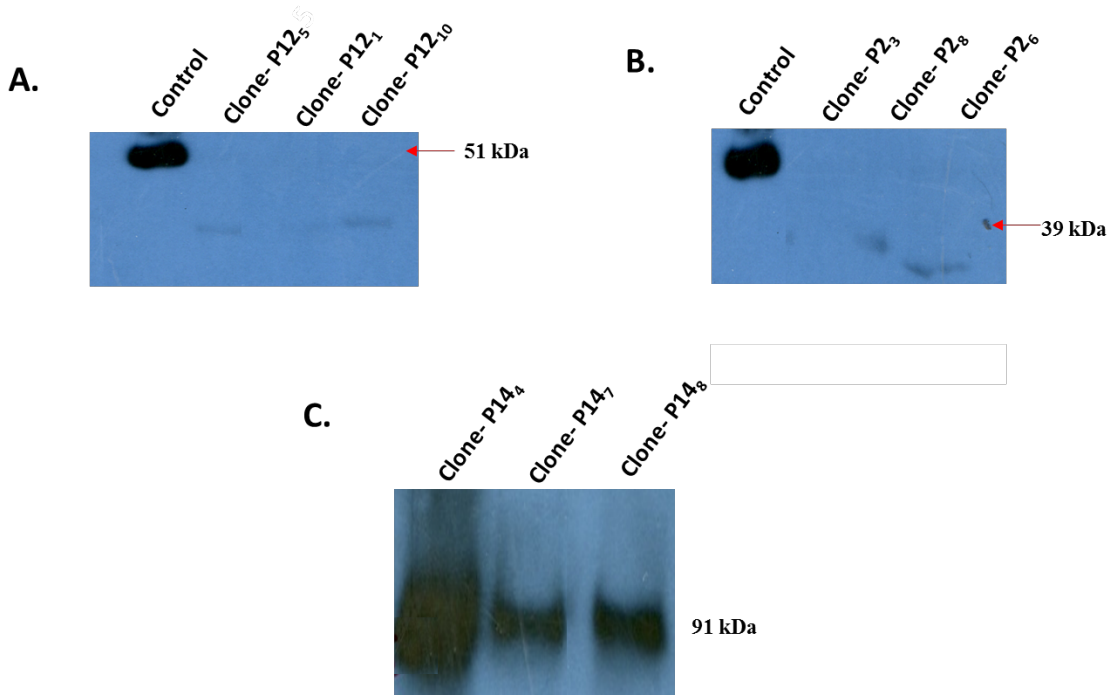

Figure 24. Expression of RING finger proteins in *Leishmania donovani.*
A. Western blot analysis for *Ld*PEX12 expressed in *Leishmania donovani*. Lane 1 shows control (Histag-*Ld*PEX12 expressed in *E. coli*) and lane 2-3 shows no expression of *Ld*PEX12 in the indicated clones of *L. donovani* transfected by pRP-H-BSD- LdPEX12. **B.** Western blot analysis for *Ld*PEX2 expressed in *Leishmania donovani*. Lane 1 shows control (Histag-*Ld*PEX12 expressed in *E. coli*) and lane 2-3 shows no expression of *Ld*PEX2 in the indicated clones of *L. donovani* transfected by pRP-H-BSD- LdPEX2. **C.** Western blot analysis for FLAG tagged *Ld*PEX14 expressed in *Leishmania donovani*, lane 1-3 shows expression of *Ld*PEX14 in the indicated clones of *L. donovani* transfected by pRP-H-BSD-flag-*Ld*PEX14.

In a study conducted on *T. brucei,* it was shown that PEX10 and PEX12 were targeted to glycosomes by observing their colocalization with glycosomal proteins [17]. Therefore, overexpression of *Ld*PEX2 and *Ld*PEX12 proved lethal to the parasite as they might hamper the functioning of the glycosome (Figure 25B, 25D). The hydrophobic nature of these proteins may play a role in this lethal phenotype as they tend to remain associated with the lipid bilayer and

blocking target of proteins to the glycosome or assembly of glycosome itself. Therefore, a regulatable system such as a fusion of ddFKBP (destabilizing domain of FKBP12 protein) could help prevent overexpression of proteins and prevent lethality due to overexpressed proteins [187]. In stark contrast, *Ld*PEX14 is an essential protein for glycosomes; unregulated expression of this protein was not lethal to the parasite (Figure 25C).

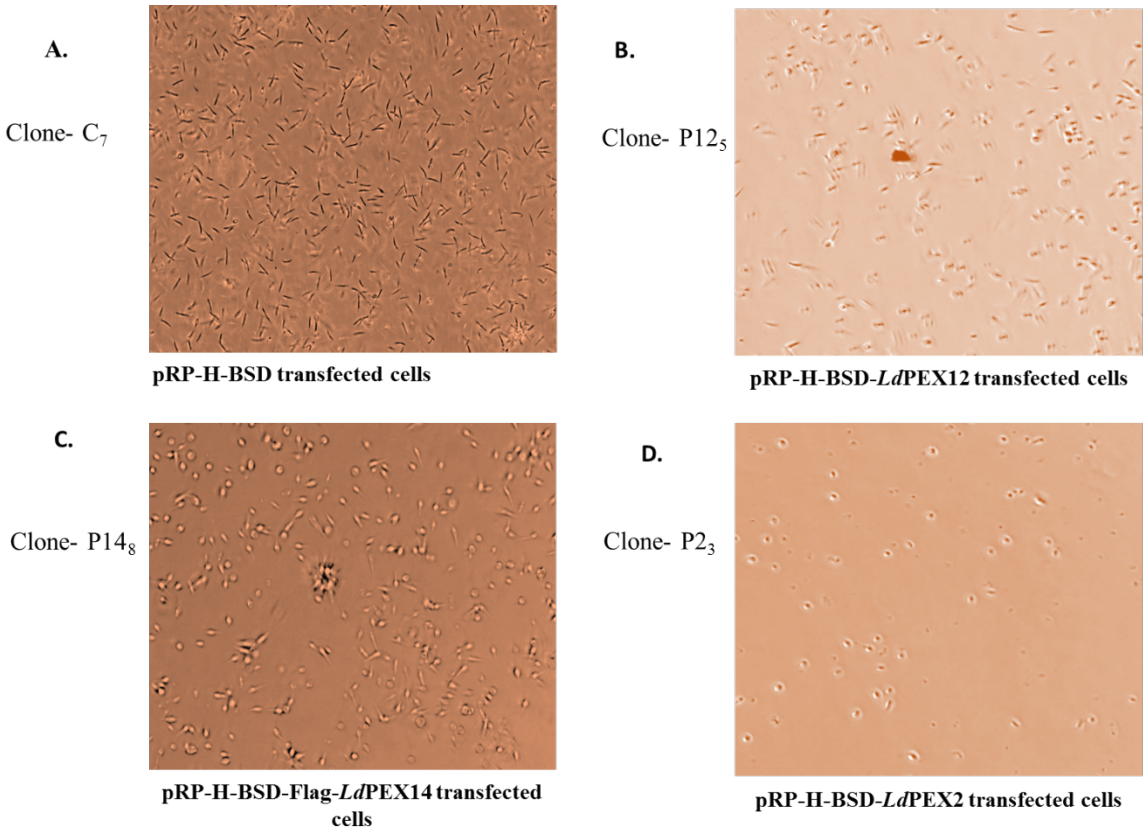

Figure 25. Cell growth and morphology of *Leishmania donovani* transfected with pRP-H-BSD, pRP-H-BSD-*Ld*PEX12, pRP-H-BSD-*Ld*PEX2 and pRP-H-BSD-flag-*Ld*PEX14.
The images were taken with an Evos XL core microscope on day 21 of cloning. **A.** Parasites transfected with pRP-H-BSD on day 21 of the transfection were in elongated ovoid cell shape. **B.** Parasites transfected with pRP-H-BSD-*Ld*PEX12 were either dead or round, indicating stress. **C.** Parasites transfected with pRP-H-BSD-flag-*Ld*PEX14 were in a maximal elongated ovoid shape, while few dead cells were observed. **D.** Parasite transfected with pRP-H-BSD-*Ld*PEX2 on day 21 of the transfection displayed a reduced total parasite count and were round, indicating stress.

Summary and Discussion

Compartmentalization of glycolysis inside glycosomes makes it an essential organelle in trypanosomatids [156, 188], as compared to peroxisomes of yeast, mammals and plants. Necessary glycosomal proteins are post translationally translocated into the glycosome, as it lacks genetic material and protein translation machinery of its own. The extreme difference between peroxisomes and glycosomes could be exploited for developing drugs against these parasites. A previous study on PEX10 and PEX12 RING finger proteins in *T. brucei* has shown that depletion of these genes by RNA interference resulted in partial mis-localization of various glycosomal matrix proteins to the cytosol [17]. Hence, it also affected the growth of both mammalian bloodstream form and procyclic insect form of trypanosomes [156]. PEX10 and PEX12 were also analyzed for topology and cross species targeting and it was found that these PEX RING finger proteins are crucial for protein import/export cycling [17].

Though these proteins have been found in trypanosomatids and other eukaryotes (yeast and mammals), the level of PEX sequence identity among the organism is low, indicating a divergent evolution of glycosomes from peroxisomes [11, 12, 17]. For example, PEX10 shares 53-65% sequence identity among trypanosomatids, but only 26-28% identity between themselves and humans, and 25-26% between themselves and *S. cerevisiae* [156]. While, for PEX12 trypanosomatid sequences share 40-57% sequence identity between themselves and comparatively low identity, 18-20% with humans and 13-17% with *S. cerevisiae* [156]. Similarly, *T. brucei* and *S. cerevisiae* PEX14 shares only 32% [188] and 35% [189] sequence identity with the human PEX14. However, considering the vast differences in the sequence identity between the mentioned organisms, the presence of specific motifs across species supports the possibility of some common putative functions. Cargo protein loaded-PEX5 among plant (*Arabidopsis*) [190], yeast (*S.*

cerevisiae) [189], human [189], and trypanosomatid (*T. brucei, L. donovani*)[20, 188] have been found to interact with PEX14 at the docking/translocation site and is suggested to induce pore formation in the peroxisomal/glycosomal membrane to transport cargo protein inside the organelle.

However, some PEX proteins have been found responsible for different biochemical activity across various organisms. In a knockout study of PEX19, most organisms exhibited peroxisomal loss, but in *Yarrowia* it led to the formation of peroxisome like organelles that replaced the role of peroxisomes [191]. This shows that the role of PEX protein may differ across species, hence the nuances of *Leishmania donovani* PEX proteins may differ from another organisms. Therefore, when studying these proteins, confirmation of their functional activity using *in vitro* experiments in *Leishmania donovani* is still a key endeavor.

As a step to characterize and map the interactions (putative motifs) of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 via *in vitro* assays, expression and purification of these recombinant proteins was performed. Expression of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 recombinant proteins in *E. coli* initially encountered problems of low or negligible expression, which was in accordance with another study in which all attempts to purify full length PEX2, PEX10 and PEX12 recombinant proteins of yeast were unsuccessful [16]. Our attempt to express *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 in *E. coli* were successful, and we were able to optimize the expression conditions by varying the media (Terrific broth and Luria-Bertani), concentration of IPTG, induction time and temperature. Improved protein production was observed when induction time was decreased from three to two hours in Terrific broth medium. Varying IPTG concentration and temperature did not produce any drastic effect on induction. Use of benzyl alcohol as an osmolyte was an important factor that

improved the induction level of these proteins, likely acting as a chemical chaperone that helps proper protein folding and prevent large aggregation of proteins [193].

Simultaneously our attempt to express these proteins in *Leishmania* were carried. But unavailability of specific antibodies along with low expression of endogenous proteins makes it a daunting task to purify these RING finger proteins from *Leishmania*. Therefore, we attempted to express and purify these proteins (using Histag) in *Leishmania donovani*, using the pRP vector which was modified to be able to integrate into the ribosomal RNA locus and provide stable protein expression. The expression of *Ld*PEX2 and *Ld*PEX12 proved to be lethal to the parasite. This could be due to unavailability of regulatable system for protein expression in the pRP-H-BSD plasmid used[187], or targeting of these proteins to glycosomes may have resulted in their interaction with other RING proteins and hence interfering with the metabolic functions of the glycosome or assembly of glycosome itself [17]. This maybe the reason that we observed "clumpy" phenotype (Figure 25) [192] when *Ld*PEX2 and *Ld*PEX12 were expressed compared to when *Ld*PEX14 was expressed in *Leishmania* cells.

Hydrophobicity plots of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 predicted at least one transmembrane domain in each of the proteins. In mammals, PEX12 contains two transmembrane domains [194], in yeast PEX12 has a single transmembrane domain [17], whereas in *T. brucei* PEX12 is predicted to have two transmembrane domains [156]. Similarly, the transmembrane domain predicted for *Ld*PEX12 by Phobius suggested two membrane spanning regions. This indicates that RING finger proteins, are to some extent, similar in topology among typanosomatid orthologues and other organisms. The interaction energy of hydrophobic regions in membrane spanning portions of these recombinant proteins are very high [195]. The hydrophobicity of *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 proteins posed challenges for us in purification and

characterization of these proteins. Urea and detergents were used to extract these proteins from inclusion bodies and keep them soluble, resulting in denaturation of proteins. These issues were resolved by synthesizing proteoliposomes. During the process of proteoliposome preparation, proteins were refolded while the detergent and urea were dialyzed out; this provided them energetically favorable environment embed into hydrophobic regions of lipids and maintain a folded state [196].

This system of embedding PEX proteins into a proteoliposome membrane allowed us to perform *in vitro* studies, that allowed us to better characterize these proteins and relate how these proteins might be behaving in glycosomes. Dye leakage assay demonstrated that proteoliposomes formed were closed nonporous vesicles entrapping FITC-dextran. Further, preliminary results showed that the incorporation of these proteins into proteoliposomes showed the tendency of these *Ld*PEX2, *Ld*PEX10 and *Ld*PEX12 to remain in hydrophobic environment. Results from alkaline carbonate extraction of *Ld*PEX10 suggests that it behaves as an integral membrane protein. This is in accordance with *in vivo* experiment conducted on TbPEX10 and TbPEX12 in *T. brucei*, which demonstrated that they behaved as an integral membrane protein [17]. In contrast to *T. brucei* [17], study of RING finger proteins by gene knockdown was not possible in *L. donovani* as it lacks an RNAi machinery [197]. Therefore, genome editing by homologous recombination or through CRISPR (clustered regularly interspaced short palindromic repeat)_Cas9 could be employed in future to study functions of RING finger proteins in *L. donovani* [198].

Further studies would be needed to determine the exact orientation of these proteins. The proteoliposome model of RING finger proteins will allow us to predict protein-protein interactions with other partner proteins, like PEX5 [151, 164]. RING finger proteins PEX2, PEX10 and PEX12 have been demonstrated to possess ubiquitin-protein isopeptide ligase activity (E3s ligases) [16,

173]. Once the orientation is elucidated, these RING finger proteins embedded in proteoliposomes could be used to validate the putative enzymatic activity of the zinc binding motif present in RING finger domain. It would also allow us to explore the PTSI and PTS2- dependent glycosomal matrix protein import/export cycle, as these E3s ligases are responsible for recycling of PTSI and PTS2 receptor proteins through ubiquitination signalling [16, 17, 172]. An alternative approach could be purification of recombinant *Leishmania* RING finger domains and deducing its ubiquitination enzyme activity by *in vitro* ubiquitination assays [16].

In a nutshell, this current study instills more insight into how these proteins would behave in a glycosomal membrane. We were able to shed some light on the hydrophobic nature of these integral membrane proteins. Purification of the *Leishmania* PEX2, PEX10 and PEX12 proteins, and a functional proteoliposome model will allow the exploration of the essential role that they play in glycosomal import cycle. Understanding the role of these RING proteins will not only decipher protein-protein interactions vital for organisms survival, but also reveal more about the glycosome biogenesis which may eventually offer the possibility of developing inhibitors for protein-protein interactions [199] that could precisely target the *Leishmania* parasites.

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