Insight into the physiology of human rhomboid protease RHBDL4: proteolytic mechanism and cellular role

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Master of Science

DEDICATION

To my Family and Friends

You are my inspiration!

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Thinking back to the my very first day in this lab, I believe I've grown a lot and so very thankful to all the knowledge and experiences that I will always treasure in my heart. I cannot thank enough each and every one who have in any way supported and contributed to the accomplishment of this thesis.

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This brings me last but not the least to my family and friends, you have always been there every step of the way – I can't thank you enough! *Mom, Dad,* thank you for all the sacrifices; *Sher,* thanks for enduring all the ranting and always boosting my confidence! *Pierre,* you are amazing! Thank you for being so patient, helping me in any way you can – you're my shining armor babe; My besties, *Chelle, Sassa, Maude, Ming, Kevin, Gizelle,* you're craziness kept me sane throughout! Thanks for all your love and support; My vitzes, *Bessie & Jannelle,* you have supported me since 2008 – thank you! My *kasamas* (you know who you are, particularly, *Renzo, Zhar, Jayson, Elesser, Sheryl, Marie, Malcolm, Kuya Joey*) thanks for always being there! STP!!

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ABSTRACT

A highly conserved class of serine intramembrane proteases defines rhomboid proteases. In a determining event over a decade ago, the bacterial rhomboid GlpG was named the first intramembrane protease to be crystallized. Hence, not only has advancement in rhomboid research mitigated the dispute surrounding the notion of a proteolytic reaction occurring in a plane of the membrane, but the implications of this clade of proteases in health and diseases have helped govern our conceptions of many important cellular processes. With regards to human rhomboid proteases, the path to progress remains however protracted with only few substrates identified and a myriad of *in vitro* evidence for their cellular roles requiring substantial physiological basis.

In our attempt to understand the relevance of endoplasmic reticulum (ER)-residing human rhomboid protease RHBDL4, the most structurally resembling mammalian homologue to GlpG, we identified RHBDL4 as a novel amyloid precursor protein (APP) cleaving enzyme yielding distinct APP N-terminal and C-terminal fragments. APP can undergo amyloidogenic processing by β- and γ-secretases producing Aβ peptides, which are one of the major hallmarks of Alzheimer's disease (AD). In the ER, RHBDL4 cleaves APP multiple times in the ectodomain thereby precluding amyloidogenic processing of APP. Furthermore, unpublished data from our lab revealed elevated RHBDL4 mRNA and protein expression in post-mortem brain tissue from AD dementia cases as compared to healthy controls. To gain deeper insight into the role of RHBDL4 in AD, we further characterized the proteolytic mechanism of the enzyme through analyses of APP mutants. Although ectodomain cleavages have been described before in intramembrane proteases' substrates, we were still intrigued as to how RHBDL4 exert its actions on both transmembrane sequences (TMS) and ectodomains. Our findings allude to the idea of positively-charged, destabilizing amino acid residues in the APP TMS conferring cleavage specificity. We are also the first to investigate the proteolytic mechanism of a human rhomboid in a cell-based expression

system with a physiological substrate. As such, expanding our knowledge on RHBDL4-mediated APP processing could be pivotal in deciphering the etiology of AD and might contribute to development of more efficacious RHBDL4 inhibitors.

Previous work has demonstrated RHBDL4 involvement in cancer although a detailed understanding of its key role is still unaddressed. Interestingly, it has been well-established that most cancer cells manifest excessive glucose uptake and glucose transporter 1 (GLUT1) expression while overexpression of RHBDL4 has been found in certain human cancer tissues. Upon cultivating mouse embryonic fibroblasts (MEFs) lacking Rhbdl4, we made a striking observation that the pH indicator, phenol red in the cell culture media did not turn to yellow, indicating a lesser degree of acidification as compared to wild type. Indeed, our data show diminished glucose consumption and glycolytic flux rendering less lactate secretion in Rhbdl4-deficient MEFs. We further demonstrate that dysregulation in cellular glucose metabolism is consequent to impaired Glut1 trafficking to cell surface. Not only are we the first to characterize Rhbdl4-deficient mice, but currently the sole to provide an *in vivo* evidence for a potential role of RHBDL4 as gatekeeper in the ER and modulator of cellular glucose metabolism.

Overall, in this thesis, we advanced our knowledge of the recently described, non-amyloidogenic RHBDL4-mediated APP processing pathway and elucidated a novel putative physiological function of RHBDL4 that could make headway towards further enlightenment of certain diseases, such as AD and cancer.

ABRÉGÉ

Une classe très conservée de protéases intramembranaires à sérine définit les protéases rhomboïdes. Il y a plus de 10 ans, GlpG, un rhomboïde bactérien, a été désigné comme la première protéase intramembranaire à être cristallisée. Par conséquent, non seulement les progrès de la recherche sur les rhomboïdes ont atténué la controverse entourant la notion de réaction protéolytique survenant dans un plan de la membrane, mais les implications de ce groupe de protéases dans la santé et la maladie ont contribué à régir nos conceptions de nombreux processus cellulaires importants. En ce qui concerne les protéases rhomboïdes humaines, la voie du progrès reste cependant longue, avec seulement quelques substrats identifiés et une myriade de preuves *in vitro* de leurs rôles cellulaires nécessitant une base physiologique substantielle.

Dans notre tentative de comprendre la pertinence de la protéase rhomboïde humaine RHBDL4 résidant dans le réticulum endoplasmique (RE), l'homologue mammifère le plus structurellement resemblant à la GlpG, nous avons récemment identifié RHBDL4 comme une nouvelle enzyme clivante de la protéine précurseur de l'amyloïde (APP) donnant des fragments. N- et C- terminaux distincts. L'APP peut subir un traitement amyloïdogène par les β- et les γ-sécrétases produisant les peptides bêta amyloïde (Aβ), qui font parties des principales caractéristiques de la maladie d'Alzheimer (MA). Dans le RE, RHBDL4 coupe l'APP à plusieurs reprises dans l'ectodomaine, empêchant ainsi le traitement amyloïdogène de l'APP. En outre, des données non publiées provenant de notre laboratoire ont révélé une expression élevée de l'ARNm et des protéines RHBDL4 dans le tissu cérébral post-mortem provenant de cas de démence MA par rapport aux témoins sains. Pour mieux comprendre le rôle de RHBDL4 dans la maladie d'Alzheimer, nous avons également caractérisé le mécanisme protéolytique de l'enzyme par le biais d'analyses de variantes d'APP synthétisés. Bien que des clivages d'ectodomaines aient déjà été décrits dans les substrats de protéases intramembranaires, nous étions toujours intrigués par la

manière dont RHBDL4 exerce ses actions sur les séquences transmembranaires (STM) et les ectodomaines. Nos résultats font allusion à l'idée de résidus d'acides aminés déstabilisants chargés positivement dans l'APP STM conférant une spécificité de clivage. Nous sommes également les premiers à étudier le mécanisme protéolytique d'un rhomboïde humain dans un système d'expression basé sur des cellules avec un substrat physiologique. En tant que tel, élargir nos connaissances sur le traitement de l'APP médiée par RHBDL4 pourrait être essentiel pour déchiffrer l'étiologie de la MA et pourrait contribuer à la mise au point d'inhibiteurs de la RHBDL4 plus efficaces.

Des travaux antérieurs ont démontré l'implication de RHBDL4 dans le cancer, bien qu'une compréhension détaillée de son rôle clé ne soit toujours pas abordée. Fait intéressant, il est bien établi que la plupart des cellules cancéreuses manifestent une absorption excessive de glucose et une expression du transporteur du glucose 1 (Glut1), tandis que la surexpression de RHBDL4 a été constatée dans certains tissus cancéreux humains. Après avoir cultivé des fibroblastes embryonnaires de souris (MEFs) dépourvus de Rhbdl4, nous avons fait une observation frappante: le rouge phénol, un indicateur de pH, dans le milieu de culture cellulaire ne jaunissait pas, indiquant un degré d'acidification moindre par rapport au type sauvage. En effet, nos données montrent une diminution de la consommation de glucose et un flux glycolytique entraînant une réduction de la sécrétion de lactate dans les MEFs déficients en Rhbdl4. Nous démontrons en outre que la dérégulation du métabolisme du glucose cellulaire est consécutive à une altération du trafic de Glut1 vers la surface cellulaire. Non seulement nous sommes les premiers à caractériser les souris déficientes en Rhbdl4, mais nous sommes actuellement les seuls à pouvoir fournir une preuve *in vivo* du rôle potentiel de RHBDL4 en tant que contrôleur dans le RE et modulateur du métabolisme du glucose cellulaire.

Globalement, dans cette thèse, nous avons approfondi nos connaissances sur la voie de traitement non amyloïdogène de l'APP par RHBDL4 récemment décrite et avons élucidé une nouvelle fonction physiologique putative de RHBDL4 qui pourrait permettre d'éclairer davantage certaines maladies, telles que la MA et le cancer.

AUTHOR CONTRIBUTIONS

This thesis is composed of two manuscripts wherein the former was published in *Biological Chemistry* in 2018 (permitted by the journal to reprint) while the latter will be submitted in an altered form for publication. I am the sole first author of Manuscript I and co-first author of Manuscript II. The authors contribution is listed in detail below:

Manuscript I: Alternative processing pathway of APP reveals two distinct cleavage modes for rhomboid protease RHBDL4

Sherilyn Junelle Recinto, Sandra Paschkowsky, Lisa Marie Munter

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I designed and performed the majority of the experiments, analyzed data, illustrated the figures and wrote the first draft of the manuscript. SP contributed to Figure 2D, helped in the analysis and illustration of figures and revised the manuscript. LMM led research, designed experiments and revised the manuscript. All authors approved the final version of the manuscript.

Manuscript II: Human rhomboid protease RHBDL4 as a modulator of cellular glucose metabolism

Sherilyn Junelle Recinto*, Sandra Paschkowsky*, Lisa Marie Munter
*equal contribution as co-first authors

I performed the experiments shown in figures 2 to 7, analyzed data, illustrated the figures and wrote the first draft of the manuscript. SP made the initial observation regarding the color of the cell culture media, initiated the project (performed experiments in Figure 1A-D, contributed to Figure 1F, 2A and 2B, and analyzed data), revised the manuscript. LMM led research, designed experiments and revised the manuscript. All authors approved the final version of the manuscript.

Moreover, I contributed to a manuscript published in *Journal of Biological Chemistry* during my time in the lab (attached as appendix A).

Appendix A: Membrane cholesterol as a regulator of human rhomboid protease RHBDL4

Sandra Paschkowsky, Sherilyn Junelle Recinto, Jason C. Young, Ana-Nicoleta Bondar, Lisa Marie Munter

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I cloned all APP and RHBDL4 cholesterol-binding deficient mutants, contributed to Figure 4F and 4I, and revised the manuscript. SP designed and performed all experiments, analyzed the data, illustrated the figures and wrote the manuscript. JCY provided the initial homology model and contributed to the discussion; ANB provided the RHBDL4 homology model; LMM led research, designed experiments and contributed to the manuscript. All authors approved the final version of this manuscript.

SUMMARY OF ORIGINAL CONTRIBUTIONS

i. I designed and cloned all the APP mutants (Manuscript I).

ii. I showed that the substrate transmembrane sequence confers cleavage specificity of RHBDL4 (Manuscript I).

iii. I showed that destabilizing amino acid residue in the APP transmembrane sequence (TMS) redirect an ectodomain shedding to within or near the TMS by RHBDL4 (Manuscript I).

iv. Upon co-expression of APP mutants containing positively-charged amino acid in TMS with active RHBDL4, I performed inhibitor treatments to show that the RHBDL4-mediated APP C-terminal fragments previously identified were not generated at all (Manuscript I).

v. Through western blot analyses, I showed that APP ectodomain backfolding to the membrane is an unlikely mechanism for how RHBDL4 cleaves APP within its linker region (Manuscript I).

vi. Co-expression of active RHBDL4 with C-terminal domain truncated APP revealed that the cytoplasmic tail of APP is not necessary for RHBDL4-mediated APP processing (Manuscript I).

vii. Using automated, electrochemical NovaBio Analyzer, I measured glucose and lactate levels in supernatant from cultivated MEFs and found that Rhbdl4-deficient MEFs consume less glucose and secrete less lactate during normoxic and hypoxia-mimicking conditions (Manuscript II).

viii. Analyses of Glut1 protein levels demonstrated less Glut1 in Rhbdl4-deficient mouse embryonic fibroblasts (MEFs) and cerebral endothelial cells as compared to wild type during normoxic and hypoxia-mimicking conditions (Manuscript II).

ix. I performed qPCR to measure *Glut1* mRNA expression during normoxic and hypoxia-mimicking conditions and concluded that Rhbdl4-deficient MEFs have impaired posttranscriptional regulation of Glut1 (Manuscript II).

x. I showed that Rhbdl4-deficient MEFs are dependent on glutamine as source of energy and carbon intermediates (Manuscript II).

xi. I provided the first characterization of Rhbdl4-deficient mice: serum glucose, lipids, total protein concentrations; total body weights; Rhbdl4 tissue distribution (Manuscript II).

xii. By performing glucose tolerance tests on 2-4 months old male and female mice, I concluded that there is no systemic glucose metabolic deficit upon loss of Rhbdl4 (Manuscript II).

xiii. Using cell surface biotinylation and immunocytochemistry, I showed that Rhbdl4 abrogation led to deregulated Glut1 trafficking to the cell surface (Manuscript II).

I. GENERAL INTRODUCTION

1. Alzheimer's disease

It is hardly surprising that the World Alzheimer report 2015 estimated someone in the world will develop dementia every 3 seconds. With the aging population and age as the biggest risk factor, the estimated 46.8 million people worldwide living with dementia in 2015 is expected to double every 20 years (http://www.worldalzreport2015.org/). Alzheimer's disease (AD) remains one of the main challenges of the 21st century with over 100 clinical trials that failed. The lack of curative and preventative treatment strategies further calls to the imminent need to understand the disease etiology (Y. J. Wang, 2014).

1.1 Disease pathology

Initially discovered by Alois Alzheimer, presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFT) are the pathological hallmarks of the disease (Alzheimer, 1907; Braak & Braak, 1991). They are believed to be the initial drivers of synaptic loss and neurodegeneration causing cognitive deficit and memory loss (DeKosky & Scheff, 1990). The plaques consist of aggregation-prone, oligomeric amyloid-β (Aβ) peptides (Masters, Simms, et al., 1985) while accumulation of hyperphosphorylated microtubules-associated tau proteins beget NFT (Grundke-Iqbal, Iqbal, Quinlan, et al., 1986; Grundke-Iqbal, Iqbal, Tung, et al., 1986; Masters, Multhaup, et al., 1985). Immunolabelling for β-amyloid revealed that distinct morphological attributes of amyloid plaques occur at varying stages of the disease progression. Despite evidence for toxicity induced by a pathological shift to fibrillation, the role of β-amyloid plaques as neurotoxic remains a controversy. A new wave of Alzheimer's research initiated the notion of fibril-free soluble oligomeric Aβ themselves as instigator of AD neuropathology. Insoluble amyloid plaque burden is rather viewed by others as a protective mechanism to sequester these pathogenic culprits (DaRocha-Souto et al., 2011; Viola & Klein, 2015; Walsh et al., 2002).

Conversely to plaques, NFTs are found intracellularly, primarily composed of paired helical filaments recognized by Thioflavin-S dye. Aberrant misfolding and hyperphosphorylation of microtubule-associated tau proteins engenders dissociation of the tau protein from microtubules leading to destabilization of microtubules and formation of tau aggregates, and eventually NFTs (Grundke-Iqbal, Iqbal, Tung, et al., 1986; Iqbal, Gong, & Liu, 2013; Kadavath et al., 2015). There are three types of NFT, namely diffused, fibrillar intraneuronal or extraneuronal "ghost", distinguished by nuclear and cytoplasmic morphology of the neurons bearing NFTs (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). The occurrence of NFT is not limited to AD unlike amyloid plaques; frontal temporal dementia (FTD), chronic traumatic encephalopathy (CTE) and Pick's disease are amongst the neurodegenerative disease that also feature NFTs (Spillantini & Goedert, 2013). However, the number of fibrillar plaques as well as distribution correlates with the severity and duration of AD while clinicopathological studies have established that amyloid burden does not (Arriagada, Growdon, Hedley-Whyte, & Hyman, 1992; Bierer et al., 1995; Ingelsson et al., 2004). A debate as to whether NFT formation is a precursor to neuronal death or simply a surrogate marker of ongoing pathological process therefore still exists.

1.2 Structure and function of the amyloid precursor protein

Undoubtedly, it is pivotal to gain a deeper understanding of the key players involved in AD pathology in order to decipher the underlying cause of the disease onset and progression. A key neuropathological alteration outlined above is the accumulation of aggregation-prone, neurotoxic Aβ peptides derived from sequential cleavages of the amyloid precursor protein (APP). George Glenner first purified Aβ peptides (Glenner & Wong, 1984) in 1984, however, until now, we are still lacking the complete crystal structure of its precursor protein – a hurdle in the search for physiological functions of cleavage product and precursor. Located on chromosome 21, *APP* encodes a type I transmembrane glycoprotein with a large extracellular N-terminal domain (NTD)

consisting of individual folding units and a short cytoplasmic C-terminal domain (CTD) acting as a hub for multiple protein interactions. Constituted of about 70% of the entire amino acid sequence, the APP ectodomain comprising of E1 and carbohydrate domains have been structurally characterized in the last years. Both domains are formed of complex α helices and β-pleated structures linked by an unstructured and flexible acidic region. E1 is further subdivided into the growth-factor like domain (GFLD) and copper-binding domain (CuBD). Meanwhile, the latter comprises of the E2 domain and the linker region, which is N-terminally located to Aβ sequence as well as devoid of any complex secondary structures [reviewed in (Reinhard, Hebert, & De Strooper, 2005)] (see Figure 1).

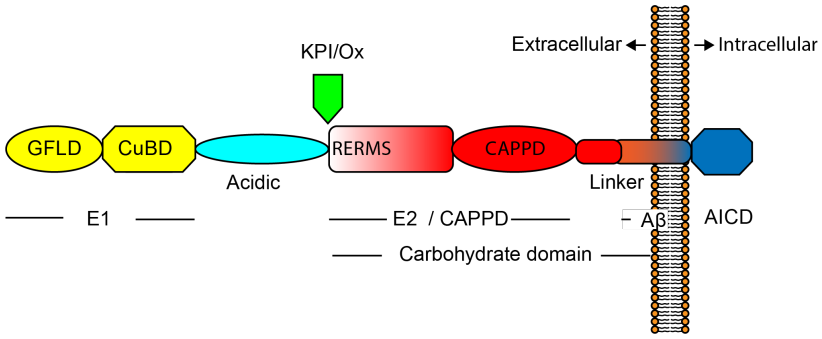

Figure 1: Structure of amyloid precursor protein
Illustration of the amyloid precursor protein (APP) comprising of a large extracellular N-terminal domain, subdivided into E1 (yellow) and E2 (red), and short cytoplasmic C-terminal domain (dark blue). Three predominant APP isoforms are APP 695, 751 and 770. The latter has both KPI and Ox-2 domains (green) while APP 751 has only KPI and APP 695 does not contain KPI nor Ox-2. GFLD: Growth-factor-like domain; CuBD: Copper-binding domain; KPI: Kunitz protease inhibitor; CAPPD: Central APP binding domain; AICD: APP intracellular domain. Modified from Reinhard 2005 (Reinhard et al., 2005).

Although the notion of APP dimerization is still heavily disputed, the hydrophobic patch adjacent to the heparin-binding site in E1 potentially used as a dimerization interface and the antiparallel dimer formation of E2 in solution strongly push forth the possibility of an oligomerization state of the extracellular domain. It was proposed that dimeric form of APP could regulate cell-cell adhesion whereas APP monomer may participate in growth factor signaling either as a receptor when cell-bound or ligand subsequent to α-secretase cleavage (Mohammadi,

Olsen, & Ibrahimi, 2005; Y. Wang & Ha, 2004). The "GxxxG" motifs in APP transmembrane sequence may further provide a junction for dimerization (Munter et al., 2007). These binding sites in APP incentivized to employ a protein-protein interaction network strategy to uncover its cellular function. In the midst of over 200 interacting partners are an array of extracellular matrix integral membrane proteins and cell adhesion molecules essential for neurite outgrowth, axonal pathfinding and synapse structure (Perreau et al., 2010). Despite the high regard allocated to APP in the context of Alzheimer's, APP null mice exhibit only subtle defects, including reduced brain and body weight as well as grip strength, CNS-specific synaptic phenotype, and increased susceptibility to seizures, traumatic brain injury as well as acute hypoxia (Z. W. Li et al., 1996; H. Zheng et al., 1995). The molecular underpinning of APP physiology still awaits to be entirely unravelled as our current understanding remains mainly speculative requiring *in vivo* validation.

1.3 Classical APP processing and the amyloid hypothesis

The pre-symptomatic stage of the disease is defined by molecular changes occurring twenty years prior to any overt cognitive and memory deficits. One of the earliest alterations detected is the accumulation of Aβ peptides through amyloidogenic processing of APP. This process debuts with the ectodomain shedding of APP by β-site cleaving enzyme 1 (BACE-1) at the amino-terminus of the Aβ sequence, thereby releasing soluble APPβ (sAPPβ). The membrane-bound remnant (β-CTF) is sequentially cleaved by γ-secretase, a multiprotein complex comprising either presenilin 1 or 2 (PSEN1/2) at the active centre. Consequently, APP intracellular domain (AICD) is released while Aβ peptides with differing lengths are secreted depending on the initial cleavage site of γ-secretase. Aβ of 1-40 and 1-42 amino acid long are the most commonly produced. Since BACE1 is higher expressed in endocytic compartments, amyloidogenic processing subcellularly occurs in endosomes upon APP retranslocation from the cell surface or direct trafficking (Kinoshita et al., 2003). Alternatively, cell surface APP can be processed by α-secretase, with the cleavage

occurring within the Aβ region, leading to the liberation of soluble APPα (sAPPα) and subsequent cleavage of the remaining C-terminal fragment (α-CTF) by γ-secretase. As opposed to Aβ, soluble p3 fragment is produced and secreted [reviewed in (De Strooper, 2010)] (See Figure 2)

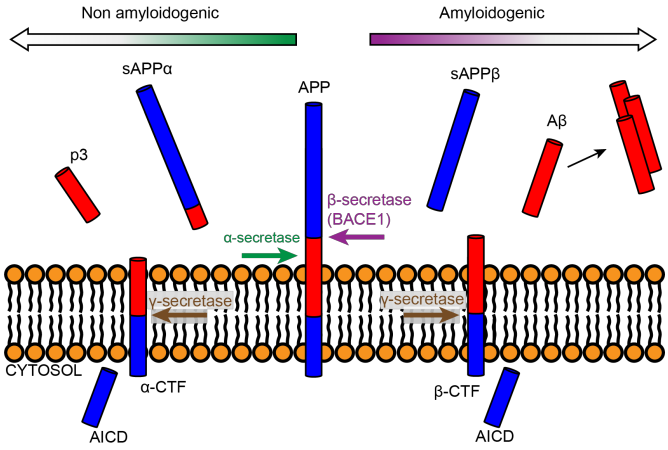

Figure 2: Classical APP processing by α-, β- and γ-secretases
Cleavage of amyloid precursor protein (APP) by α- or β-secretase generates sAPPα or sAPPβ, respectively. Remaining membrane-bound stub (α-CTF or β-CTF) is sequentially processed by γ-secretase, liberating APP intracellular domain (AICD) as well as p3 or Aβ fragment, respectively. The former is termed non-amyloidogenic processing (green) while the latter is known as amyloidogenic processing (purple).

Since APP has a relatively fast turn-over rate (Herreman et al., 2003) the study of its cleavage products becomes even more enticing and paramount. Many focused on the structural investigation of Aβ peptides to elucidate the agency of their said toxicity; indeed about 40 structures have been identified (Morgan, Colombres, Nunez, & Inestrosa, 2004). The presence of α helices in Aβ40/42 instead of β-strand structures which are associated with insolubility and plaque generation is quite striking and alludes to the notion of a conformational switch underlying the amyloidogenic properties of Aβ fibrils. While excessive amyloidosis and toxicity often go hand in hand, cleavage products from the non-amyloidogenic pathway correlate with neuroprotective effects. sAPPα can rescue long-term potentiation deficits as well as protects against neuronal death during transient ischaemia and acute hypoxia (Hefter et al., 2016; Ring et al., 2007; Smith-Swintosky et al., 1994; Xiong et al., 2017). Further *in vitro* studies revealed its role in activating

survival pathways (G. Cheng, Yu, Zhou, & Mattson, 2002; Milosch et al., 2014) and inhibiting molecules involved in apoptotic pathways (Gralle, Botelho, & Wouters, 2009). Lastly, the unstable nature of AICD makes it challenging to study, however, consistent reports with use of AICD-overexpressing cells demonstrated AICD to possess transcriptional activity (Cao & Sudhof, 2001). With regards to recent investigations and our own, it is also now evident the existence of other, non-canonical APP processing pathways, giving rise to novel cleavage products (Jefferson et al., 2011; Paschkowsky, 2018; Willem et al., 2015; Zhang et al., 2015).

The primacy of APP and its amyloidogenic processing in the development of AD is centred on the view that Aβ is the chief perpetrator of the detrimental molecular cascade impeding memory function and cognitive abilities. This is known as the amyloid hypothesis. Although still elusive, Aβ was proposed as an upstream regulator of oxidative damage (Deshpande, Mina, Glabe, & Busciglio, 2006; Kanski, Aksenova, & Butterfield, 2002), inflammation (El Khoury et al., 2007) and NFT formation (Hernandez & Avila, 2008; M. S. Lee et al., 2000). Indeed, inherited mutations in *APP* and *PSEN1/2* genes causing early-onset familial AD either increase the overall Aβ levels or Aβ42:Aβ40 ratio [reviewed in (Cacace, Sleegers, & Van Broeckhoven, 2016)]. The occurrence of a protective missense mutation in an Islandic cohort (A673T) proximal to the β-secretase cleavage site in APP, which attenuates Aβ production (Jonsson et al., 2012), further substantiates the amyloid hypothesis. However, a mere 1% of AD cases are familial while the rest seems to have multiple facets that cannot simply be reduced to the amyloid hypothesis. To date, only a single clinical trial revealed promising results, the Aβ-targeting antibody aducanumab, while most Aβ-targeting therapies were not conducive (Sevigny et al., 2016).

1.4 Multifactorial character of AD

Pre-symptomatic changes that precede the manifestation of clinical symptoms also includes cerebrovascular dysregulation. A Rotterdam study of 1, 730 participants aged 55 years and older

reported a negative association between cerebral blood flow velocity and likelihood of developing dementia. The study further claimed that cerebral hypoperfusion occurs before and contributes to the onset of cognitive decline (Ruitenberg et al., 2005). This is consistent with the two-hit vascular hypothesis of AD [reviewed in (Zlokovic, 2011)] wherein injury to the brain microvasculature would compromise the blood-brain-barrier (BBB) integrity, causing diminished cerebral blood flow, and consequently, initiate an Aβ-dependent or independent mechanisms of neurotoxicity. In addition to cerebral hypoperfusion, nearly 50% reduction in cerebral glucose utilization occurred in patients with mild cognitive impairment and in presymptomatic subjects with genetic predisposition to develop AD (Mosconi, 2005; Mosconi et al., 2006). Similarly, glucose transporters (GLUTs) 1 and 3, responsible for blood glucose uptake into the brain, were downregulated in AD patients (Simpson, Chundu, Davies-Hill, Honer, & Davies, 1994). Certain transgenic mouse models of AD also exhibit both cerebral hypoperfusion and hypometabolism. For instance, cerebrovascular dysfunctions, including stenoses, BBB leakage, loss of vascular smooth muscle cells and decreased GLUT1 expression, were demonstrated in mice overexpressing the human APP with the two familial APP mutations, namely swedish (K670N/M671L) and arctic (E693G). Furthermore, these pathological changes precede the onset of Aβ plaque pathology (Merlini, Meyer, Ulmann-Schuler, & Nitsch, 2011). Cerebral hypometabolism has been linked to alterations in Aβ clearance. Upon deletion of one *Glut1* allele in mice overexpressing the swedish mutation, Aβ peptides depositions in the cortex and hippocampus aggravated compared to the same transgenic mouse model of AD with functional Glut1. More importantly, APP and β-secretase expression levels were similar between the two strains, hinting to cerebral hypometabolism-mediated exacerbation of Aβ load consequent to perturbed clearance (Winkler et al., 2015). The implication of impaired Aβ clearance in sporadic AD cases emerge in more recent findings. The major cerebral cholesterol carrier conferring a high risk factor for late-onset AD,

apolipoprotein E ε4 (ApoE ε4), has also been associated in Aβ efflux from the brain (C. C. Liu, Liu, Kanekiyo, Xu, & Bu, 2013). Interestingly, glucose hypometabolism was detected in pre-symptomatic ApoE ε4 carriers (Reiman et al., 2005). Moreover, cerebral glucose metabolism is thought to be a pivotal player in posttranslational regulation of tau. A marked decrease in GLUT1 and GLUT3 levels in AD brains correlated to diminished intraneuronal glucose levels, rendering a downregulation in O-GlcNAcylation while promoting its hyperphosphorylation (F. Liu, Iqbal, Grundke-Iqbal, Hart, & Gong, 2004; Y. Liu, Liu, Iqbal, Grundke-Iqbal, & Gong, 2008). Cerebral hypometabolism as an early pathological event that precedes, and likely induces AD pathology is another evolving line of AD research.

2. Cancer

Unlike Alzheimer's disease, accessible cancer treatments ranging from conventional to more innovative strategies including immunotherapy, hormonal therapy and stem cell transplants, can improve patients' prognosis and accentuate the likelihood of survival. This may be ascribed to the rapidly and widely developing breadth of knowledge in cancer biology. Nevertheless, as one of the leading causes of death before 70 years old, cancer still poses a significant burden on society (Bray et al., 2018). Tumorigenesis remains a perplexing concept that is not sufficiently understood to develop effective, personalized therapy for the various forms of cancer.

2.1 Cancer genetics

Regardless of continual cell division and differentiation to repopulate the tissues and organs, the human body maintains a constant weight. A network of overlapping molecular mechanisms oversees this enormous production of cells by ensuring an equal balance of proliferation and programmed death, termed apoptosis. Only when the intricate control becomes haywire resulting in irrepressible cellular multiplicity, giving rise to cells irresponsive to homeostatic feedback mechanisms and thereby growing autonomously, is said the beginning of the carcinogenic process.

This is known as neoplasia. The genes encoding critical players in the regulation of the equilibrium between cell division and apoptosis oftentimes harbor a gain- or loss-of-function mutations rendering them the causative agent in the carcinogenic process. Oncogenes are defined as those that have acquired mutations engendering increase activity of the proteins they code for whereas mutation-induced inactivation of the gene is a characteristic of tumor suppressor genes [reviewed in (Bertram, 2000)]. Combinatorial approaches involving functional cloning, linkage analyses, positional cloning and mutational analyses of genetically predisposed individuals permitted the identification of multiple oncogenes, including the first one discovered now referred to as *RAS*, and tumor suppressor genes, such as retinoblastoma (*RB*) (E. Y. Lee & Muller, 2010). Converse to oncogenes that exhibit a dominant mutation, loss of function only manifest when both alleles of tumor suppressor genes are damaged.

Merely a handful of mutations are inherited while most are spontaneous due to chemical damage to DNA or induced by exogenous carcinogenic agents. DNA damage itself is not sufficient instigator of carcinogenesis, DNA replication and subsequent cell division are prerequisites to acquire and maintain such change in DNA. At least five crucial regulatory genes must harbor mutations in a cell to evade homeostatic control and proliferate autonomously due to a robust DNA repair system. The process of clonal expansion is sustained to garner more mutations in the process and become more adaptive to self-governance. This is translatable clinically as to increased proliferation rate, ability to invade neighboring normal tissue and metastasis [reviewed in (Bertram, 2000)].

2.2 Hallmarks of metabolic reprogramming

2.2.1 Dysregulation of cellular bioenergetics

Glucose is the principal nutrients important for biosynthesis and survival in mammalian cells. Breakdown of this nutrient provides carbon intermediates as building blocks of many

macromolecules while electron carriers generated from further oxidation of their carbon skeletons fuel ATP production or provide reducing power for biosynthetic reactions. Almost a century ago, Otto Warburg observed an ample of lactate production by cancer cells in normoxic environment with a marked increased glucose consumption. He initially postulated that tumors have impaired mitochondrial function and thereby resort to aerobic glycolysis for continued ATP generation (Warburg, 1956). This particular feature of tumor cells has been validated in various human cancers using fluorodeoxyglucose-positron emission tomography (FDG-PET) and nowadays, coined the *Warburg effect* (Almuhaideb, Papathanasiou, & Bomanji, 2011). However, it is also now evident that the premise of upregulated glycolysis as a result of a dysfunctional mitochondria was misconceived. Most cancer cells still rely on oxidative phosphorylation to generate ATP. Instead, it is the activation of oncogenes and loss of tumor-suppressors genes that provoke the high glycolytic rates [reviewed in (Pavlova & Thompson, 2016)].

Extracellular stimuli, such as growth factors, regulate nutrient uptake necessary for cell survival and proliferation [reviewed in (Thompson, 2011)]. Hence, depletion of growth factors could reduce cellular glucose consumption necessary to maintain bioenergetics. Such decline in glucose uptake can be rescued upon combined expression of cell surface GLUT1 and hexokinase, the first enzyme in the glycolytic pathway (Rathmell et al., 2003). In the case of cancer cells, oncogenic mutations devoid them of the external requirements for nutrient uptake. Genetic alterations in key enzymes involved in growth-factor signalling, for instance, phosphatidylinositol-3-kinase (PI3K)-Akt and its negative regulators phosphatase and tensin homolog (PTEN) and inositol polyphosphate-4-phosphatase (INPP4B), were identified in diverse cancer types [reviewed in (Vogelstein & Kinzler, 2004)]. *GLUT1* mRNA expression and cell surface protein translocation can be induced through PI3K-Akt pathway (Barthel et al., 1999; Wieman, Wofford, & Rathmell, 2007). In addition, both chief points of regulation in glycolytic pathway, hexokinase and

phosphofructokinase, are potentiated by Akt and thus, retains glucose intracellularly upon phosphorylation and advances glycolysis (Deprez, Vertommen, Alessi, Hue, & Rider, 1997; Gottlob et al., 2001). Enhanced *GLUT1* transcription is mediated by the first oncogene identified, *RAS*, facilitating cellular glucose uptake (Murakami et al., 1992).

2.2.2 Utilization of opportunistic nutrient acquisition modes

Due to increased nutrient consumption and limited vascular supply, tumor cells encounter metabolically unfavorable conditions. They, however, are able to survive and proliferate owing to acquisition of mutations permitting the use of opportunistic modes to obtain necessary nutrients. For instance, mutation in *Ras* stimulates macropinocytosis to scavenge extracellular macromolecules. Upon engulfment, free amino acids can be liberated through lysosomal degradation (Commisso et al., 2013). Moreover, Kras-driven pancreatic cancer cells also espouse on phagocytosis of entire living cells and apoptotic cellular bodies to amass free amino acids in nutrient-depleted environment (Kamphorst et al., 2015). In order to overcome the deficit in certain fatty acids under hypoxic conditions, tumor cells adopt mechanisms to readily import lysophospholipids from their surrounding or induce the release of stored lipids from neighboring cells (Kamphorst et al., 2013).

2.2.3 Upregulated biosynthesis of macromolecules

Tumors exhibit high glycolytic rates not only due to mutations but also tumorigenesis requires an exorbitant need for precursor molecules and NADPH used in anabolic pathways relative to ATP demand of the cells. Glucose and various glycolytic intermediates provide the building blocks of the biosynthetic reactions while ongoing tricyclic acid cycle coupled to electron transport chain produces ATP and NADH, repressing glycolysis. As a result, cancer cells acquire mechanisms to favor increased consumption of glucose, free amino and fatty acids while maintaining low yet sufficient ATP synthesis [reviewed in (DeBerardinis & Chandel, 2016)]. Mutations in Ras

stimulates the pentose phosphate pathway (PPP) which generates NADPH and ribose-5-phosphate – the structural component of nucleotides – from glucose-6-phosphate branching off from the first step in glycolysis (Ying et al., 2012). Moreover, cancer cells convert excess intermediates that were not utilized for anabolism to lactate via lactate dehydrogenase (LDH) thereby renewing sufficient pool of NAD^+ to perpetuate glycolysis. Excessive LDH and pyruvate dehydrogenase kinase 1 (PDK1) expression is triggered by hypoxia as well as oncogenes (Papandreou, Cairns, Fontana, Lim, & Denko, 2006). Converse to non-lipogenic normal adult cells wherein *de novo* fatty acid synthesis remains limited, it is accentuated during tumorigenesis. This permits cancer cells to expedite the production of lipid bilayers as well as adopt cellular membranes comprising of more saturated fatty acids resistant to oxidative damage as means to prolong survival rate (Rysman et al., 2010). Consequently, targeting the various metabolic reprogramming cancer cells utilize may provide a framework for cancer therapy enabling to mitigate tumorigenesis and avert metastasis.

3. Mammalian glucose transporter

As discussed in section 2, glucose oxidation is the principal source of carbon backbone and energy reserve used by mammalian cells. Glucose uptake is therefore a key step in glucose metabolism. This ignited a research interest on proteins with glucose-carrying capacity across tissue barriers.

3.1 Concentrative and facilitative glucose transporter family

In 1965, the concentrative sodium glucose transporters (SGLT) were discovered as the first major family of glucose transporters (Bosackova & Crane, 1965). Expressed at brush border cells of small intestine and kidney, absorption of glucose across epithelial cells through SGLT are coupled with cotransport of Na^+ ions. The ATPase pump maintains the Na^+ ions electrochemical gradient which drives the accumulation of glucose into the cell. To date, there are 12 different SGLT types responsible for intracellular uptake of carbohydrates, amino acids, carboxylic acids and certain

ions [reviewed in (Simmons, 2011)]. Soon thereafter, another significant glucose transporter family, termed GLUTs, was identified which belong to the larger family of solute carriers 2A (SLC2A) (Mueckler et al., 1985). Converse to SGLT, glucose exchange across cell membrane through GLUTs is bidirectional and energy-independent. The inward and outward movement of GLUT are kinetically distinct, but both rely on a glucose concentration gradient (Carruthers, 1990). These facilitative integral membrane proteins are alternatively spliced giving rise to 14 various types, subdivided into three classes. Similar to SGLTs, all GLUT isoforms comprise of 12 transmembrane domains connected by short cytoplasmic loops (see Figure 3). The intracellular N- and C-termini regions are isoform-specific, which may confer the differences in kinetics and subcellular localization. Among the different classes, class I, which consists of GLUT1-4, is the most heavily studied. Virtually, every cell in the body expresses at least one type of GLUT, with varying affinity for glucose and distinct regulatory mechanisms [reviewed in (Mueckler & Thorens, 2013)].

In the late 1900s, one of the first membrane transporters purified and cloned was GLUT1 (Mueckler et al., 1985). It is ubiquitously distributed but predominantly expressed in endothelial cells of blood-tissue barriers where it is responsible for basal glucose uptake given its high affinity for the monosaccharide (Koranyi et al., 1991; Yeh, Lin, & Fu, 2008). Other members of class I transporters, with the exception of GLUT2, have also a greater affinity for glucose relative to other sugar molecules. GLUT3 and GLUT4, however, exhibit a more targeted tissue expression; the former is abundantly expressed specifically in neurons while the latter in adipose tissue as well as skeletal and cardiac muscle. A unique feature of GLUT4 is the ability to accumulate in intracellular vesicles creating a pool that can be readily released upon insulin or exercise stimulation (Leino, Gerhart, van Bueren, McCall, & Drewes, 1997) [reviewed in (Huang & Czech, 2007)]. GLUT2 is predominant in pancreatic β-cells and hepatocytes with low affinity to glucose (Thorens, Cheng,

Brown, & Lodish, 1990). Liver-specific inactivation of Glut2 in adult mice nonetheless downregulates glucose uptake and these mice develop glucose intolerance in the long run due to reduced pancreatic insulin secretion (Seyer et al., 2013). This suggests for a role of hepatic GLUT2 in β-cell glucose competence albeit the mechanism is rather elusive.

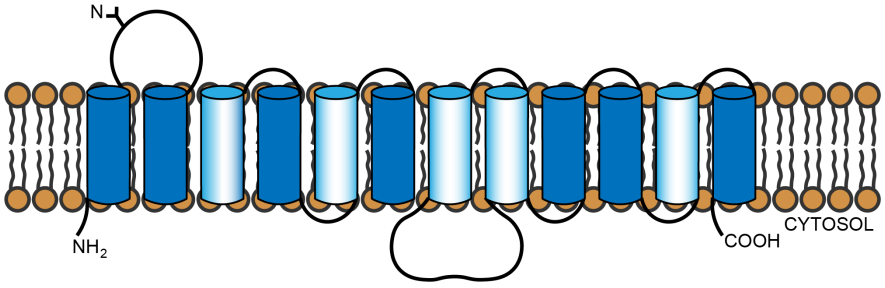

Figure 3: Structure of glucose transporter 1 (GLUT1)
Glucose transporters consist of 12 membrane-spanning helices (blue cylinders) with intracellular N- and C-termini and short loops. All also have a single N-glycosylation site, but the localization differs amongst the three classes.

By taking advantage of conserved carbohydrates transporter signatures, a wide array of novel GLUT-like genes was discovered. Until now, however, very little is known about these GLUTs although some have been associated with particular diseases. For instance, single nucleotide polymorphisms (SNPs) identified in *GLUT9* were linked with high plasma uric acid levels, suggesting its implication in renal disorders (S. Li et al., 2007). *GLUT12* perinuclear and cell membrane expression were observed in human prostate and breast cancer cell lines (Macheda, Rogers, & Best, 2005) .

3.2 GLUT1

3.2.1 Regulation at genomic level and disease implication

GLUT1 has gained increasing attention over the years as both the lack of and surplus are detrimental to health. As discussed in section 1.4, diminished cerebral glucose uptake was frequently shown in conjunction with lower GLUT1 expression at the BBB. In 1991, *De Vivo* and colleagues described two children with reduced cerebrospinal glucose concentration in addition to experiencing various movement and neurological disorders. They suspected that glucose exchange

across the BBB of these children was impaired (De Vivo et al., 1991). It was later found in 1998 that these patients only had one functional GLUT1 allele and since then, the disease was termed GLUT1 deficiency syndrome (G1D). Majority of affected individuals harbor either missense or loss-of-function mutations distributed throughout the *GLUT1* gene localized in chromosome 1 and behaved as a cellular codominant trait with haploinsufficiency (Seidner et al., 1998). About 90% of G1D patients exhibit developmental encephalopathy with seizures of varying types. Presently, successful disease-modifying therapies are still out of reach [reviewed in (Pascual, Wang, & De Vivo, 2015)].

Deregulated expression of GLUT1 is also a recurrent theme in cancer metabolism. It was initially portrayed in 1987 when it was discovered that Ras and Src oncogenes activate the hypoxia inducible factor 1 alpha (HIF1α), which subsequently induces the expression of target genes including *Glut1* leading to a consequent increase in glucose consumption in transformed malignant rat fibroblasts (Chen, Pore, Behrooz, Ismail-Beigi, & Maity, 2001; Flier, Mueckler, Usher, & Lodish, 1987). Moreover, injection with GLUT1 antisense vector in cancer cells *in vitro* and *in vivo* attenuated tumorigenesis, demonstrating GLUT1 involvement in early tumor growth, invasiveness and metastasis (Ito et al., 2002; Noguchi et al., 2000). The succeeding years of cancer research corroborated the overexpression of GLUT1 in various human tumors, including breast and colorectal cancers (Brown & Wahl, 1993; Haber et al., 1998; Yamamoto et al., 1990). Additionally, estrogen and epidermal growth factors promote Glut1-dependent breast cancer cell growth possibly via stimulation of their respective signaling pathways (Rivenzon-Segal, Boldin-Adamsky, Seger, Seger, & Degani, 2003; Wilding, Lippman, & Gelmann, 1988).

3.2.2 Posttranscriptional regulation

Despite a meager understanding of the implication of GLUT1 posttranslational regulation in cancer, several studies provided vast insights into how GLUT1 is modified and translocated from

the ER to the cell membrane. Tomoichiro Asano reported in 1991 the existence of a single N-glycosylation site at asparagine 45 in the first cytoplasmic loop of GLUT1. He subsequently demonstrated not only defective glycosylation upon mutating this site, but also decreased cell surface expression as well as higher turnover rates (Asano et al., 1991; Asano et al., 1993). However, such findings were not noted in more recent studies. Instead, some claimed that N-glycosylation of GLUT1 is critical in maintaining its high affinity for glucose; disruption led to a weaker transporter activity (Samih et al., 2003). Furthermore, it was established 30 years ago that Protein Kinase C (PKC) activation rapidly promotes glucose uptake and was believed to be through phosphorylation of GLUT1 (Witters, Vater, & Lienhard, 1985). Recent reports further corroborated the involvement of PKC signaling, as well as the PI3K-Akt pathway in inducing cell surface translocation of GLUT1 (E. E. Lee et al., 2015; Wieman et al., 2007).

GLUT1 is also being recycled continuously or upon growth factors withdrawal from endocytic compartments. Interestingly, the loss of C-terminal PDZ-binding domain of GLUT1 or its PDZ-binding protein, G-alpha interacting protein (GAIP), hindered cell surface expression while prompted rapid turnover of intracellular GLUT1 (Wieman et al., 2009). Moreover, intracellular newly synthesized GLUT1 can alternatively undergo ER-associated degradation (ERAD) with the aid of Derlin-3 which possibly forms a passageway in ER membranes for ubiquitinated ERAD substrates to reach the proteasome. Structural homology model indicated that derlins possess a rhomboid core and thereby are regarded distant members of the rhomboid superfamily. Colorectal cancer exhibit hypermethylation-induced *Derlin 3* inactivation, that is associated with upregulation of GLUT1 (Lopez-Serra et al., 2014; Oda et al., 2006).

Due to the widespread expression of GLUT1 and the discovery of diverse, distinct yet interweaved modes of regulation, we have only scratched the surface. It is possible that the relative roles of the different pathways are complementary or that other, still unknown, regulation takes

priority in specific cell types. Despite these unaddressed notions, unravelling GLUT1 regulation will open novel therapeutic avenues for both AD, wherein GLUT1 expression declines in cerebral microvasculature, and cancer, such that tumors exhibit overexpression of GLUT1, in addition to the rarer G1D syndrome.

4. Rhomboid proteases

The notion of intramembrane proteases (IMPs) which are able to cleave substrates in a hydrophobic environment arose from the study of amyloidogenic APP processing. Comprised of a third of all synthesized proteins, IMPs fulfill a wide array of functions involving lipid homeostasis, cell differentiation and apoptosis (Almen, Nordstrom, Fredriksson, & Schioth, 2009) [reviewed in (McCarthy, Coleman-Vaughan, & McCarthy, 2017). Due to their diversity and functional redundancy, this clade of proteases was classified according to their mechanism of action rather than their specific roles. Four different types of IMPs have been identified thus far: site-2-protease (S2P)-type zinc metalloproteases, aspartic, serine and most recent glutamic intramembrane proteases [reviewed in (McCarthy et al., 2017)]. For several IMPs, their physiological substrates have been elucidated as well as their subcellular localization. However, a wide gap in our understanding of most IMPs still exists, including the family of intramembrane serine rhomboid proteases which this thesis is profoundly focused on.

4.1 History of rhomboid superfamily

The discovery of serine intramembrane rhomboid proteases dates back prior to the introduction of the notion of intramembrane proteolysis. In 1984, Nusslein-Volhard & Wieschaus first described a *D. melanogaster* with a pointed skeleton head and deletion of a median portion of all denticle bands caused by a mutation in the rhomboid-1 gene on chromosome 3 (Nusslein-Volhard, Wieschaus, & Kluding, 1984). Early studies not only elucidated the role of rhomboid-1 in neural development in flies, but sequence analysis of the gene highlighted it to be a transmembrane

protein lacking protease motifs (Bier, Jan, & Jan, 1990). It was only in early 2000s when Lee *et al*., demonstrated that rhomboid-1 cleaves an epidermal growth factor (EGF) receptor ligand in Drosophila, namely Spitz, that rhomboids are regarded to possess catalytic properties (Golembo, Raz, & Shilo, 1996; J. R. Lee, Urban, Garvey, & Freeman, 2001; Wasserman, Urban, & Freeman, 2000). Mutational analyses further revealed that its catalytic residues consist of a serine-histidine dyad, distinct from a catalytic triad described for classical serine proteases like chymotrypsin (Lemberg et al., 2005; Urban, Lee, & Freeman, 2001; Y. Wang, Zhang, & Ha, 2006). The five other rhomboids in flies that were later on discovered possess similar properties, hinting for evolutionary conservation (Wasserman et al., 2000). Through genome-wide sequencing, orthologs of rhomboids were found in all branches of life (M. K. Lemberg & M. Freeman, 2007b; Lemberg et al., 2005), corroborating that these proteases are conserved down to the least common ancestors, and thereby denotes that they carry essential functions.

4.2 Rhomboid: structural characterization, gating strategies and substrate specificity

More than a decade ago, the unravelling of the crystal structure of the bacterial *E.coli* rhomboid GlpG did not only ignite major progress on structure-function analyses of the enzyme, but also marked a revolutionary discovery as the first intramembrane protease to be crystallized. It confirmed that rhomboid proteases bear an active center with a catalytic dyad comprising of serine at position 201 in transmembrane sequence (TMS) 4 and in hydrogen bond distance to histidine 254 in TMS6. A third interacting partner which is usually present in soluble serine proteases is not necessary for the catalytic reaction in rhomboid proteases (Y. Wang et al., 2006). As opposed to catalytic triad, the nucleophilic attacked by serine 201 occurs simultaneously with its deprotonation mediated by histidine 254 upon binding of the ligand (Uritsky, Shokhen, & Albeck, 2012, 2016). Asparagine 154 in TMS2 is not in hydrogen bond distance to histidine (Y. Wang et al., 2006), further substantiating its lack of direct participation in the catalytic reaction. Mutating

this residue nonetheless compromised the enzyme activity (Cho, Dickey, & Urban, 2016). Asparagine was therefore thought to play a role in stabilization of an oxyanion formed during the transition state.

The crystal structure of GlpG not only confirmed the presence of a rhomboid core, defined by six membrane-spanning helices, but also revealed that the active center lies 10Å deep within the plane of the membrane and is covered by loop 5 (Y. Wang et al., 2006). This loop was described as a "cap" that can readily open to the extracellular side permitting access of water into the cavity in close proximity to the active site, essential for enhanced peptide bond hydrolysis (Y. Zhou, Moin, Urban, & Zhang, 2012; Zoll et al., 2014). Immediately linked to loop 5 is the TMS5 whereby several structural analyses revealed a lateral gating mechanism for substrate entry mediated by movement of TMS5 away from TMS2 (Baker, Young, Feng, Shi, & Urban, 2007; Ben-Shem, Fass, & Bibi, 2007; Wu et al., 2006). Nevertheless, inhibition of the lateral motion via covalent cross linkage of TMS2 and TMS5 failed to impede GlpG activity (Xue & Ha, 2013). Alternatively, a two-stage rhomboid proteolysis was proposed following inhibition kinetics experiments. Substrate-mimicking peptidyl aldehydes non-competitively inhibit the binding to GlpG of a non-physiological substrate, TatA, suggesting two distinct substrate-bound complexes. The first was termed "interrogation complex" formed by the lateral attachment of substrate TMS to the gate-open enzyme. Subsequently, the transfer to the active site of the substrate TMS inclined to unfolding constitutes the "scission complex" thereby concluding a successful cleavage of the substrate (Cho et al., 2016).

With regards to the substrate specificity, Strisovsky et *al.* illustrated that substrates require a TMS for binding as well as a recognition motif to direct cleavage. Moreover, the recognition motif residing adjacent or within the substrate TMS requires the presence of helix-destabilizing residues in the substrate TMS. Cleavages outside the transmembrane were also reported; the

distance of the recognition motif from the substrate TMS dictates the cleavage efficiency (Strisovsky, Sharpe, & Freeman, 2009). The notion of a requirement of recognition motif was challenged by Moin *et al.* They claimed that true substrates possess intrinsically unstable helices recognized by rhomboid proteases through membrane immersion alone (Moin & Urban, 2012). Consequently, the membrane environment is the chief controller of cleavage site specificity. Perturbations in the membrane as well as alterations in its lipid composition indeed abated rhomboid specificity, rendering the cleavage of non-substrates (Urban & Moin, 2014).

Lastly, rhomboid proteolysis is not dependent on ATP but is rather kinetically-driven whereby the membrane environment restrains gate opening. As such, other proteases are 100-fold more efficient compared to rhomboids, which takes about 2.5 min to complete a single proteolytic event. Moreover, it was shown that substrate affinity plays very little role, if any, in governing catalytic reaction; all rhomboids display little to no affinity for their substrates (Dickey, Baker, Cho, & Urban, 2013). Conversely, Arutyunova et al. proposed the existence of an intramembrane exosite through rhomboid oligomerization, which can significantly enhance the enzyme affinity for the natural substrate (E. Arutyunova et al., 2014).

4.3 Mammalian active rhomboid homologues

Since the initial description of rhomboid proteases, they have been linked to an array of cellular functions spanning from parasitic invasion, growth factor cell signaling, mitochondrial dynamics and membrane protein quality control. The current view on the physiology of the mammalian homologues, although still limited, is expanding as more substrates emerge. Their implications in health and disease further aid in uncovering their biological functions. Presently, there are 14 mammalian rhomboid family members comprising of 5 proteolytically active and 9 pseudo-proteases (Bergbold & Lemberg, 2013). They can also be subdivided based on their topologies: two secretase-types with either 6 + 1 TMD structure or solely the rhomboid core; the PARL-type

possessing 1 + 6 TMD structure thereby shifting the position of catalytic residues to TMS5 and TMS7; and lastly, the iRhom-types characterized by a large globular domain in L1 loops and changes in the active site rendering them catalytically inactive (M. K. Lemberg & M. Freeman, 2007b) (see Figure 4).

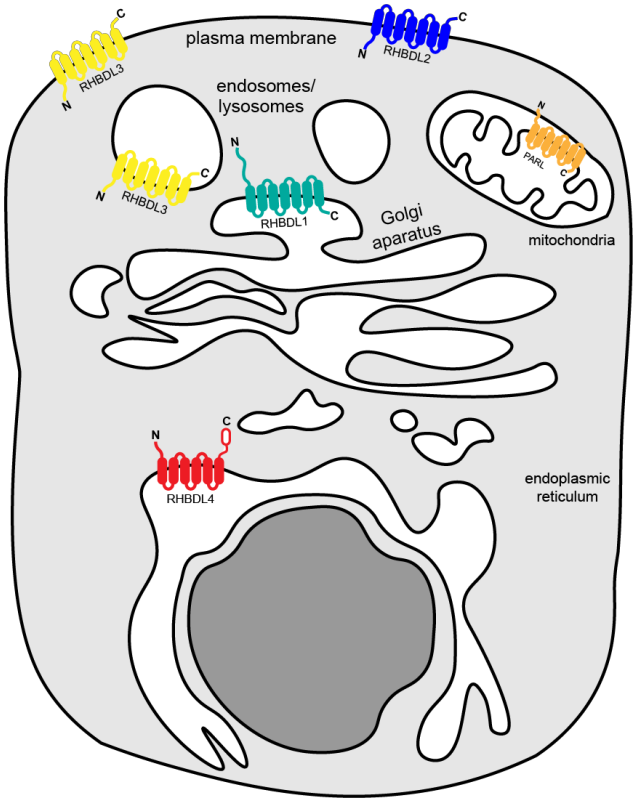

Figure 4: Subcellular localization and structure of active mammalian rhomboid proteases
Amongst the five active rhomboid proteases, four reside throughout the secretory endomembrane system while PARL (orange) has a strict mitochondria localization. RHBDL1-3 and PARL possess the rhomboid core with additional transmembrane domain at the C-terminus (6 + 1 topology) and N-terminus (1 + 6 topology), respectively. The plasma membrane is where RHBDL2 (blue) and RHBDL3 (yellow) localize. Endo-lysosomal localizations have also been reported for RHBDL3. RHBDL1 (green) is located within the Golgi apparatus. RHBDL4 (red) is an ER-resident protease with only six membrane-spanning helices. Modified from Lastun 2016 (Lastun, Grieve, & Freeman, 2016).

4.4 RHBDL4

Our interest in pursuing rhomboid substrates paved way to the discovery of an alternative, non-amyloidogenic APP processing pathway mediated by the secretase-type B clade of mammalian rhomboids, more commonly known as rhomboid-related protein 4 (RHBDL4) (Paschkowsky,

Hamze, Oestereich, & Munter, 2016). This protease is encoded by *RHBDD1* gene located in chromosome 2. Contrasting evidence however arose regarding its subcellular localization. Despite compelling reports demonstrating that RHBDL4 is exclusively localized to the ER (Fleig et al., 2012; Wunderle et al., 2016), one group reported a mitochondria localization of RHBDL4 (Y. Wang et al., 2008), thereafter the enzyme was found in the plasma membrane and cytoplasm by the same group (W. Song et al., 2015). Hence, the question of whether RHBDL4 traffics to various subcellular compartments is oftentimes raised. RHBDL4 is nonetheless more widely accepted as an exclusive ER-resident intramembrane protease.

4.4.1 Identification of its substrates and proposed functions

Over the course of eight years, the field of RHBDL4 research flourished with a diversity of substrates identified which put forward different theories regarding its cellular roles. The pro-apoptotic factor, Bcl-2 interacting killer (BIK), was the first protein shown to be processed by RHBDL4 engendering its rapid ubiquitin-mediated proteasomal degradation (Y. Wang et al., 2008). Subsequent lines of evidence reinforce its link to apoptosis. RHBDL4 upregulation induces transcriptional activation of the anti-apoptotic factor Bcl-3 via stimulation of c-Jun, a component of the transcription factor complex activator protein 1 (Ren et al., 2013). Contrary, RHBDL4 silencing in spermatogonia cells accentuated apoptosis and prevents differentiation into spermatids (Y. Wang et al., 2009) while knockdown in human hepatoma cell line halted proliferation and cell cycle (X. N. Liu et al., 2013). Additionally, Fleig *et al.* implicated RHBDL4 in ER-associated degradation (ERAD) of type I and multi-pass membrane-spanning proteins containing basic amino acid residues in the TMS, termed degron motif. The ubiquitin interacting motif in the C-terminal domain of RHBDL4 was shown indispensable for recognizing these ERAD substrates. Subsequently, RHBDL4 interacts with p97 to extract the membrane-bound remnants for degradation via the proteasome in the cytosol (Fleig et al., 2012).

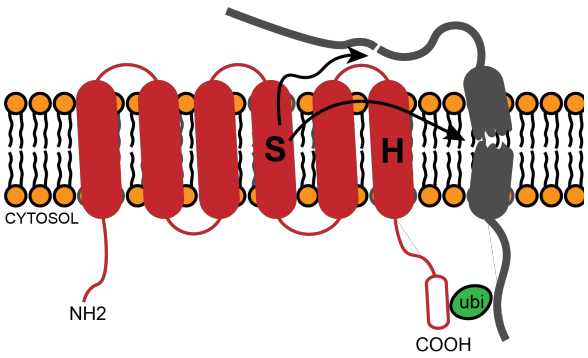

Figure 5: Cleavage by RHBDL4 via a serine-histidine catalytic dyad
RHBDL4 (red) can act as both sheddase and intramembrane protease whereby it cleaves its substrate (gray) either at the ectodomain or within the transmembrane sequence. It binds ubiquitinated ERAD substrates via its cytosolic ubiquitin-interaction motif (green) Modified from Lastun 2016 (Lastun et al., 2016).

In more recent findings, the EGF signaling pathway was likewise associated to RHBDL4 whereby pro-transforming growth factor alpha (pro-TGFα) was unveiled as a novel substrate of RHBDL4 (W. Song et al., 2015). This notion was challenged by Wunderle *et al.*: RHBDL4 mediates the release of TGFα by regulating the ER exit of pro-TGFα to the cell surface where it is then processed by ADAM family of metalloproteases and not by the ER-resident RHBDL4 (Wunderle et al., 2016). Lastly, the most novel RHBDL4 substrate reported is APP. In 2016, our group elucidated N- and C- terminal fragments (NTFs and CTFs) derived from ectodomain cleavages of APP by RHBDL4 *in vitro* (Paschkowsky et al., 2016). Although the physiological relevance of this said pathway as well as its metabolites remains to be addressed, this robust enzyme-substrate pair can bestow an efficient tool to study the proteolytic mechanism of RHBDL4 (see Figure 5).

4.4.2 RHBDL4 in health and disease

Most notably, our findings allude to a potential role of RHBDL4 in AD pathogenesis. Cell culture experiments revealed lower Aβ levels upon co-expression of RHBDL4 with APP; however, RHBDL4 expression was found higher in AD diseased patients compared to healthy controls (Paschkowsky, 2018). It has long been proposed that dysregulation in lipid metabolism could

contribute to AD. Accordingly, membrane lipid composition influences the processivity of γ-secretases, a key enzyme in the amyloidogenic APP processing [reviewed in (Paschkowsky, Oestereich, & Munter, 2018)]. We also recently demonstrated that cholesterol modulates RHBDL4 activity through direct interaction with the protein (Paschkowsky, Recinto, Young, Bondar, & Munter, 2018). Whether RHBDL4 has a protective or detrimental nature in the context of AD is still speculative and therefore warrants further scrutiny. Aside from AD, RHBDL4 has been linked to cancer. Upregulation of RHBDL4 was shown to be common in glioblastoma stem cells, colorectal and breast cancer tissues (Miao et al., 2017; W. Song et al., 2015; Stangeland et al., 2015; M. Zhang et al., 2018; X. Zhang et al., 2018). Consequently, these highlight the potential significance of RHBDL4 as a prognostic marker and/or novel therapeutic target in cancer. Further *in vivo* validation however awaits as there are currently limited data analysis on Rhbdl4-deficient mice which are solely taken from the open source mouse phenotyping consortium.

5. Aim of this thesis

Mammalian rhomboid proteases have gained increasing recognition due to their implications in health and disease. The limited knowledge on their physiological substrates remains a hurdle in understanding their mechanism of action and cellular roles. As APP currently being the best known physiological substrate for human rhomboid RHBDL4, we aimed to utilize this substrate-enzyme pair as a tool to further characterize the proteolytic mechanism of the enzyme through mutational analyses of APP. Considering a positive correlation between RHBDL4 expression and either risk to AD or cancer progression, development of inhibitors could be of importance. Unfortunately, all existing ones are either non-specific or exhibit low potency *in vivo*. A thorough investigation of how RHBDL4 cleaves its substrates could henceforth alleviate present roadblocks in drug design.

The high conservation of rhomboids across kingdoms propelled us to envisage that they must carry essential functions. In the second chapter, we thus aimed to decipher the physiological

role of RHBDL4 using *in vitro* and *in vivo* models. Not only bacterial rhomboid, GluP, has been shown involved in glucose transport and cell division, but RHBDL4 overexpression has also been found in certain cancer tissues, which manifest an aberrant glucose metabolic profile. Independent to its APP-cleaving properties, initial observation of the cell culture media of mouse embryonic fibroblasts lacking Rhbdl4 indeed led us to postulate for its contribution in glucose metabolism. This study sought to introduce a novel player in metabolism and substantiate RHBDL4 as potential therapeutic target for cancer.

II. EXPERIMENTAL SECTION

MANUSCRIPT 1: An alternative processing pathway of APP reveals two distinct cleavage modes for rhomboid protease RHBDL4

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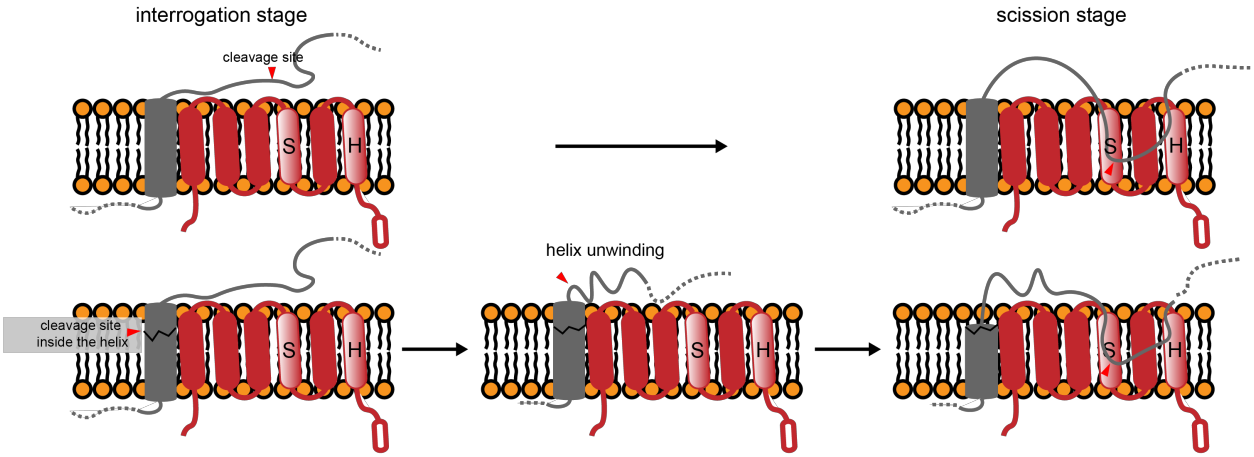

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1.1 Abstract

Since the first genetic description of a rhomboid in *Drosophila melanogaster*, tremendous efforts were geared towards elucidating the proteolytic mechanism of this particular class of intramembrane proteases. In particular, mammalian rhomboid proteases sparked our interest and we aimed to investigate the human homologue RHBDL4. In light of our recent finding of the amyloid precursor protein (APP) family as efficient substrates of RHBDL4, we were enticed to further study the specific proteolytic mechanism of this enzyme by comparing cleavage patterns of wild type APP and APP TMS chimeras. Here, we demonstrate that the introduction of positively charged amino acid residues in the TMS redirects the RHBDL4-mediated cleavage of APP from its ectodomain closer towards the TMS, possibly inducing an ER-associated degradation (ERAD) of the substrate. In addition, we concluded that the cytoplasmic tail and proposed palmitoylation sites in the ectodomain of APP are not essential for the RHBDL4-mediated APP processing. In summary, our previously identified APP ectodomain cleavages by RHBDL4 are a subsidiary mechanism to the proposed RHBDL4-mediated ERAD of substrates likely through a single cleavage near or within the TMS.

1.2 Introduction

Rhomboid proteases are an ancient class of intramembrane serine proteases that is highly conserved throughout all kingdoms of life (Kinch & Grishin, 2013; M. Lemberg & M. Freeman, 2007). Among the 14 mammalian rhomboid family members are five proteases that contain a catalytically active serine-histidine dyad (Urban et al., 2001; Y. Wang et al., 2006). Although the physiological functions of these proteolytically active rhomboids are still poorly understood, their recent implications in various diseases substantiate the quest for their physiological substrates. Human mitochondrial presenilin-associated rhomboid-like protein (PARL) has been associated with Parkinson's disease (Shi et al., 2011) and type II diabetes (Walder et al., 2005), while

endoplasmic reticulum (ER)-resident rhomboid-related protein 4 (RHBDL4) has been linked to colorectal cancer (Miao et al., 2017; W Song et al., 2015), glioblastoma (Stangeland et al., 2015) and a particular sensory-motor neuropathy (Fleig et al., 2012). In the pursuit of identifying substrates for human rhomboids, our group found that RHBDL4 efficiently cleaves the amyloid precursor protein (APP), a key player in Alzheimer's disease pathogenesis. We demonstrated an alternative, non-amyloidogenic APP processing pathway leading to the emergence of specific RHBDL4-derived N-terminal and C-terminal fragments (Paschkowsky et al., 2016). Knockdown of RHBDL4 in HEK 293T cells diminished endogenous APP cleavage fragments indicating that RHBDL4 and APP constitute a physiologically relevant enzyme-substrate pair. Furthermore, all cleavages identified so far are thought to be within the APP ectodomain, thereby prompting us to further investigate this intriguing cleavage mechanism. An earlier study by Fleig *et al*. postulated the relevance of RHBDL4 in the degradation of predestined type I or multi-pass transmembrane proteins in the ER as well as implicated the ubiquitin interacting motif present in the C-terminal domain of RHBDL4 in this degradation pathway (Fleig et al., 2012). As such, we exploited these previous findings which identified RHBDL4 substrates (MPZ-L170R) and non-substrates (MPZ), and generated different APP chimeric constructs, substituting the APP TMS with the TMS of either MPZ-L170R or MPZ. Furthermore, we studied the impact of the APP C-terminal domain on RHBDL4-mediated APP processing as well as the influence of a proposed back folding of the APP ectodomain to the membrane induced by palmitoylation at specific cysteines residues (Bhattacharyya, Barren, & Kovacs, 2013). Here we show that RHBDL4 may have two distinct cleavage modes: predominating cleavage in the TMS or in the juxtamembrane region, which possibly depends on the composition of the substrate transmembrane sequence and a second mode where APP gets cleaved multiple times in its ectodomain, potentially induced by specific environmental settings.

1.3 Results

1.3.1 Membrane-bound substrates of RHBDL4 can undergo two distinct proteolytic cleavage modes

Rhomboid proteases have been originally described as intramembrane proteases, thus they are assumed to predominantly cleave their substrates in the transmembrane sequence (TMS) (M. K. Lemberg & M. Freeman, 2007a). However, we recently identified the human rhomboid protease RHBDL4 and APP as enzyme-substrate pair and showed that RHBDL4 cleaves APP multiple times in its ectodomain (Paschkowsky et al., 2016). These cleavages generate 70-73 kDa N-terminal fragments (NTFs) and 10-25 kDa C-terminal fragments (CTFs) (Paschkowsky et al., 2016). This rather unexpected finding challenged us to further investigate the specific substrate recognition mechanism of RHBDL4 for APP. As such, we aimed to analyse RHBDL4-mediated processing of different APP TMS mutants. Previously, RHBDL4 was demonstrated to process membrane-bound proteins encompassing TMS predestined for endoplasmic reticulum-associated degradation (ERAD) (Fleig et al., 2012). We took advantage of these proposed substrates to generate APP mutants wherein the TMS of wild type APP was exchanged for the TMS of a proposed RHBDL4 substrate or non-substrate (Fig. 1A). In this regard, we mainly focused on myelin protein zero (MPZ), which is a key player in myelination of peripheral neurons (Marrosu et al., 1998). A single amino acid exchange from leucine to arginine in the TMS of MPZ, namely MPZ L170R, induces a genetic predisposition to Charcot-Marie-Tooth disease, a hereditary sensory and motor neuropathy (Numakura, Lin, Ikegami, Guldberg, & Hayasaka, 2002). Contrary to wild type MPZ, the variant was shown to be cleaved by RHBDL4. We thus analyzed the processing of APP containing either the MPZ TMS or MPZ L170R TMS variant by RHBDL4.

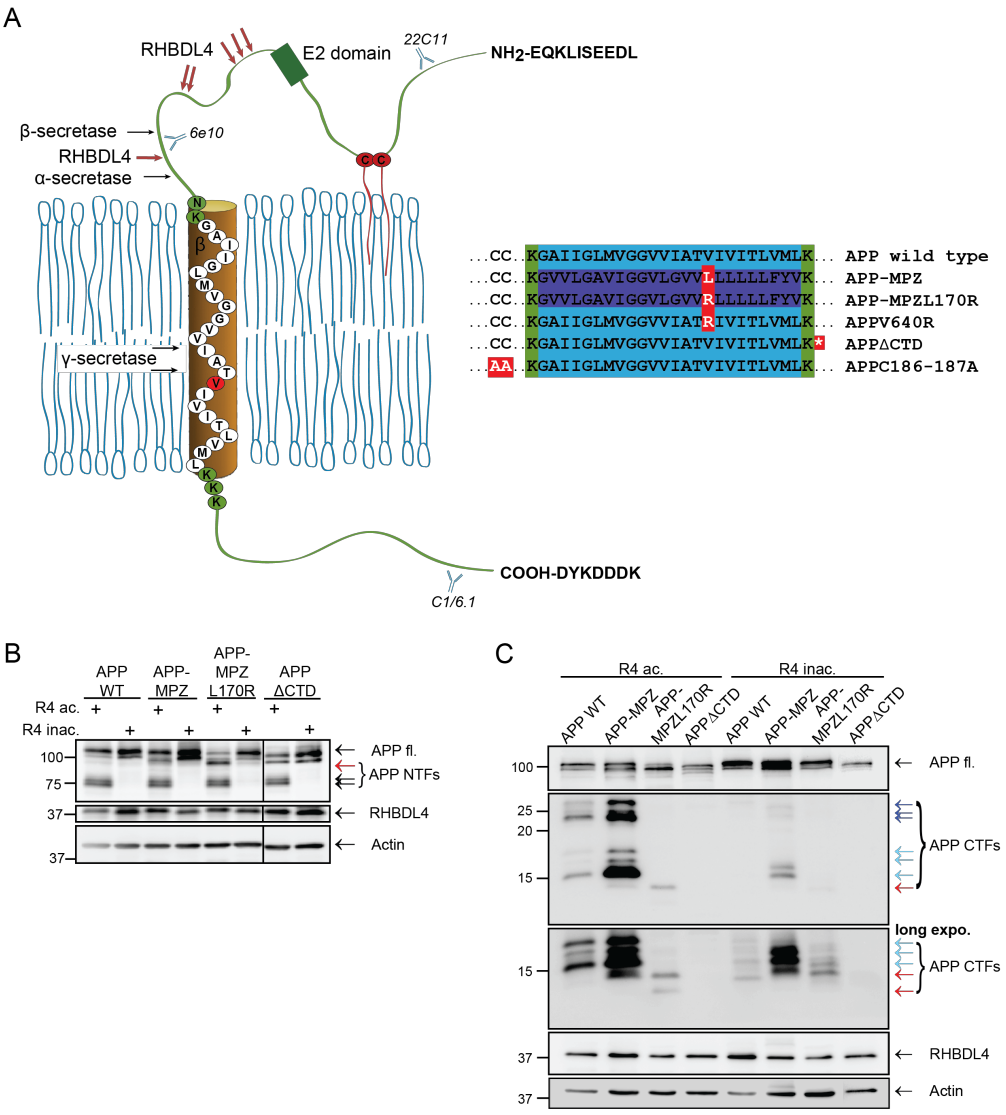

Figure 1: RHBDL4 exhibits a dual cleavage mode mechanism

A) Schematic of APP wild type (WT) with detailed transmembrane sequence and indicated cysteines residues at position 186 and 187 in the ectodomain, encircled in red. Black arrows indicate cleavage sites of canonical proteases involved in APP processing while red arrows point to RHBDL4 cleavage sites reported in Paschkowsky et al., 2016. Furthermore, shown on the right is a partial amino acid sequence of APP encompassing mutations in transmembrane region, ectodomain or deletion of the cytoplasmic tail. Antibodies used in this study are indicated in the schematic. All constructs express a C-terminal Flag and N-terminal Myc tag.

B) Analysis of full-length APP (APP fl.) and N-terminal fragments (APP NTFs) in HEK 293T cells co-expressing active (R4 ac.) or inactive RHBDL4 (R4 inac.). APP wild type, chimeric mutants (APP-MPZ and MPZ L170R) or APP lacking the C-terminal domain (APPΔCTD) co-expressed with RHBDL4 show a reduction in APP fl. levels and occurrence of 70-73 kDa NTFs (black arrows). Red arrow indicates a novel RHBDL4-specific larger NTF. APP full-length and NTFs were detected with 22C11, RHBDL4 was stained with anti-RHBDL4 and β-actin was used as loading control. Shown is a representative western blot of three independent experiments.

C) Comparison of RHBDL4-derived APP CTF pattern between WT and different variants. All CTFs derived from overexpression of an APP construct are labelled with anti-Flag antibody (2nd and 3rd panel). Detection of APP fl by

6E10 antibody. Co-expression of APP wild type and APP-MPZ with active RHBDL4 generated 15-27 kDa CTFs (indicated by dark and light blue arrows), red arrow indicates a novel smaller fragment for APP-MPZL170R. RHBDL4 was detected with anti-flag; β-actin was used as loading control. Shown is a representative western blot of three independent experiments.

In accordance with our previous report (Paschkowsky et al., 2016), co-expression of wild type APP with active RHBDL4 in comparison to inactive led to a decrease in full length APP and a concomitant increase in 70-73 kDa NTFs (Fig. 1B). Similar results were obtained upon co-expression of RHBDL4 with different APP TMS mutants; production of the 70-73 kDa NTFs was observed for all mutants (Fig. 1B). However, cleavage of APP-MPZ L170R generated an additional, larger fragment running around 90 kDa, in very close proximity to immature full length APP (indicated by a red arrow). This suggests the occurrence of an additional cleavage site for this mutant as compared to APP WT. We then proceeded to evaluate the CTFs pattern. Surprisingly, cleavage of APP-MPZ, but not APP-MPZ L170R, resulted in RHBDL4-specific CTFs in the range of 15-27 kDa similar to the wild type (note that these fragments migrate slightly higher than those described in Paschkowsky *et al*., due to the C-terminal flag tag, Fig. 1C (Paschkowsky et al., 2016)). Interestingly, APP MPZ L170R cleavage generated a fragment smaller than the thus far described, at approximately 12 kDa (indicated with red arrows in Fig. 1C). We also observed that the staining of APP CTFs derived from APP-MPZ cleavage was more pronounced upon active RHBDL4 co-expression in comparison with wild type APP. This suggests that APP-MPZ might be a better substrate for RHBDL4, which is further supported by the occurrence of APP CTFs upon co-expression with RHBDL4 inactive, implying that these fragments are derived from endogenous RHBDL4 activity. We formerly concluded, based on antibody-dependent epitope mapping, that the smallest wild type APP CTF derives from a cleavage between the cleavage sites of α- and β-secretases (Paschkowsky et al., 2016). Considering the apparent molecular weight of the newly generated small fragment of APP-MPZ L170R, RHBDL4 possibly cleaves this mutant within its TMS.

Furthermore, to investigate the relevance of the cytoplasmic APP domain, which was described to possess ubiquitination sites (El Ayadi, Stieren, Barral, & Boehning, 2012), for substrate recognition by RHBDL4, we also generated an APP mutant that lacks the C-terminal domain (APPΔCTD), but should still be anchored to the membrane (Fig. 1A). Co-expression with active RHBDL4 showed a decrease in full length APPΔCTD together with corresponding occurrence of NTFs similar to wild type APP (Fig. 1B). Thus, our results revealed that the APP cytoplasmic tail, and therefore potential ubiquitination of APP, is not required for RHBDL4-mediated APP ectodomain processing. APP-MPZ and APPΔCTD mutants are recognized and cleaved by RHBDL4 in the ectodomain; however, substitution with the TMS of MPZ L170R altered the cleavage mechanism of RHBDL4, instigating the formation of novel NTFs and CTFs.

1.3.2 Single amino acid substitution in the APP TMS circumvents initial cleavages in the ectodomain by RHBDL4

The differences in cleavage pattern observed between APP-MPZ and APP-MPZ L170R pertain to the subsequent question as to what drives the change in RHBDL4 processivity. Fleig *et al*. demonstrated that the presence of two positively charged amino acid residues in the TMS promotes RHBDL4-mediated ERAD of the membrane-bound substrate, possibly through destabilization of the helix embedded within the lipid bilayer (Fleig et al., 2012). Consequently, we generated an APP transmembrane point mutant, substituting the valine at position 640 with an arginine (APP V640R) and thereby mimicking the position of the arginine residue in the MPZ variant L170R (Fig. 1A). We hypothesize that APP V640R is degraded in the ER by RHBDL4 through a single cleavage near or within the TMS as opposed to the previously described, multiple RHBDL4 cleavages in the APP ectodomain. As expected, we observed the 70-73 kDa NTFs for APP V640R, but interestingly, we also detected an additional, novel NTF fragment at approximately 90 kDa similarly to MPZ L170R (indicated by red arrow in Fig. 2A). Detection of the corresponding CTFs

of APP V640R revealed that RHBDL4-specific fragments as seen for wild type APP were absent in this variant. However, a small fragment at approximately 12 kDa migrating at the same molecular weight as the upper small CTF from APP-MPZ L170R was detected in lysates from cells co-expressing the active but not inactive RHBDL4 **(**indicated by the upper red arrow in Fig. 2B**)**. The absence of the large RHBDL4-mediated APP CTFs for the V640R variant suggests that either the RHBDL4 cleavage pattern was altered or the mutation affects the overall stability of these CTFs, which may lead to faster degradation. To test if potential large CTFs from APP V640R were quickly degraded by the proteasome, cells were treated with the proteasomal inhibitor MG132. As shown in Figure 2C, the treatment enhanced the levels of induced myeloid leukemia cell differentiation protein 1 (Mcl-1), suggesting inhibition of the proteasomal degradation pathway. However, no enrichment in RHBDL4-specific 15-27 kDa CTFs was observed in cells expressing the APP variants. Hence, APP V640R and APP-MPZ L170R variants are likely processed differently by RHBDL4 compared to wild type APP. Strikingly, we observed elevated levels of the small CTFs derived from the APP V640R and APP-MPZ L170R variants upon MG132 treatment (indicated by the upper red arrow), suggesting proteasomal degradation for these fragments. Notably, the smallest CTF detected in lysates of cells overexpressing wild type APP with either active or inactive RHBDL4 (indicated by the pink arrow in Fig 2B-D), possibly generated from γ-secretase cleavage within APP TMS (Wolfe, 2012), ran about the same molecular weight as the novel fragment from APP V640R and APP-MPZ L170R cleavages (Fig. 2B-D). We therefore tested whether γ-secretase activity is required in the generation of the observed novel fragment. As expected, γ-secretase inhibition enhanced the signals for α/β-CTF (indicated by yellow arrow in Fig. 2D). However, the 12 kDa fragment of either APP mutants was not affected by γ-secretase inhibition indicating that they are indeed derived from RHBDL4 activity (Fig. 2D). Please note that we observed faint signals for APP CTFs upon co-expression of

wild type APP with inactive RHBDL4, suggesting that they are derived from endogenous RHBDL4 activity. This, however, was not the case for the APP mutants (Fig. 2D) implying that the two cleavage modes apply not only to overexpressed RHBDL4, but also endogenous RHBDL4. Consequently, the exchange of a single amino acid in the TMS of the RHBDL4 substrate APP seemingly triggers a change in the RHBDL4 cleavage mode. While wild type APP and APP-MPZ may predominantly be cleaved in their ectodomains generating large C-terminal fragments, APP V640R or APP MPZ L170R may redirect the RHBDL4 cleavage site towards the juxtamembrane region/TMS, precluding initial ectodomain cleavages.

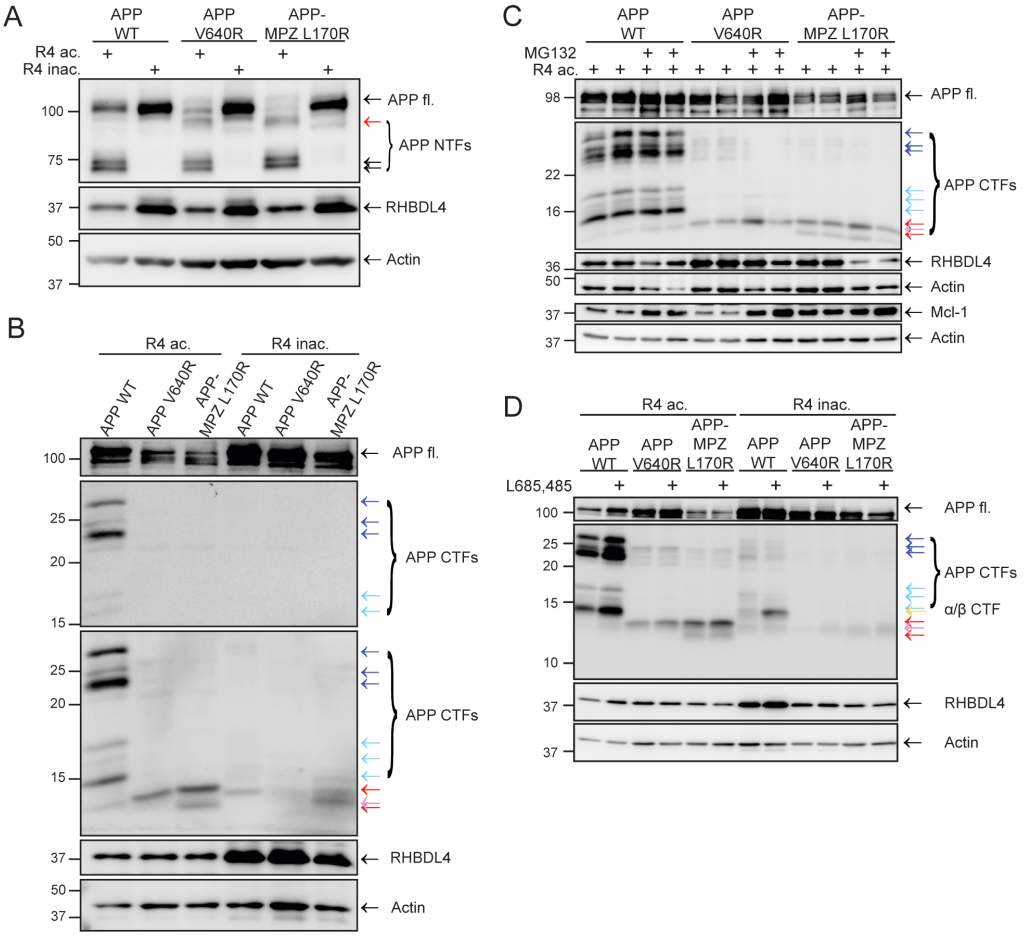

Figure 2: The substrate transmembrane sequence confers different cleavage modes of RHBDL4
A) Co-transfection of APP wild type, APP V640R or APP-MPZ L170R together with active or inactive RHBDL4 in HEK 293T cells. APP fl and 70-73 kDa NTFs are indicated by black arrows; novel large NTF is indicated by a red arrow (detection with 22C11). RHBDL4 was stained with anti-RHBDL4; β-actin was used as loading control. Shown is a representative western blot of four independent experiments.

B) Analysis of RHBDL4-specific CTF cleavage pattern. Co-expression of RHBDL4 active and inactive together with APP wild type, APP V640R or APP-MPZL170R. Dark and light blue arrows indicate previously published RHBDL4-derived 15-27 kDa CTFs. Red arrows indicate RHBDL4-specific smaller CTFs that are C1/6.1- but not 6E10-reactive. Pink arrow indicates an additional smaller fragment that was only detected for WT and ran likely between the two CTFs generated from the APP variants. APP fl labelled with C1/6.1, RHBDL4 detected with anti-RHBDL4 and β-actin used as loading control. Shown is a representative western blot of four independent experiments.
C) 30h post transfection cells co-expressing APP variants and RHBDL4 were treated with MG132 for 16 h. As expected, treatment increased Mcl-1 levels in all samples. No effect on 15-27 kDa RHBDL4-specific CTFs (indicated by dark and light blue arrows) was observed; however, novel C1/6.1-reactive CTFs (indicated by red arrows) accumulated upon proteasome inhibition. APP fl labelled with C1/6.1, RHBDL4 detected with anti-RHBDL4 and β-actin used as loading control. Shown is a representative western blot of three independent experiments.
D) HEK 293T cells co-expressing RHBDL4 and APP variants were treated for 12h with a γ-secretase inhibitor (L685,485). Accumulation of α/β-CTF (indicated by a yellow arrow) but no effect on RHBDL4-derived CTFs was observed. APP fl and CTFs detected with C1/6.1, RHBDL4 stained with anti-flag; β-actin used as loading control. Shown is a representative western blot of four independent experiments.

1.3.3 Cysteine residues possibly implicated in the APP ectodomain back folding to the membrane are not required for RHBDL4-mediated processing

The RHBDL4-mediated APP ectodomain cleavages between the TMS and E2 domain (Fig. 1A) suggest that this region must gain access to the active center of RHBDL4 through a still unknown mechanism (Paschkowsky et al., 2016). Notably, the structure of the E2 domain was solved, but no structure was determined thus far for the linker region between E2 and the TMS. We therefore hypothesized that the proposed flexibility of this linker region would facilitate a close proximity of the APP ectodomain to the membrane surface, for instance, by back folding (Kaden, Munter, Reif, & Multhaup, 2012; Reinhard et al., 2005). Interestingly, the notion of back folding has been studied in APP wherein the ectodomain has been described to anchor to the membrane upon palmitoylation of two critical cysteines (C186 and 187) in the ectodomain (Bhattacharyya et al., 2013). As such, we exchanged the cysteine residues to alanine (C186-187A) to potentially abolish palmitoylation at these sites, and consequently, we anticipated cleavage abrogation if back folding through these cysteine residues would be important (Fig. 1A). However, the APP variant C186-187A was processed similarly as the wild type APP based on NTF as well as CTF generation suggesting that those ectodomain cysteine residues may not play a major role in RHBDL4-mediated APP processing (Fig. 3A, B).

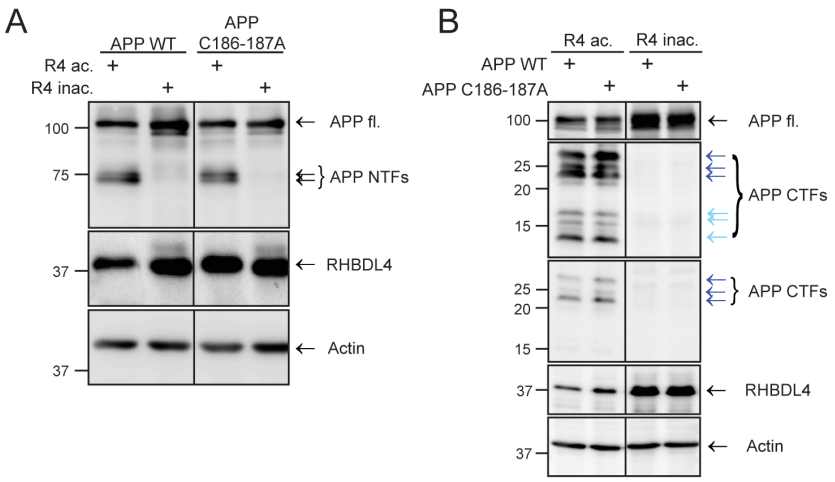

Figure 3: Cysteine mutants do not affect RHBDL4-mediated ectodomain cleavages of APP
A) Upon co-expression of APP C186-187A with active RHBDL4, similar NTFs as compared to WT and reduced APP fl were detected with 22C11. RHBDL4 stained with anti-flag; β-actin used as loading control. Shown is a representative western blot of three independent experiments.
B) Co-expression of RHBDL4 active and APP C186-187A resulted in the generation of 15-27 kDa CTFs, similar to WT. RHBDL4-specific large CTFs were labelled using 6E10 (Panel 3) and C1/6.1 stained large and small CTFs (Panel 2; dark and light blue arrows, respectively). Detection of RHBDL4 with anti-RHBDL4; β-actin used as loading control. Shown is a representative western blot of three independent experiments.

1.4 Discussion

While bacterial rhomboid homologues were the first intramembrane proteases to be crystallised (Y. Wang et al., 2006), research on mammalian rhomboid proteases is still at its infancy. Nevertheless, several research groups, including ours, recently elucidated potential physiological functions of several human homologues by identifying novel substrates and further linking them to diseases (Adrain, Zettl, Christova, Taylor, & Freeman, 2012; T. L. Cheng et al., 2014; Christova, Adrain, Bambrough, Ibrahim, & Freeman, 2013; Johnson et al., 2017; McIlwain et al., 2012; Paschkowsky et al., 2016; Shi et al., 2011; W Song et al., 2015). The description of RHBDL4-mediated APP processing as a novel APP processing pathway does not only potentially link rhomboid proteases to Alzheimer's disease, but could also shed light on the elusive physiological functions of APP (Muller, Deller, & Korte, 2017; Paschkowsky et al., 2016). Using antibody-based

epitope mapping, we previously concluded that RHBDL4-mediated cleavages predominantly occur in the APP ectodomain, a rather unexpected result, considering that intramembrane proteases have the unique ability to cleave membrane proteins within their transmembrane helices. It is, however, noteworthy to highlight that a crystal structure of a bacterial rhomboid displayed an active site exposed to the lumen and cleavage can take place outside of the transmembrane domain depending on the position of the substrate recognition motif (Wang et al., 2006; Strisovsky et al., 2009). Consequently, we sought to unravel the mechanism as to how RHBDL4 cleaves APP. In this regard, it is important to note that a previous publication implicated RHBDL4 in ERAD (Fleig et al., 2012), alluding to the importance of the ubiquitin interacting motif of RHBDL4 and the acquisition of helix destabilizing residues in the substrate TMS as prerequisites for RHBDL4-mediated ERAD substrate recognition.

Intrigued by this data, we generated a chimeric APP construct containing the TMS of a proposed RHBDL4 substrate (APP-MPZ L170R), assuming that RHBDL4-mediated APP processing would still take place, or containing a TMS that does not undergo RHBDL4-mediated ERAD (APP-MPZ), anticipating that RHBDL4 would not cleave this APP chimera. Unexpectedly, however, we observed two potentially distinct cleavage modes of RHBDL4: APP-MPZ was processed similar to wild type APP in the ectodomain while APP-MPZ L170R showed generation of NTFs, but absence of previously reported RHBDL4-specific APP CTFs. Interestingly, APP-MPZ L170R generated two smaller CTFs distinct from the previous APP CTFs reported. Based on the size of the smallest fragment as well as the evident lack of the 6E10 epitope, we propose that RHBDL4 initially cleaves in very close proximity to or within the transmembrane domain of this mutant and subsequently cleaves the soluble ectodomain to generate the 70-73 kDa NTFs. Fleig *et al.* demonstrated that the CMT disease-associated leucine to arginine mutation in the MPZ TMS induces RHBDL4-mediated ERAD of the protein, whereas the wild type form of MPZ is not

cleaved nor processed through ERAD by RHBDL4 (Fleig et al., 2012). Hence, it is possible that RHBDL4 preferentially processes its substrate by cleaving within the TMS, similar to other intramembrane proteases, and solely specific conditions would trigger ectodomain cleavages. Accordingly, APP processing by RHBDL4 is perhaps subsidiary, occurring depending on the availability of unsaturated enzyme or in a disease setting. It is therefore enticing to decipher the key driver of the RHBDL4-APP processing pathway. Moreover, it will be intriguing to investigate for possibly naturally occurring variants that result from mutations in the TMS of APP, rendering the APP TMS a prime substrate for RHBDL4.

The differences in cleavage pattern observed between the APP variants, APP-MPZ and MPZ L170R, may be attributed to certain characteristics of the amino acids encompassing the transmembrane domain. Fleig *et al.* proposed that the occurrence of at least two basic amino acids (e.g. arginine and lysine, also termed "degron" motif) in the transmembrane domain could increase the susceptibility of the membrane-bound substrate for RHBDL4-triggered turnover in the ER, perhaps due to helix-destabilizing effects of these amino acids (Bonifacino, Suzuki, & Klausner, 1990; Fleig et al., 2012). Although, our knowledge regarding mammalian rhomboid proteases and their substrates is limited to date, their bacterial homologues have been crystallized and well-characterized (E Arutyunova et al., 2014; Lemieux, Fischer, Cherney, Bateman, & James, 2007; Strisovsky & Freeman, 2014; Strisovsky et al., 2009; Urban & Baker, 2008; Y. Wang et al., 2006). These known bacterial rhomboids were shown to cleave non-physiological substrates containing helix-destabilizing residues wherein mutations reduced the processing efficiency of the enzyme (Strisovsky et al., 2009). Considering the structural similarity between bacterial rhomboids and RHBDL4 (M. K. Lemberg & M. Freeman, 2007b), their substrate recognition mechanisms likely intersect. For GlpG, a two-stage catalytic mechanism was proposed: TMS 2 and 5 form an exosite, which retains the substrate with a partially unfolded TMS and therefore increases the likelihood of

transfer to the scissile complex (Strisovsky, 2016). As such, we substituted the APP valine at position 640 to an arginine, mimicking the mutation L170R in MPZ to potentially destabilize the APP TMS in a similar manner. Strikingly, the previously described CTFs derived from ectodomain cleavages were abolished, similar to what we had observed for APP-MPZ L170R. In addition, we again detected 70-73 kDa large NTFs and one smaller CTF, which was enriched upon MG132 treatment. We therefore suspect that these APP variants containing a "degron" motif are rapidly degraded by the proteasome, likely via RHBDL4-mediated ERAD. Furthermore, our findings contribute to the general idea that unstable helical substrate transmembrane regions are a pivotal instigator of rhomboid protease cleavage (Fleig et al., 2012; Strisovsky et al., 2009; Urban & Freeman, 2003). Alternatively, arginine residues were proposed to stabilize TMS sequences and it is possible that APP V640R interacts more tightly with RHBDL4 prompting its cleavage within or near the membrane. The burial of a positively-charged arginine residue may reduce the energy cost of membrane insertion by snorkeling the side chain into the headgroup region of the membrane and by local deformation of the lipid bilayer (Ulmschneider et al., 2017). However, Ulmschneider *et al*. also suggest that due to its membrane composition, the membrane fluidity of the ER (where the RHBDL4-mediated processing takes place) may be decreased. As a result, it might not be able to accommodate the burial of an arginine, which may ultimately increase the energy cost. Consequently, the interaction between RHBDL4 and arginine-containing APP variants in the ER membrane may not necessarily be more favorable than wild type APP. Thus, a stabilized TMS is less likely to be the reason why RHBDL4 preferentially cleaves within or near the TMS of these variants as opposed to the ectodomain of wild type APP.

We also hypothesize that the dual cleavage mode of RHBDL4 may give further insight to a potential physiological role of this enzyme. It was previously shown that rhomboid pseudo-protease, iRhom2 is important in guiding the tumor necrosis factor-alpha converting enzyme

(TACE) out of the ER (Adrain et al., 2012; Christova et al., 2013; McIlwain et al., 2012). Similarly, RHBDL4 might modulate the ER exit of protein, in particular APP, a concept that previously has been proposed for RHBDL4 (Wunderle et al., 2016). In this regard, turnover of immature, misfolded APP could be promoted by specific conditions. We have evidence that the membrane composition might impact on RHBDL4 activity, specifically membrane cholesterol (Paschkowsky, Recinto, et al., 2018). This could be in line with various observations that the membrane lipid composition can confer activity and specificity to intramembrane proteases (Paschkowsky, Oestereich, & Munter, 2017).

Lastly, we investigated whether specific domains in APP mediate substrate recognition and affect enzyme processivity. The cytoplasmic tail of APP (APPΔCTD) has multiple protein-protein interaction domains and is thought to bridge interacting proteins (De Strooper & Annaert, 2000; Minopoli et al., 2001). Furthermore, polyubiquitination of the intracellular domain was demonstrated to be important in regulating APP trafficking to the plasma membrane (El Ayadi et al., 2012). Our findings, however, revealed that the C-terminal domain is not necessary for RHBDL4-mediated APP processing as the levels of mature C-terminally truncated APP diminished upon co-expression with the active protease and similarly, RHBDL4-derived NTFs were detected. Notably, we did not observe the occurrence of CTFs as they are likely too small to be detected in our gel system. Likewise, we demonstrated that RHBDL4-mediated APP processing does not require cysteine residues at position 186 and 187 that were previously reported to induce back folding through palmitoylation, allowing anchoring of the APP ectodomain to the membrane (Bhattacharyya et al., 2013). Please note, that other possible posttranslational modifications that could induce such folding were not exploited in this study. However, based on our present findings, back folding of the APP ectodomain to the membrane is a rather unlikely explanation as to how RHBDL4 cleaves APP within its linker region. Nevertheless, another idea arose: RHBDL4 could

have a higher propensity to interact with unstructured, highly flexible regions that mediate ectodomain cleavages. Ultimately, this would suggest a potential chaperone-like activity of RHBDL4 similar to Derlins or iRhoms (Adrain et al., 2012; Christova et al., 2013; Lemberg & Adrain, 2016).

1.5 Conclusion

In our present study, we demonstrated that RHBDL4 exhibits two distinct cleavage modes that are potentially distinguished based on the composition of the substrate transmembrane sequence. Elucidating the proteolytic mechanisms of RHBDL4 could provide insight into its regulatory mechanisms, which could be exploited for novel therapeutic approaches, especially with regard to the implications of RHBDL4 in various diseases.

1.6 Methods

1.6.1 DNA constructs

Plasmid pCMV6 containing cDNA encoding for human RHBDL4 with a C-terminal myc and flag tag was obtained from OriGene, USA. Inactive RHBDL4 S144A was generated by site directed mutagenesis using the forward primer 5'-GCTGTAGGTTTCGCAGGAGTTTTGTTT-'3 and reverse primer 5'-AAACAAAACTCCTGCGAAACCTACAGC-'3. APP695 in pcDNA3.1 (Invitrogen) was provided as a kind gift of Dr. Claus Pietrzik, Johannes Gutenberg University, Mainz, Germany, and was tagged at the N-terminus with myc and at the C terminus with flag tag, which served as a template for all APP mutants described. APP mutants were cloned using gene blocks purchased from IDT. Gene blocks were dissolved in H_2O at a concentration of 10ng/µL of which 4ng/µL were used in a restriction enzymatic reaction with EcoRI and XbaI, at 37°C for 16-18 h. Enzymes were subsequently heat inactivated at 65°C for 20 mins and stored at -20°C for later use. Simultaneously, 1 µg of pcDNA3.1 N-myc APP695 C-flag plasmid was digested by the same restriction enzymes at 37° for 60 mins followed by alkaline phosphatase treatment to avoid

re-ligation of the vector, heat inactivation and purification from gels. 50 ng of vector were ligated in different ratios with digested gene blocks using T4 DNA ligase (NEB) at room temperature overnight (16-18h). After transformation into Top10 cells via heat shock, cells were plated and incubated overnight at 37°C. DNA constructs of single colonies were then purified (Qiagen) and full sequences confirmed using DNA sequencing by Genome Quebec.

1.6.2 Cell culture and transfection

HEK293T cells, passaged at 80-90% confluency, were used for all transient transfections. Cells were cultured in Dulbecco´s modified Eagle medium (DMEM) containing 4.5 g/l glucose, 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate (Wisent) and supplemented with 10% FCS (Wisent), at 37°C and 5% CO_2. For 6-well or 12-well plates, 6 x 10^5 cells/well or 2 x 10^5 cells/well were seeded, respectively, and 24 h later, transiently transfected with 2 µg DNA per 4 µL polethylenimine (PEI) or 1 µg DNA per 2 µL PEI, respectively. For co-transfections, a 5:1 ratio of APP to RHBDL4 was used. 36 h post-transfection, cells were lysed with TNE-lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, and complete protease inhibitors, Roche) and prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 6 x SDS sample buffer (2 M Tris/HCl pH 6.8, 20% SDS, 100% glycerol, bromophenol blue, 10% β-mercaptoethanol) was added to the samples for a final concentration of 1 x.

1.6.3 Inhibitor treatments

20 h or 36 h post-transfection, cells were treated with 2 µM proteasomal inhibitor MG132 for 16 h or 1 µM γ-secretase inhibitor L685,485 (Tocris) for 12 h, respectively. DMSO was used as vehicle control. Cells were then lysed according to the aforementioned procedure. 4 x LDS sample buffer (with 10% β-mercaptoethanol, Invitrogen) was added to the lysates for a final concentration of 1 x.

1.6.4 Western blot and data analysis

Samples were separated on 10% (for NTFs) and 15% (for CTFs) tris-glycine gels by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes with transfer buffer containing 10% ethanol. The following primary antibodies were used: 22C11 (Millipore), 6E10 (Covance), C1/6.1 (Biolegend), anti-RHBDD1 (Sigma), mouse-anti-myc (9B11, Cell Signaling), mouse-anti-β-actin (8H10D10, Cell Signaling), rabbit-anti-Flag (D6W5B, cell signaling). Horseradish peroxidase (HRP)-coupled secondary antibodies directed against mouse or rabbit IgG were purchased from Promega. Chemiluminescent images were acquired using the ImageQuant LAS 500 or 600 system (GE Healthcare).

1.7 Acknowledgements

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MANUSCRIPT 2: Human rhomboid RHBDL4 as a modulator of cellular glucose metabolism

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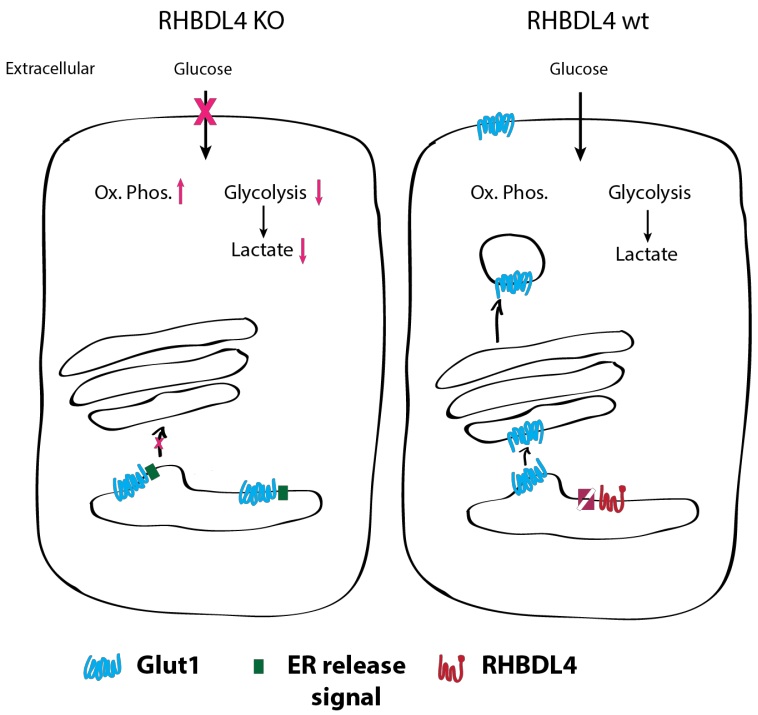
To be submitted for publication



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2.1 Abstract

Insight into the mammalian rhomboid protease RHBDL4 has only recently advanced owing to significant breakthroughs in identifying their substrates and potential cellular roles. Nevertheless, many of the proposed functions have been demonstrated in an overexpression cell system and have not been confirmed *in vivo.* We found that abrogation of the enzyme retained glucose transporter 1 (GLUT1) in the ER, thereby compromising cellular glucose metabolism. While overall systemic metabolic profile of Rhbdl4-deficient mice remained unaltered, endothelial cells in their cerebral microvessels exhibited diminished GLUT1 levels. Collectively, we present a physiological role of RHBDL4 in regulating the trafficking of GLUT1 to the cell surface.

2.2 Introduction

Rhomboid proteases are serine intramembrane proteases initially discovered in 1984 by Nusslein-Volhard & Wieschaus (Nusslein-Volhard et al., 1984). A determining turning point in the study of rhomboids was the solving of the crystal structure of the bacterial GlpG more than a decade ago (Y. Wang et al., 2006). Presently, bacterial rhomboids were shown valuable players in quorum-signal transduction pathway (Rather, Ding, Baca-DeLancey, & Siddiqui, 1999), glucose transport and cell division (Mesak, Mesak, & Dahl, 2004), antibiotic resistance and host parasitic invasion (Kateete et al., 2012). In particular, rhomboid GluP acts as a glucose exporter wherein loss of GluP impaired sporulation and cell division of *Bacillus subtilis*. Notably, bacterial cells deficient in GluP were also less efficient at importing radiolabelled glucose as compared to wild type (Mesak et al., 2004). Rhomboid proteases are believed to have been, later on, acquired by archaea and eukaryotes through various horizontal gene transfer (Koonin et al., 2003).

Enticed by this highly conserved, large family of intramembrane proteases, we draw an even closer look to the human rhomboid protease structurally most closely related to bacterial rhomboids. Rhomboid-related protein-4 (RHBDL4) possesses six membrane-spanning helices

with cytoplasmic N- and C-terminal domains residing in the endoplasmic reticulum (ER). RHBDL4 was reported to participate in key cellular pathways essential for protein quality control and cell proliferation, namely ER-associated degradation (ERAD) and epidermal-growth factor (EGF) signaling, respectively (Fleig et al., 2012; Wunderle et al., 2016). Earlier studies also postulated its overexpression in glioblastoma, breast and colorectal human cancer tissues (Miao et al., 2017; W. Song et al., 2015; Stangeland et al., 2015; M. Zhang et al., 2018; X. Zhang et al., 2018). Meanwhile, recent findings from our lab implicated RHBDL4 in Alzheimer's disease (AD). A major hallmark of AD pathology is the production of amyloid β (Aβ) peptides from sequential cleavages of the amyloid precursor protein (APP) by β- and γ-secretases. We demonstrated an efficient, non-amyloidogenic processing of APP mediated by RHBDL4 *in vitro* (Paschkowsky et al., 2016). Independently, we made the surprising observation that the colour of the cell culture media from Rhbdl4-deficient and wild type cells are different, rendering us to hypothesize an involvement of RHBDL4 in glucose metabolism as well.

Mammalian cells are heavily dependent on glucose oxidation as main source of carbon skeletons and ATP production under oxygen-deprived condition. Consequently, glucose uptake is considered the rate-limiting step in glucose metabolism. Glucose exchange across cell membrane is mediated by two families of glucose transporters: concentrative sodium glucose transporters (SGLT) and facilitative glucose transporter (GLUT). The latter belongs to a larger family of solute carriers and has a more widespread tissue expression [reviewed in (Mueckler & Thorens, 2013)]. The first GLUT purified and one of the most extensively studied homologues is GLUT1 (Mueckler et al., 1985). Given its high affinity for glucose as well as its ubiquitous distribution, GLUT1 is regarded responsible for basal glucose uptake, predominantly in endothelial cells of blood-tissue barriers (Koranyi et al., 1991; Yeh et al., 2008). A wide array of studies established different modes of regulations at both genomic and proteomic levels. Hypoxia induces transcriptional activation of

GLUT1 while stimulation of protein kinase C (PKC) and phosphatidylinositol 3-kinase-Akt (PI3K-Akt) signaling pathway affect its cell surface translocation (Chen et al., 2001; Flier et al., 1987; E. E. Lee et al., 2015; Wieman et al., 2007). The precise mechanism of these pathways and how they intertwine are yet to be discerned. Furthermore, downregulation of GLUT1 expression was associated in AD (Simpson et al., 1994) while its upregulation is observed in tumors (Brown & Wahl, 1993; Haber et al., 1998; Yamamoto et al., 1990)..

In the present study, we introduce a novel, putative function of RHBDL4 in glucose metabolism. Immortalized mouse embryonic fibroblasts (MEFs) lacking Rhbdl4 manifest diminished glucose consumption as well as reduced glycolytic flux compared to wild type cells. We further observe that total and cell surface Glut1 levels are reduced in Rhbdl4-deficient MEFs without changes in mRNA expression. However, Rhbdl4-deficient mice do not exhibit overt signs of systemic metabolic dysregulation. Isolation of cerebrovascular endothelial cells from these animals suggested *in vivo* evidence for a compromised Glut1 regulation. Our observations thus raised the intriguing possibility that ER-resident RHBDL4 controls the trafficking of GLUT1 to the cell surface in a cell type-specific manner through a still unidentified mechanism of action.

2.3 Results

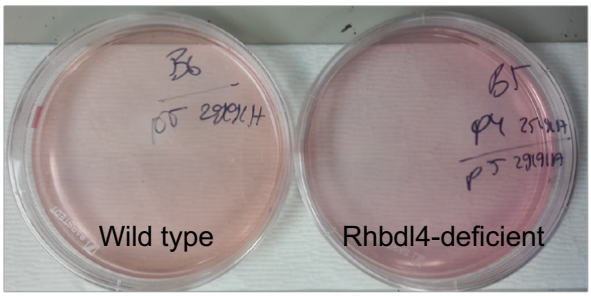
2.3.1 Rhbdl4-deficient MEFs exhibit reduced glycolytic flux and diminished total Glut1 levels without changes in mRNA expression

Owing to a deliberate scrutiny, we noticed that the cell culture supernatant of cultivated mouse embryonic fibroblast (MEF) lacking the rhomboid protease Rhbdl4 did not change in colour after several days of cultivation as compared to that of wild type MEF (Fig. 1A). Cultivating immortalized eukaryotic cells requires the presence of nutrients in the extracellular media for adequate growth and survival. As such, wild type MEFs, similar to most mammalian proliferating cells, readily consume extracellular glucose and release excess lactate as by-product through

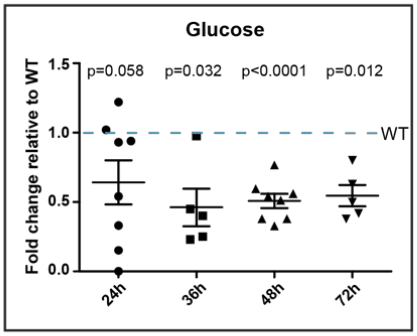
aerobic glycolysis [reviewed in (Jones & Bianchi, 2015)], engendering a downward shift in pH towards more acidic conditions as indicated by a shift of the phenol red towards yellow. Strikingly, we did not discern a change in color of the pH indicator in the cell culture supernatant of cultivated Rhbdl4-deficient cells within the span of seven days, thereby suggesting a lesser degree of acidification. To confirm our predictions, using an automated, electrochemical Nova BioProfile analyzer, we measured glucose and lactate concentrations in the cell culture supernatant of Rhbdl4-deficient and wild type MEFs cultivated for 24, 36, 48 and 72 hours. Rhbdl4-deficient MEFs consumed significantly about 2-fold less glucose than wild type at indicated time points (Fig. 1B). As expected, a similar effect was seen regarding lactate production wherein the difference in lactate release of Rhbdl4-deficient MEFs relative to wild type was most pronounced at earlier time points (Fig. 1C).

Upon uptake, cells metabolize glucose in a process known as glycolysis, to generate carbon intermediates for various biosynthetic reactions aside from providing energy reserves. In an oxygen-rich environment, the majority of ATP is synthesized from the glycolytic end-product, pyruvate, via oxidative phosphorylation in the mitochondria. In the case of oxygen deprivation, known as hypoxia, this energy-producing process is compromised rendering an upregulation of the glycolytic flux. The increase in pyruvate can be modulated by accentuating its conversion to lactate, which thus leads to an immense lactate secretion [reviewed in (J. Zheng, 2012)]. To discriminate potential disturbances in these cellular processes in Rhbdl4-deficient MEFs, we performed live cell metabolic measurements and determined oxygen consumption as well as extracellular acidification rates (OCR and ECAR) via a seahorse assay. As shown in Figure 1D, Rhbdl4-deficient MEFs depicted a significantly elevated OCR to ECAR ratio relative to wild type. Considering ECAR is reflective of glycolytic rate, our finding further insinuates the abating glycolysis and dysregulation of glucose metabolism consequent to the deletion of Rhbdl4.

A


Wild type
Rhbdl4-deficient

B


Glucose
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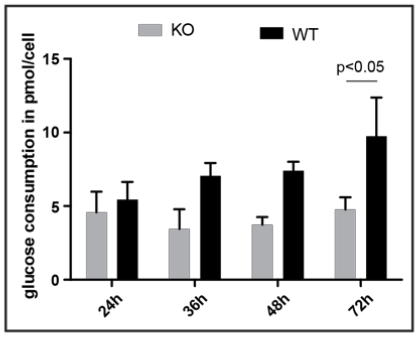
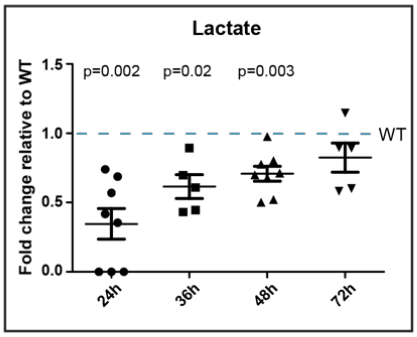
p=0.032
p<0.0001
p=0.012
WT
24h
36h
48h
72h


KO
WT
glucose consumption in pmol/cell
p<0.05
24h
36h
48h
72h

C


Lactate
Fold change relative to WT
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p=0.02
p=0.003
WT
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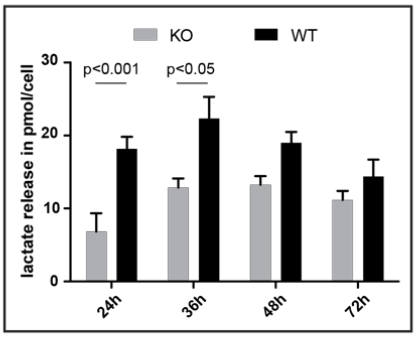
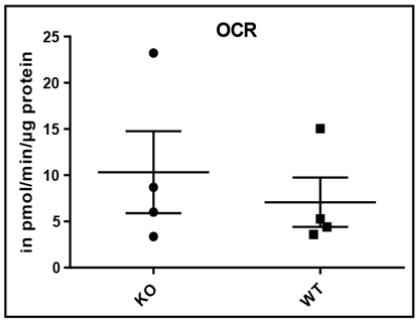
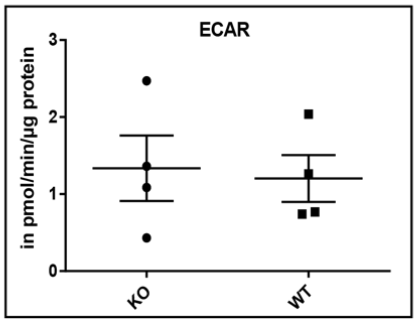
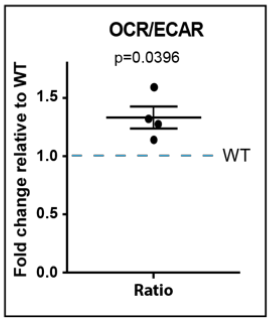
36h
48h
72h


KO
WT
lactate release in pmol/cell
p<0.001
p<0.05
24h
36h
48h
72h

D


OCR
in pmol/min/µg protein
KO
WT


ECAR
in pmol/min/µg protein
KO
WT


OCR/ECAR
p=0.0396
Fold change relative to WT
WT
Ratio

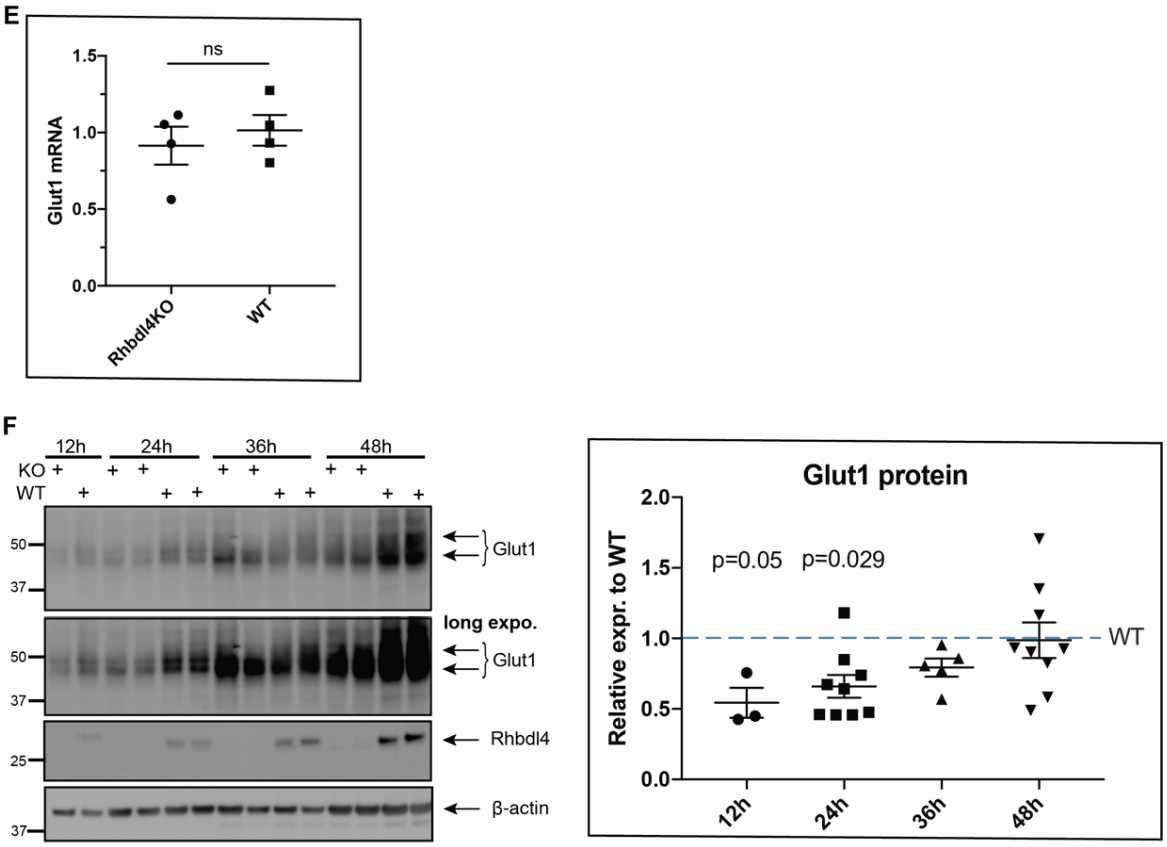


Figure 1: Rhbdl4-deficient MEFs display lower glucose uptake, glycolytic flux and Glut1 protein expression
A) Shown is an image of wild type (B6, left) and Rhbdl4-deficient (B5, right) MEFs 7 days after initial seeding in DMEM with 4.5 g/L glucose and 0.584 g/L glutamine, supplemented with 10% FBS. A change in the color of the cell culture media indicates a downward shift in pH due to excess production of lactate.
B, C) Supernatants of MEFs were collected 24, 36, 48 and 72 h post-seeding. Shown are the fold change in glucose consumption and lactate release of Rhbdl4-deficient MEFs relative to wild type (set to 1) as well as the relative concentrations in pmol/cell ± SEM, n=5,8. One-sample, t-test was conducted on the fold change data after a non-significant Shapiro-Wilks test while concentrations in pmol/cell were analyzed by unpaired, two-tailed t-test; p-values reported.
D) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured via seahorse analysis. Mean concentration as well as fold change in OCR to ECR ratio of Rhbdl4-deficient relative to wild type (set to 1) ± SEM are displayed, n=3. One sample, t-test was performed on the fold change data after a non-significant Shapiro-Wilks test; p-value reported.
E) Glut1 mRNA expression normalized to reference genes, RSP18 and PICALM. Mean ± SEM is displayed, n=4. Two-tailed, unpaired t-test performed after a non-significant Shapiro-Wilks test; p-values reported.
F) Glut1 protein levels following 12, 24, 36 and 48 hours normalized to β-actin. Shown are the fold change in Glut1 protein expression of Rhbdl4-deficient MEFs relative to wild type (set to 1) ± SEM, n=3-9. One-sample, t-test was conducted on the fold change data after a non-significant Shapiro-Wilks test; p-values reported. Shown is a representative western blot probed with anti-Glut1, anti-Rhbld4 and β-actin used as loading control.
Please note that initial observations and all data presented in figures 1A-1D were generated by SP.

Consecutively, we were intrigued whether the low glucose consumption can be attributed to insufficient expression of glucose transporters. Since GLUT1 governs basal glucose uptake and is widely expressed in most cell types, including fibroblasts, we performed quantitative real-time PCR to ascertain *Glut1* mRNA levels from wild type and Rhbdl4-deficient MEFs. Figure 1E displays the mean expression of *Glut1* in both cell types normalized to multiples reference genes chosen; analysis yielded a non-significant difference in mRNA levels. However, western blot analyses revealed that Rhbdl4-deficient MEFs exhibit weaker signals corresponding to endogenous Glut1 protein as well as fainter bands at the higher molecular weight, most likely to be ascribed to the glycosylated mature form of Glut1 (Fig. 1F). Subsequent quantification revealed a significantly diminished Glut1 protein expression as compared to wild type. Notably, the extent in the variability between the two cell types attenuates over time (Fig. 1F). Overall, our data alludes to a defect in posttranscriptional regulation of Glut1 that possibly underlies the decrease in glucose consumption, thereby diminishing the glycolytic flux as a consequence of Rhbdl4 deficiency.

2.3.2 Similar alterations in glucose metabolism of Rhbdl4-deficient MEFs transpired under hypoxia-mimicking condition

Cells subjected to hypoxia are known to induce GLUT1 transcription through the stabilization of hypoxia inducible factor 1 alpha (HIF1α) (Hayashi et al., 2004). Furthermore, lack of particular rhomboid proteases in yeast *Schizosaccharomyces pombe* and fungi, namely Rbd2 and RbdA, halted cell growth in hypoxic environment (Hwang et al., 2016; Vaknin et al., 2016). Accordingly, we investigated whether deletion of Rhbdl4 results in an altered response under hypoxia-mimicking conditions. Cobalt chloride ($CoCl_2$) is an inorganic salt commonly used to mimic hypoxia by stabilizing HIF1α, which under normoxic condition is rapidly degraded via the proteasome. In response to $CoCl_2$ treatment, HIF1α translocates to the nucleus whereby it binds to hypoxia response elements (HREs) and promotes the expression of target genes, including

GLUT1 (Yuan, Hilliard, Ferguson, & Millhorn, 2003). Glucose consumption and lactate release were determined 24 h following treatment with either $CoCl_2$ or H_2O as vehicle control. $CoCl_2$ exacerbated glucose consumption and lactate secretion compared to vehicle as anticipated in wild type cells. Interestingly, the responsiveness to hypoxia-mimicking conditions was comparable between both cell types although a trend towards lower glucose consumption and lactate production was noted for Rhbdl4-deficient MEFs (Fig. 2A and B). We subsequently contrasted Glut1 mRNA and protein expression for Rhbdl4-deficient and wild type MEFs treated with either vehicle or $CoCl_2$. Consistent with earlier reports, *Glut1* transcription was induced upon $CoCl_2$ treatment in both cell types (Fig. 2C). Accordingly, we observed an increase in Glut1 protein levels compared to vehicle treatment (Fig. 2D and 2E). It was striking that Rhbdl4-deficient MEFs displayed a significantly greater induction of *Glut1* mRNA than wild type, but no difference under basal condition was observed (Fig. 2C, see also Fig. 1E). Nonetheless, Glut1 protein levels were significantly reduced in MEFs lacking RHBDL4 compared to wild type despite the elevated mRNA expression in $CoCl_2$-treated condition (Fig. 2D and 2E). Another HIF1α-regulated gene related to glucose metabolism is the pyruvate dehydrogenase kinase 1 (PDK1). PDK1 inactivates the mitochondrial multienzyme complex, pyruvate dehydrogenase, which catalyzes the decarboxylation of pyruvate to synthesize acetyl-coA necessary for cellular respiration. Earlier studies demonstrated a buildup of glycolytic metabolites as a result of enhanced expression of PDK1 triggered by hypoxia or oxidative stress (Kim, Tchernyshyov, Semenza, & Dang, 2006; Papandreou et al., 2006). As such, we analyzed endogenous Pdk1 protein levels in response to $CoCl_2$ treatment in MEFs. Vehicle-treated wild type and Rhbdl4-deficient MEFs had comparable protein expression of Pdk1. Consistent with other reports, $CoCl_2$ significantly augmented Pdk1 levels both cell types although Rhbdl4-deficient MEFs exhibited lower Pdk1 levels as compared to wild type under hypoxia-mimicking condition (Figure 2D and 2F). We ultimately suggest that,

even during hypoxia, Rhbdl4 deficiency impairs cellular glucose metabolism due to deregulated posttranscriptional regulation of Glut1.

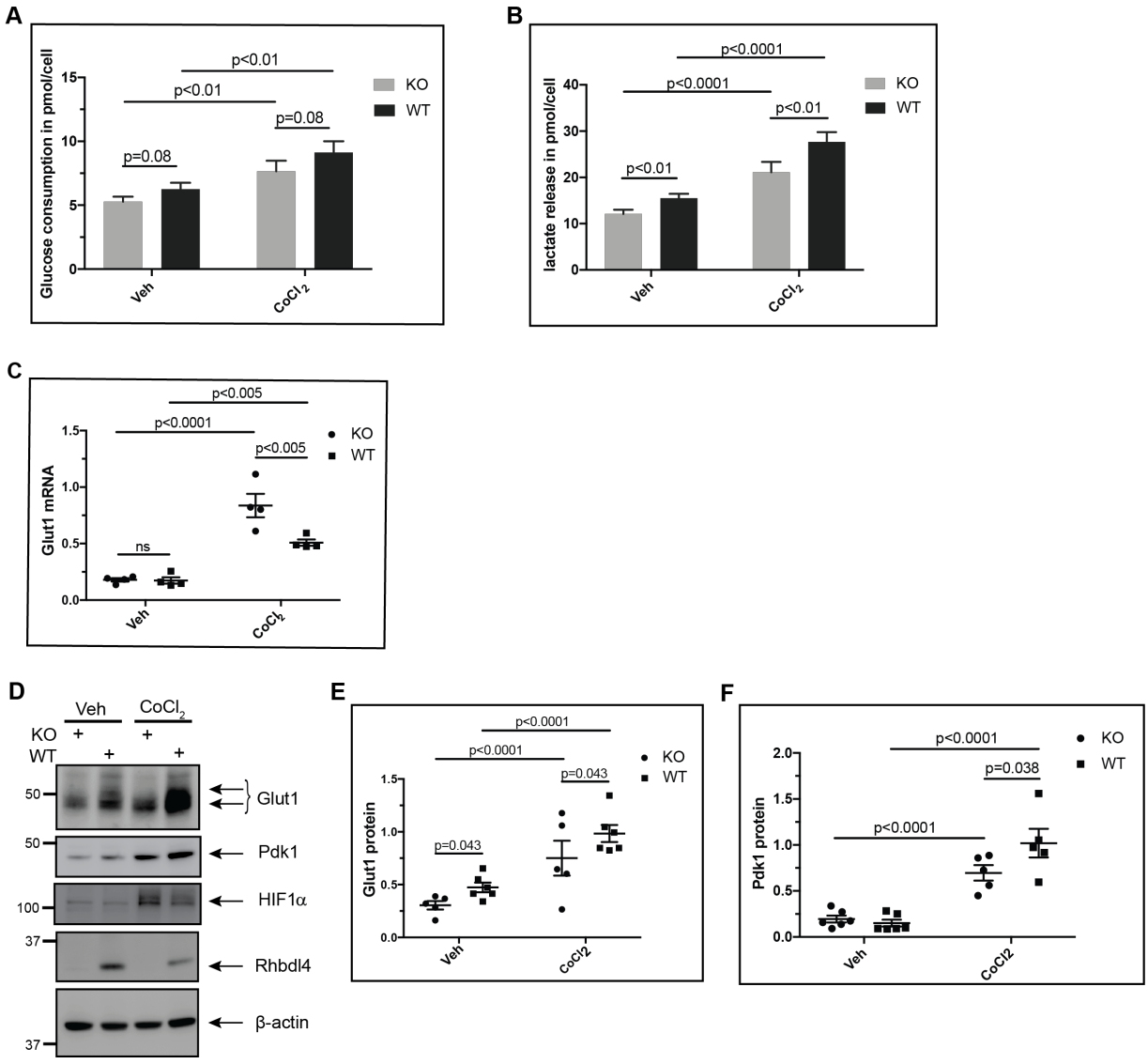


Figure 2: Rhbdl4-deficient MEFs exhibit deregulated glucose metabolism even under hypoxia-mimicking condition

Cells were either treated with H_2O (as vehicle control) or 100 µM $CoCl_2$ for 24 h.

A, B) Supernatants of MEFs were collected 24 h post-seeding. Shown is the glucose consumption and lactate release concentrations in pmol/cell ± SEM, n=9. Between-subject, two-way ANOVA conducted after a non-significant Shapiro-Wilks test; p-values reported.

C) Glut1 mRNA expression normalized to reference genes, RSP18 and PICALM. Mean ± SEM is displayed, n=4. Between-subject, two-way ANOVA performed after a non-significant Shapiro-Wilks test; p-values reported.

D) Shown is a representative western blot of 5 independent experiments stained with Glut1, Pdk1, Hif1α, Rhbdl4 and β-actin used as loading control.

E) Shown is mean expression of Glut1 protein normalized to β-actin ± SEM, n=5. Between-subject, two-way ANOVA conducted after a non-significant Shapiro-Wilks test; p-values reported.
F) Shown is mean expression of Pdk1 protein normalized to β-actin ± SEM, n=5. Between-subject, two-way ANOVA conducted after a non-significant Shapiro-Wilks test; p-values reported.

2.3.3 Metabolism of Rhbdl4-deficient MEFs distinct to wild type

Since glucose is the primary carbon and energy source that most proliferating eukaryotic cells utilize for cell growth and proliferation, we were perplexed as to whether the Rhbdl4-deficient MEFs are dependent on glucose at all. We initially addressed the possible impairments in proliferation upon deletion of the *Rhbdl4* gene by monitoring the number of adherent cells cultivated in media containing glucose, lipids and amino acids at 24 and 48 hours following a 24-hour period of cell growth. The lack of Rhbdl4 did neither slow down nor accelerate the proliferation of these cells as there were similar numbers of adherent wild type and Rhbdl4-deficient MEFs present at indicated timepoints determined with an automated cell counter (Fig. 3A). Meanwhile, when we deprived the cells of glucose, neither cell type proliferated to the same extent as when kept in the "full" media. Rhbdl4-deficient MEFs in glucose-deprived media, nonetheless, continued to slowly proliferate whereas wild type cells simply reached a plateau phase after 48 h (Fig. 3B). These staggering observations raise the likelihood of another, more indispensable nutrient on which Rhbdl4-deficient MEFs live on. We performed similar experiments using lipid-depleted media. Strikingly, similar results as with glucose-deprived media were obtained (Fig. 3C); proliferation of Rhbdl4-deficient MEFs is less contingent on the availability of extracellular glucose or lipids than wild type cells. The proliferation of these cells, however, declined following a 48 h cultivation in glutamine-depleted media similar to that of wild type (Fig. 4D). Albeit to a lesser degree, glutamine is a non-essential amino acid that is also used by mammalian cells for energy generation and biosynthetic reactions owing to its anaplerotic properties (DeBerardinis et al., 2007). The diminution in the demand for glucose and lipids while

enhanced susceptibility to amino acid deprivation, particularly glutamine, thus stipulate a change in metabolism upon abrogation of Rhbdl4 in MEFs.

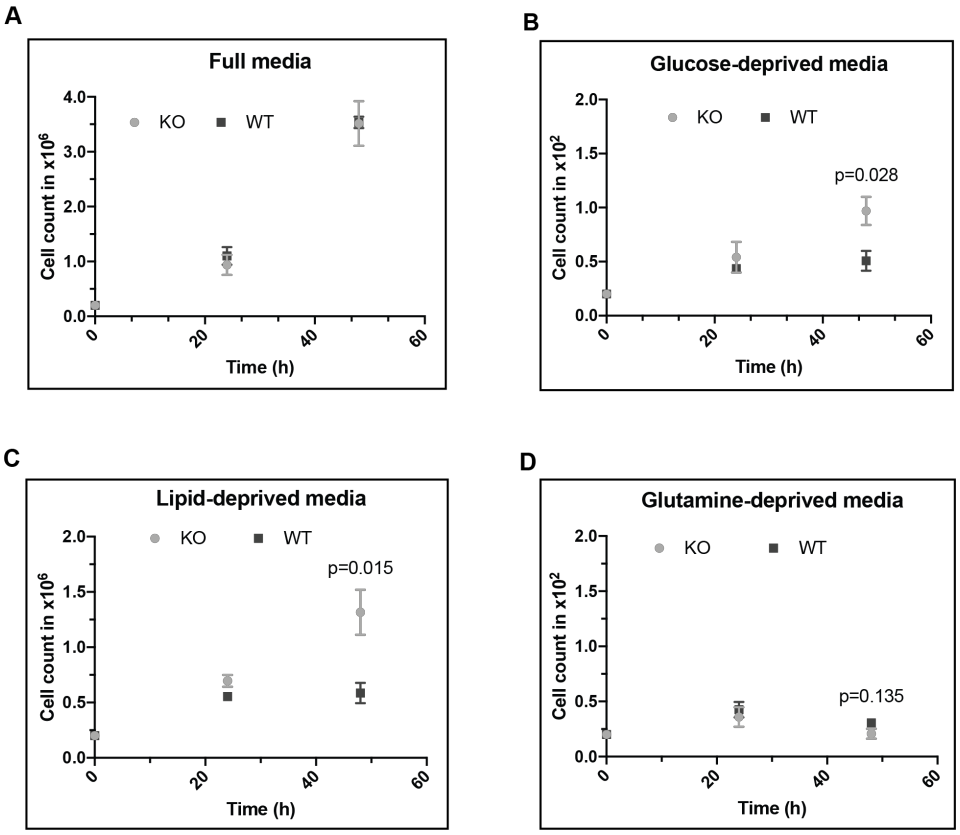


Figure 3: Glutamine is an essential source of energy reserve and carbon intermediates of Rhbdl4-deficient MEFs as opposed to glucose and lipids

Displayed is mean cell count in $x10^6 \pm$ SEM at 24 h and 48 h following a 24-hour period of cell growth, n=4,5. Rhbdl4-deficient and wild type MEFs are cultivated in cell culture media containing 4.5 g/L glucose and 0.584 g/L glutamine, supplemented with 10% FBS (A), depleted of glucose (B), supplemented with lipid-deprived serum instead of FBS (C) and depleted of glutamine (D). Two-tailed, unpaired t-test performed for each time point and condition after a non-significant Shapiro-Wilks test; p-values reported.

2.3.4 Characterization of Rhbdl4-deficient mice

Currently, only available *in vivo* data on Rhbdl4-deficient mice are taken from the open source mouse phenotyping consortium (www.mousephenotype.org). We therefore had a closer look on the Rhbdl4 distribution in certain mouse tissues, mainly those that are metabolically active (Z. Wang et al., 2010). As demonstrated in Figure 4A, Rhbdl4 expression is highest in the liver, but the enzyme is also expressed in the heart, pancreas and to a very low extent in the brain.

Interestingly, we did not observe Rhbdl4 expression in the kidneys, which is the organ that predominantly express ATP-dependent sodium glucose transporter 2 (X. X. Wang et al., 2017). Furthermore, we analyzed serum glucose, cholesterol and protein levels of male and female mice aged between 2 to 6 months old; no salient genotype-dependent differences were observed (Fig. 4B-D). Notably, Rhbdl4-deficient older males had significantly greater serum total protein concentration compared to wild type (Fig. 4D), which comprises of albumin and γ-globulin acting in humoral immunity, and is occasionally used as a nutritional biomarker (Takegawa et al., 2019). Serum cholesterol concentration was also higher amongst male mice, in general (Fig. 4C). Lastly, we observed that the total body weight of old Rhbdl4-deficient mice is lower than that of wild type mice. This observation was, nonetheless, sex-specific (Fig. 4E).

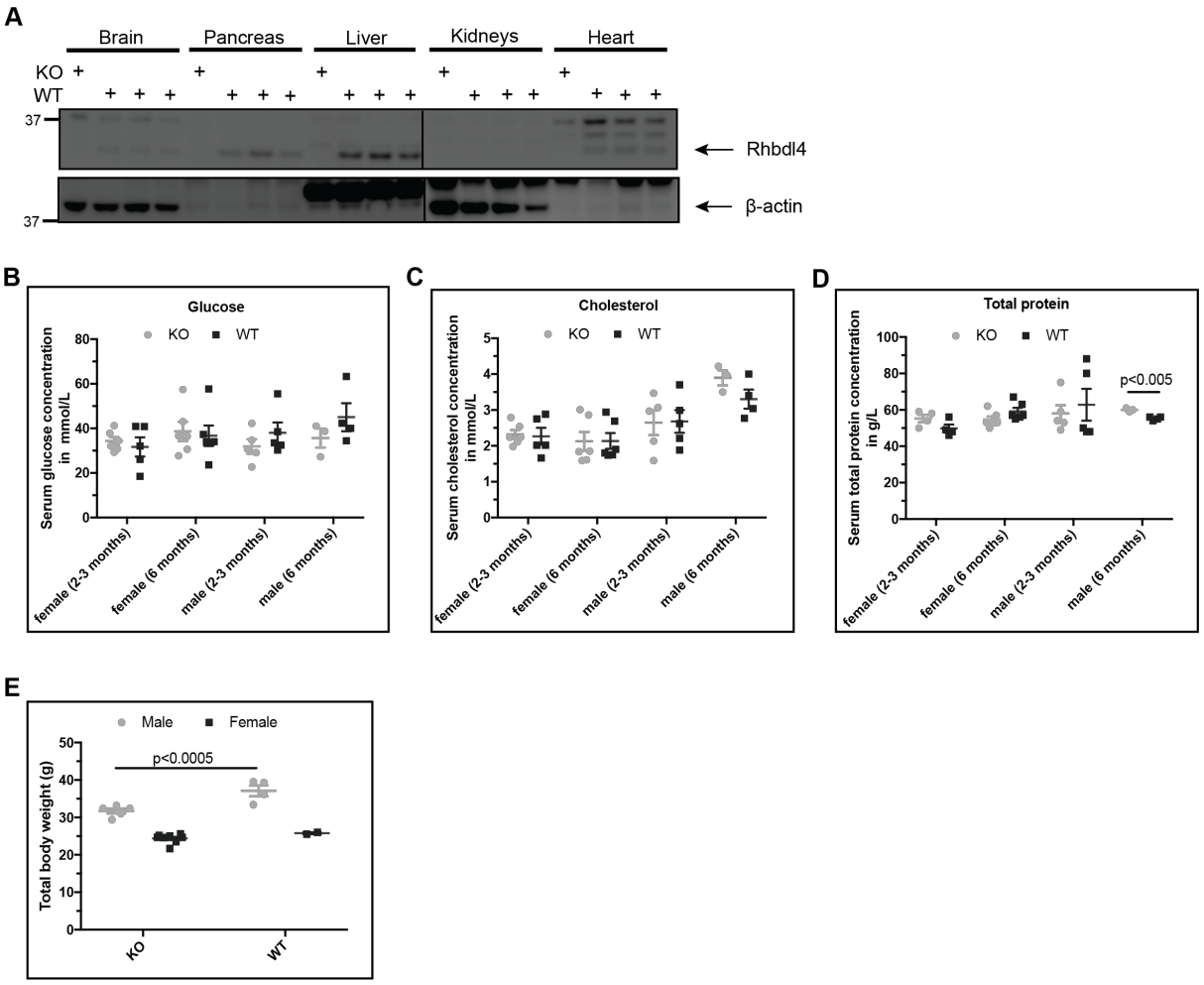


Figure 4: Characterization of Rhbdl4-deficient mice
A) Shown is a western blot probed with anti-Rhbdl4 and β-actin as loading control. Tissues isolated from 5 months old male Rhbdl4-deficient and wild type mice.
B-D) Displayed is mean serum concentration of glucose, cholesterol and total proteins in mmol/L ± SEM of 2-3- and 6-months old male and female, Rhbdl4-deficient and wild type mice, n=3-6. Two-tailed, unpaired t-test performed between genotypes after a non-significant Shapiro-Wilks test; p-values reported.
E) Displayed is mean total body weight ± SEM of 5 months old male and female, Rhbdl4-deficient and wild type mice, n=2-8. Two-tailed, unpaired t-test performed between genotypes after a non-significant Shapiro-Wilks test; p-values reported.

2.3.5 Rhbdl4-deficient mice did not show signs of metabolic syndrome but exhibited reduced endothelial Glut1 expression in the cerebrovasculature

Considering the metabolic differences depicted in wild type and Rhbdl4-deficient MEFs, we were intrigued whether mice lacking Rhbdl4 also manifest a metabolic change. Metabolic syndromes are oftentimes investigated in the context of impairments in glucose tolerance and insulin sensitivity. As such, we performed glucose tolerance tests on 2 to 4 months old, male and female mice. A physiologically-relevant 5 h starvation was initiated immediately at the onset of the scheduled daytime of the rodents. Despite oral gavage being a more physiological route of administration, we chose to inject the mice peritoneally with 2 g/kg of glucose solution to circumvent several factors influencing glucose clearance in the stomach, such as rate of gastric emptying and incretin effect. Baseline blood glucose concentration (BGC) was measured followed by measurements in 15- and 30- mins increments after glucose bolus. As illustrated in Figure 5A and B, there are no salient changes in the overall glucose tolerance profile of the transgenic animals for either sexes compared to wild type. The fasting BGC also did not gravely differ. It is, however, noteworthy to mention the faster decline in blood glucose concentration amongst females compared to males; the former returns to its basal BGC merely after an hour following glucose administration while the latter achieved basal levels in the span of two hours. Moreover, among male mice, a slightly steeper drop in BGC of wild type mice was noticeable at the last time point compared to Rhbdl4-deficient mice. This may suggest a possible shortcoming in the total glucose

uptake capacity of the transgenic animals. Nevertheless, there were no significant differences in the area under the curve, reflecting the overall changes in BGC across different time points, between Rhbdl4-deficient and wildtype mice of both sexes (Fig. 5C). Given the widespread tissue distribution of Glut1 as well as the presence of other isoforms of glucose transporters in conjunction with Glut1, these findings were not quite surprising. They are rather indicative of a Glut1-specific effect of RHBDL4 on glucose metabolism *in vivo.*

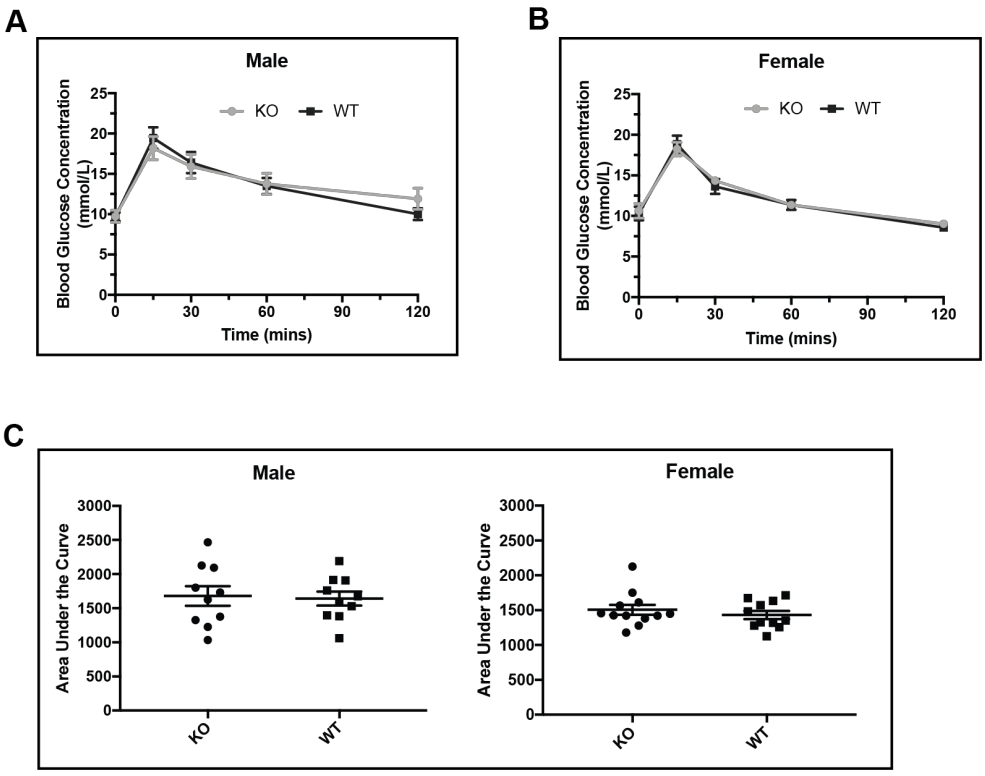


Figure 5: No systemic metabolic deficit manifested by Rhbdl4-deficient mice
Glucose tolerance test performed on 2-4 months old male and female, Rhbdl4-deficient and wild type mice, n=10, 12. Fasting blood glucose concentration determined followed by measurements in 15- and 30-mins increments after glucose administration intraperitoneally.
A, B) Displayed is mean blood glucose concentration in mmol/L ± SEM.
C) Mean area under the curve of Figures 5A and 5B ± SEM are shown. Two-tailed, unpaired t-test conducted after a non-significant Shapiro-Wilks test.

Based on the large database, the Allen brain atlas, endothelial cells lining the brain microvasculature predominantly express Glut1 over other isoforms and Rhbdl4 expression is highest in these cells compared to other cell types in the brain (Lein et al., 2006). Furthermore, a

tight regulation of glucose metabolism is pivotal for brain physiology. We therefore shifted our attention particularly to the brain, isolated cerebral microvessels from mice aging 3 to 5 months old and subsequently extracted proteins to detect Glut1 expression level by western blot. Staining for endothelial nitric oxide synthase (eNOS), which is the primary enzyme responsible for generating nitric oxide in vascular epithelium (Forstermann & Munzel, 2006), depicted stronger signal in the vascular fraction indicative of successful enrichment of endothelial cells as compared to parenchyma (Fig. 6A). The blood-brain-barrier comprises of three other major cell population aside from endothelial cells: astrocytic end-feet, microglial cells and pericytes. Interestingly, probing for Glut1 in microvessel purified from Rhbdl4-deficient mice brains demonstrated high molecular weight bands (indicated by the black arrows) and another band migrating below (indicated by the red arrow). The smaller band was detected in both the vascular and parenchymal fractions (Fig. 6A), implying that it most likely represents the 45 kDa Glut1 isoform predominant in glial cells (Yu & Ding, 1998). More importantly, quantification of the larger, endothelial-specific Glut1 isoform and subsequent normalization to β-actin revealed significantly weaker expression in Rhbdl4-deficient microvessels compared to wild type (Fig. 6B). Together, we propose that loss of Rhbdl4 contributes to the Glut1 paucity in specific cell types which may result in cellular glucose metabolic deficit without compromising overall systemic metabolism.

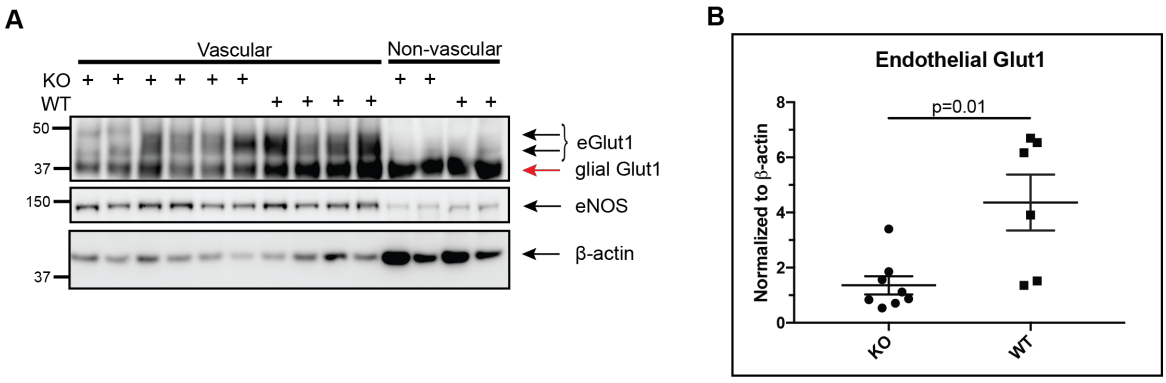


Figure 6: Rhbdl4-deficient mice exhibit less cerebral microvessel expression of Glut1

A) Cerebral microvessels isolated from 3-5 months old mice, n=6,8. Shown is western blot depicting endothelial-specific large Glut1 isoform (black arrows) and glial small Glut1 isoform (red arrow), endothelial nitric oxide synthase (eNOS) and β-actin as loading control.

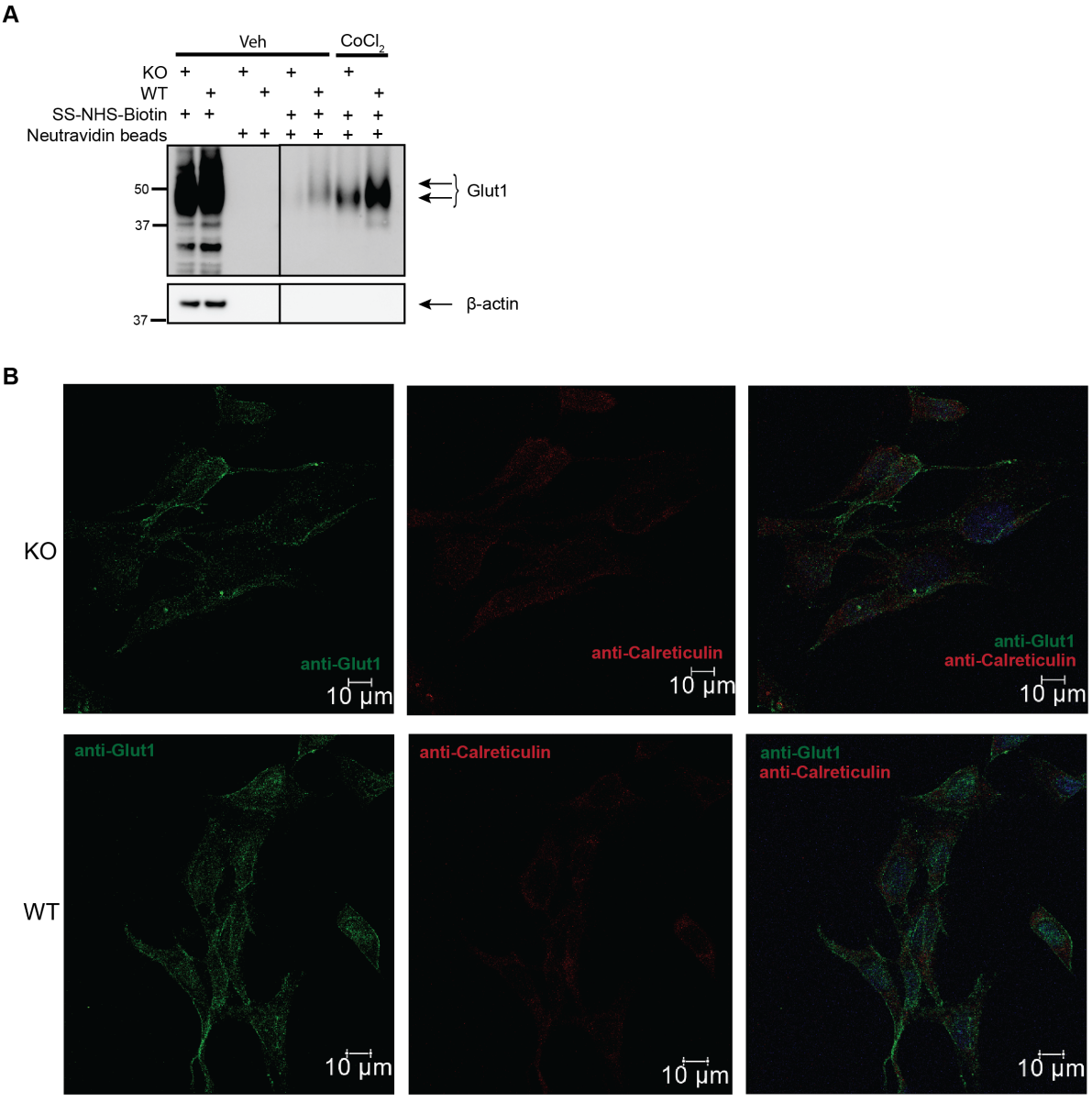
B) Mean expression of endothelial-specific Glut1 normalized to β-actin ± SEM is displayed, n=6,8. Two-tailed, unpaired t-test conducted after a non-significant Shapiro-Wilks test; p-values reported.

2.3.6 Rhbdl4 controls the ER exit of Glut1 to the cell surface in MEFs

While physiological and pathological regulation of Glut1 transcription have been extensively examined, our current understanding of its posttranscriptional regulation remains sparse. A critical growth signaling pathway consistently influencing cell surface GLUT1 protein expression is the activation of PI3K-Akt pathway (Barthel et al., 1999; Wieman et al., 2007). Nonetheless, investigation of the upstream regulators of this pathway are still underway. Herein, we introduce a possible role of RHBDL4 in regulating GLUT1 anterograde trafficking. Using a cell surface biotinylation approach, we labelled all proteins at the plasma membrane, and subsequently probed for endogenous Glut1 in wild type and Rhbdl4-deficient MEFs. Strikingly, Figure 7A illustrates lower cell surface Glut1 expression in MEFs lacking Rhbdl4 upon treatment of either H_2O or $CoCl_2$. Note that $CoCl_2$-treated cells have greater Glut1 levels at the plasma membrane due to the upregulation of Glut1 transcription described previously. As aforementioned, Rhbdl4-deficient MEFs exhibit diminished total Glut1 protein levels, mirrored by reduced Glut1 signal in the total lysate control from these cells. Furthermore, similar β-actin levels in the two cell types indicate that total amount of proteins loaded in the gel were similar and could not account for differences in Glut1 cell surface and total expression.

To further substantiate these findings, we performed immunocytochemistry to discriminate Glut1 localization between Rhbdl4-deficient and wildtype MEFs. With RHBDL4 localized to the ER, we would expect Glut1 to be retained in the ER upon RHBDL4 deficiency. Thus, we co-stained Rhbdl4-deficient and wild type MEFs for Glut1 as well as Calreticulin as ER marker. In vehicle and $CoCl_2$-treated condition, Glut1 staining was overall weaker in Rhbdl4-deficient MEFs compared to wild type. As predicted, wild type cells exhibit less co-staining of Glut1 with Calreticulin, and instead Glut1 signal more evidently delineates the ER in wild type compared to

Rhbdl4-deficient MEFs (Fig. 7B and C). Our data alludes to the idea that deregulated expression of RHBDL4 manifests either a hypo- or hyper cell surface Glut1 expression, further leading to downregulation or upregulation of glycolytic flux (Fig. 7D). Essentially, we propound a cellular role of RHBDL4 acting as gatekeeper in the ER: loss of RHBDL4 likely prevents the exit of Glut1 while overexpression of the enzyme, as in case of colorectal and breast cancer, could promote excessive Glut1 trafficking in particular cell types.



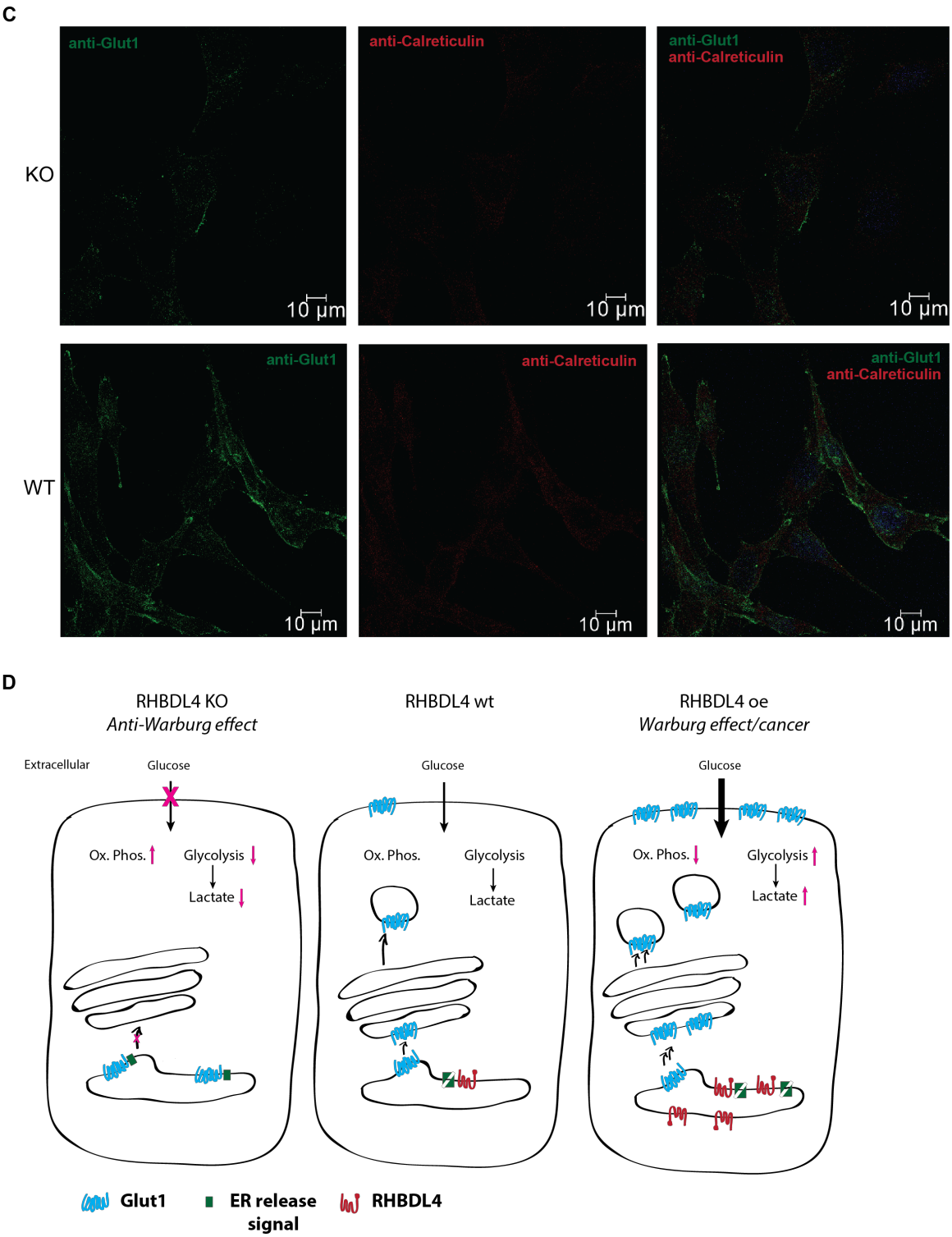


Figure 7: Total and cell surface Glut1 protein expression are reduced upon loss of Rhbdl4 in MEFs

A) Rhbdl4-deficient and wild type MEFs were either treated with H_2O (as vehicle control) or 100 µM $CoCl_2$ for 24 h. Pull down of biotinylated membrane proteins by neutravidin-beads. Shown is a representative western blot of 5 independent experiments stained with Glut1 and β-actin as loading control.

B, C) MEFs treated with H_2O as vehicle control (B) or 100 μM $CoCl_2$ for 24 h (C). Co-staining of Glut1 expression labelled with Alexa Fluor 488 (green signal) with Calreticulin fluorescently-tagged with Alexa Fluor 647 (red signal). Depicted are confocal images representative of 3 independent experiments acquired with objectives magnification of 40X.
D) Schematic of cellular glucose metabolism in cases of abrogation and overexpression of Rhbdl4. Rhbdl4 modulates cell surface translocation of Glut1.

2.4 Discussion

Our current understanding of human rhomboid proteases remains scarce in spite that the study of serine intramembrane proteases debuted over a decade ago. Additionally, most recent findings await *in vivo* validation. Multiple investigators nonetheless thrive to uncover the biological significance of these proteases. Our pursuit of identifying rhomboid substrates shed light on the role of the ER-residing human rhomboid protease RHBDL4 as an APP cleaving enzyme (Paschkowsky et al., 2016). In this present study, we now postulate another potential function of RHBDL4: a modulator of glucose metabolism likely through regulation of GLUT1 trafficking.

Upon *Rhbdl4* deletion, we observed a diminution in glucose consumption and concurrently in lactate production in mouse embryonic fibroblasts. Nevertheless, these cells did not exhibit difficulty in proliferation in nutrient-rich environment. In accordance, they were capable of sustaining growth longer than wild type cells in glucose-deprived media. Such decline in glucose dependence is substantiated by a greater tendency to recede from aerobic glycolysis compared to wild type cells as determined by seahorse analysis (see Fig. 1D). Hence, we suspect that Rhbdl4-deficient MEFs have undergone a metabolic change wherein amino acids, such as glutamine, are established as the primary source of tricyclic acid cycle intermediates fueling ATP synthesis through oxidative phosphorylation. Interestingly, the observed alterations in glucose metabolism are converse to the metabolic phenotype described a century ago, commonly known as *Warburg effect*. It describes a unique feature of autonomously, uncontrollable, proliferating cells, such as cancer cells that manifest an accelerated glycolytic rate as consequence to upregulated glucose consumption (Warburg, 1956). Intriguingly, a decreased OCR to ECAR ratio (contrary to our

seahorse analysis of Rhbdl4-deficient MEFs) is usually indicative of a Warburg effect and a cancerous like metabolic profile of a cell. Abrogation of Rhbdl4 further engendered a reduction in Glut1 total protein levels in immortalized embryonic fibroblasts while mRNA expression was unaltered. Conversely, tumorigenesis ensues from enhanced GLUT1 expression and henceforth cell surface translocation (Brown & Wahl, 1993; Haber et al., 1998; Yamamoto et al., 1990). We therefore demonstrated a contrasting opposing phenotype to cancer cells upon removal of RHBDL4. Accordingly, overexpression of the enzyme has been shown in colorectal cancer and correlates with poor patient survival rate (Miao et al., 2017; W. Song et al., 2015; M. Zhang et al., 2018). This is congruent to microarray data revealing upregulation of RHBDL4 in glioblastoma stem cells compared to neural stem cells (Stangeland et al., 2015) . RHBDL4 was also proposed to stimulate breast cancer progression through activation of the Akt-cyclin-dependent kinase (CDK2) (X. Zhang et al., 2018). Akt stimulation plays as key regulator of GLUT1 trafficking to the cell surface (Rathmell et al., 2003; Wieman et al., 2007). GLUT1 is indeed a stringent prerequisite at initial stages of mammary tumorigenesis; however, becomes dispensable following tumor growth (Wellberg et al., 2016). Consequently, not only is RHBDL4 potentially carrying a pivotal part in cancer metabolism, it may possess differential action at varying stages of the disease.

Furthermore, GLUT1 expression can be regulated by transcriptional activation upon HIF1α stabilization during hypoxia (Hayashi et al., 2004). Loss of Rhbdl4 did not alter responsiveness to $CoCl_2$-mediated Hif1α induction as determined by the increased expression of Glut1 relative to vehicle treatment for both the knockout and wild type MEFs. Nevertheless, the metabolic change in Rhbdl4-deficient MEFs was still apparent: reduced glucose consumption, lactate release and total Glut1 proteins. Surprisingly, *Glut1* mRNA expression in Rhbdl4-deficient MEFs compared to wild type cells was increased upon $CoCl_2$ treatment. This may indicate a

compensatory enhanced transcriptional activation of *Glut1* via HIF1α stemming from the still exhibited shortfall in Glut1 protein expression. Alternatively, *Rhbdl4* deletion could have further escalated the stability of HIFα in hypoxic condition thereby augmenting *Glut1* mRNA expression. An alluring finding elucidated the interference of inactive rhomboid iRhom1 in HIF1α degradation assisted by the receptor of activated protein C kinase-1 (RACK1) in an O_2-independent manner (Z. Zhou et al., 2014). Under normoxia, HIF1α is rapidly degraded through O_2-dependent prolyl hydroxylation, which is then subsequently ubiquitinated and sent to the proteasome [reviewed in (Masson & Ratcliffe, 2003)]. Modulation of HIF1α during hypoxia is rather achieved by competing interactions with molecular chaperone, heat shock protein 90 (HSP90), promoting its stabilization or RACK1 which recruits E3 ubiquitin ligase inducing proteasomal degradation of HIFα (Y. V. Liu et al., 2007; Y. V. Liu & Semenza, 2007). In addition to sequestering RACK1 and thus promoting HIF1α stabilization in O_2-restrictive environment, the ER and Golgi-resident iRhom1 has been shown to participate in transactivation of EGF receptor by controlling G-protein-coupled receptor (GPCR) (Nakagawa et al., 2005; Zou et al., 2009). The overlapping potential role in growth signal transduction of the active RHBDL4 and inactive iRhom1 pose the intriguing possibility of shared, but opposing, functions in regulating HIF1α during hypoxia, henceforth influencing glucose and cancer metabolism. HIF1α also induces the transcription of PDK1 (Kim et al., 2006; Papandreou et al., 2006) and thus we were enticed to determine its protein expression. In a similar manner as observed for Glut1, Rhbdl4-deficient MEFs exhibit lower Pdk1 levels in comparison to wild type following treatment with $CoCl_2$. The diminished repression of the pyruvate dehydrogenase complex through phosphorylation by PDK1 further corroborated the greater inclination to oxidative phosphorylation over glycolysis.

There is presently no literature regarding Rhbdl4-deficient mice besides the information available in the mouse phenotyping consortium. Herein, we provided a first characterization of mice lacking *Rhbdl4*. Consistent with our *in vitro* data, Rhbdl4 is expressed in tissues wherein glucose transporters assume an appreciable responsibility, such as brain, pancreas, liver and the heart. Converse to cell-type specific analyses, serum glucose and lipid concentration from Rhbdl4-deficient and wild type mice revealed no significant differences. Interestingly, heterozygous Glut1 mice similarly do not display alterations in blood glucose levels as compared to wild type although they exhibit hypoglycorrhachia (D. Wang et al., 2006). Earlier studies reported 50 g/L of total serum protein concentration is typical of mice from the C57BL/6 background, which is comparable to our wild type animals (Zaias, Mineau, Cray, Yoon, & Altman, 2009). However, Rhbdl4-deficient mice had significantly higher total serum protein concentration, reflecting the likelihood of a compromised immune system. This is in line with its established phenotype of anomalous immune cell production (www.mousephenotype.org). Interestingly, the elevated total protein levels were only observed amongst older males. Moreover, these animals significantly weigh less than the older wild type males. Whether there is a causative relationship between these findings remains nonetheless speculative. Remarkably, Rhbdl4-deficient mice also did not display a global glucose intolerance despite evidence for Glut1 paucity in specific cell types, including endothelial cells in the brain microvasculature and embryonic fibroblasts. Overnight fasting prior to the glucose tolerance test is not physiological in rodents as it accentuates insulin-stimulated glucose utilization due to liver glycogen depletion, which contrasts the response in humans. An advantage of prolonged fast is to reduce the variability in baseline blood glucose (Andrikopoulos, Blair, Deluca, Fam, & Proietto, 2008; Ayala, Bracy, McGuinness, & Wasserman, 2006). Fortunately, fasting blood glucose concentration did not significantly differ between wild type and Rhbdl4-deficient mice and thus it validates further our chosen more physiological method of 5 h starvation

prior to glucose load administration. Overnight fasting has nonetheless been shown useful in studies merely focusing on glucose utilization without consideration of insulin action (Bowe et al., 2014). In this study, we had only examined the effect of Rhbdl4 on Glut1 given that it is the predominant glucose transporter in our cell system. However, other glucose transporters isoforms, such as the insulin-sensitive Glut4 or Glut2 which is highly expressed in pancreatic and hepatic tissues merit further investigation in the context of Rhbdl4 abrogation. It might therefore be imperative to perform both glucose tolerance test with an overnight fast and subsequently insulin tolerance tests.

The brain is the main consumer of glucose in mammals and is dependent on glucose uptake from the blood via the BBB. Thus, dysregulation of predominant neuronal and non-neuronal glucose transporters has alarming repercussions in brain functions. Upon glucose entry into endothelial cells and astrocytic end feet lining the BBB via GLUT1, glucose diffuses throughout the extracellular fluid to more distant neuronal cells, facilitating its uptake mediated by GLUT3 (Simpson, Carruthers, & Vannucci, 2007). As alluded to previously, endothelial Glut1 in cerebral microvessels that form the BBB in mice lacking Rhbdl4 is significantly reduced in comparison to wild type. This striking result further entails a key role of RHBDL4 in cerebral glucose metabolism. Furthermore, it has been shown that cerebral glucose metabolism is impaired in patients with AD consequent to insufficient GLUT1 expression in the brain microvasculature (Mosconi, 2005; Mosconi et al., 2006; Simpson et al., 1994). Limited delivery of glucose into neurons may underlie some of the neurodegenerative aspects characterized in AD. There are nonetheless controversies regarding cellular fate of glucose during brain activity for many decades since quantification of metabolic activity in single cells *in vivo* at high spatiotemporal resolutions remains challenging. Notably, it was demonstrated that aerobic glycolysis is preferentially upregulated during brain activity contrary to resting awake brain wherein oxygen consumption

nearly equals glucose oxidation (Dienel, 2012). It will therefore be interesting to distinguish potential differences in glucose and lactate levels in the cerebrospinal fluid between Rhbdl4-deficient and wild type mice during resting and active states.

Lastly, we extend our proposition to the already existing evidence for a cellular role of rhomboid proteases in guiding the ER exit of membrane proteins (Adrain et al., 2012; Wunderle et al., 2016). This captivating notion is substantiated from cell surface biotinylation and immunocytochemistry data implying that Rhbdl4 modulates the trafficking of Glut1 to the plasma membrane in embryonic fibroblasts. Indeed, RHBDL4 has been elucidated to regulate the release of pro-transforming-growth factor alpha (pro-TGFα) from the ER, which is required for the activation of the mammalian EGF receptor (Wunderle et al., 2016). Similarly, inactive rhomboid iRhom2 was implicated in guiding the tumor necrosis factor-alpha converting enzyme (TACE), responsible for shedding EGFR ligands, out of the ER (Adrain et al., 2012; Christova et al., 2013). This chaperone-like function was also demonstrated in another class of intramembrane proteases, namely the signal peptide peptidase like (SPPL). In particular, SPPL3 was reported a key factor in regulating glycosylation at the ER wherein only properly folded and glycosylated proteins are permitted to advance in the secretory pathway (Voss et al., 2014). Accordingly, we propose that the deregulated glucose metabolism in embryonic fibroblasts emanates from aberrant translocation of Glut1 to the cell surface upon loss of Rhbdl4. This then likely results in crowding the ER with misfolded Glut1 proteins rendering their rapid turnover rate as a compensatory mechanism, which may underlie the reduction in total Glut1 levels without changes in mRNA expression or despite elevated *Glut1* transcription during hypoxia-mimicking condition. However, with the current evidence, we cannot exclude the possibility that lower Glut1 protein levels in Rhbdl4-deficient MEFs is a consequence of impaired or slowed protein synthesis. Furthermore, the metabolic switch to glutamine as a primary nutrient source of energy and carbon skeletons for anabolic reactions

could be an adaptive mechanism spurring from a restrictive glucose uptake following *Rhbdl4* deletion. In essence, it remains fundamental to evaluate whether the catalytic activity of RHBDL4 mediates its modulatory role in ER exit of Glut1 and to delve deeper into the underlying mechanism of this newly posited Glut1 regulation.

2.5 Conclusion

Here, we presented an unprecedented link between RHBDL4 and glucose metabolism, which both were previously propound as central elements in tumorigenesis. Our results demonstrated that RHBDL4 modulates cell surface GLUT1 translocation thereby influencing glycolytic flux in particular cell types. Glut1 paucity in Rhbdl4-deficient endothelial cells lining the cerebrovasculature further implicated the importance of this protease in cerebral functions. Understanding the cellular role of RHBDL4 may therefore be instrumental in exploiting novel avenues potentially conducive in the prevention and/or attenuation of metabolic diseases, such as cancer.

2.6 Methods

2.6.1 Cell culture and treatments

Immortalized embryonic fibroblasts from wild type and Rhbdl4-deficient mice (kindly provided by Dr. Matthew Freeman, Oxford University) were cultured in "full" Dulbecco´s modified Eagle medium (DMEM) containing 4.5 g/l glucose, 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate (Wisent) and supplemented with 10% FBS (Wisent), at 37°C and 5% CO_2. 2 x 10^5 - 5 x 10^5 cells/well were seeded in "full" DMEM supplemented with 10% FBS in a 6-well plate for 24 h for adequate cell proliferation. For hypoxia-mimicking experiments, 24 h treatment with 100 µM $CoCl_2$ (Sigma) or H_2O, as vehicle control, was performed. In nutrient deprivation experiments, cells were conditioned with DMEM lacking either glucose or glutamine or supplemented with lipoprotein-deprived serum (LPDS) instead of FBS for 24 and 48 hours.

2.6.2 Glucose and lactate measurements

2 x 10^5 cells per well (6-well plate) were seeded in 2 ml full media, at indicated time points the supernatant was collected, cells were trypsinized and the total amount of cells per well was determined using an automated cell counter (Biorad). The supernatant was analyzed with the Nova's BioProfile analyzer (BioProfile 400 Test Menu) for its glucose and lactate concentrations. For each timepoint, there was a media only control to enable calculation of glucose consumption and lactate release. Results obtained were transformed from mmol/l to total µmol present and lastly normalized to the total cell count, resulting in glucose consumption and lactate release in pmol per cell. Simultaneously, the other plate was lysed with TNE-lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, and complete protease inhibitors, Roche) and prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 4 x LDS sample buffer (with 10% β-mercaptoethanol, Invitrogen) was added to the samples for a final concentration of 1 x.

Seahorse analysis: $2x10^4$ cells (embryonic fibroblast from Rhbdl4-deficient or wildtype mice) were seeded one day prior to the assay run with 5 technical replicates per condition. On the day of the assay Seahorse media (XF Base Medium Minimal DMEM) was substituted with 10 mM Glucose, 2 mM Glutamine and 1 mM Sodium Butyrate and pre-warmed to 37°C. Cells were washed three times with the Seahorse media and then incubated in a non-CO_2 incubator for 1 h. For simultaneous measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), we used the Seahorse Extracellular Flux (XF) 96 Analyzer (Seahorse Bioscience, Inc, North Billerica, MA, USA), a specially designed system to perform real-time analysis of these metabolic constants. For both parameters, three independent, repeated measurements were performed over an 18 min period. Individual OCR and ECAR values were normalized to total protein load in µg determined by Bicinchoninic acid (BCA) assay (Pierce) following seahorse run.

OCR/ECAR ratios were calculated independently of the overall protein load per well. Results from 4 independent experiments are displayed.

2.6.3 Quantitative real-time PCR (qPCR)

48 h subsequent to seeding, mRNA was isolated from wild type and Rhbdl4-deficient MEFs (MACHERY-NAGEL kit) then reverse transcribed into cDNA (iScript mix). qPCR was performed using the SsoAdvanced SYBR green supermix on a Biorad CFX384Touch cycler. Primers used were: Glut1 forward (GCTTCTCCAACTGGACCTCAAAC) and reverse (ACGAGGAGCACCGTGAAGATG), RSP18 forward (CGGAAAATAGCCTTCGCCATCAC) and reverse (ATCACTCGCTCCACCTCATCCT), PICALM forward (GATGTTCACCTGCCCATTGCTTC) and reverse (TGGCTGTGCAACTGGAGAAGGA). Primer efficiency and optimal melting temperature were determined to be 90-110% and 58°C, respectively. For normalization of *Glut1* gene expression, RSPI8 and PICALM were confirmed to be stable and thus were used as reference genes. RT-qPCR was analyzed using the CFX Maestro software. Data is displayed as mean expression.

2.6.4 Western Blot

Protein lysates were separated on 4-12% bis-tris gels (Novex, Nupage, Invitrogen), ran with MES running buffer (Invitrogen) then transferred onto nitrocellulose membrane with transfer buffer containing 10% ethanol. Antibodies against Glut1 (Cell Signaling), Rhbdl4 (Sigma), eNOS (BD Biosciences), Pdk1 (Enzo) and mouse-anti-β-actin (8H10D10, Cell Signaling) were utilized. Horseradish peroxidase (HRP)-coupled secondary antibodies directed against mouse or rabbit IgG were purchased from Promega. Chemiluminescent images were acquired using the ImageQuant LAS 500 or 600 system (GE Healthcare).

2.6.5 Cell surface biotinylation

5 x 10^5 wild type and Rhbdl4-deficient MEFs were seeded and treated with either H_2O or 100 μM $CoCl_2$ for 24 h according to the procedure above. Cells were then washed twice with pre-warmed PBS containing Mg^{2+} and Ca^{2+} ions (Wisent) and incubated for 15 mins, at 37 °C and 5% CO_2. On ice, cells were incubated in 0.5 mg/mL non-permeant, Sulfo-NHS-SS-Biotin (Thermo Scientific) for 10 mins. Residual unbounded biotin was washed twice with PBS containing 10 mM glycine (BioShop). Membrane extracts were prepared by scraping cells in 200 μL TNE-lysis buffer and subsequently incubated for 30 mins at 4 °C. Fractions were spun down at 11 000 rpm for 10 mins then supernatant was collected, and protein concentration was measured using BCA assay. 100 μL total cell lysates were taken as expression controls and prepared with 1 x LDS sample buffer with 10% β-mercaptoethanol. Remaining lysates were diluted with PBS and incubated with 60 μL washed, diluted Neutravidin-beads (1:1 beads: PBS, Thermo Scientific) overnight. Samples were spun down at 1 500 x g, 3 mins repeatedly until final wash. Beads were washed twice with 400 mM NaCl and once with PBS. Using 18-gauge needle, beads were dried. 30 μL LDS sample buffer and additional 5% SDS were added then samples were boiled for 5 mins, at 65 °C.

2.6.6 Immunocytochemistry

5 x 10^4 cells were seeded in collagen-coated coverslips in DMEM, supplemented with 10% FBS. Subsequently, cells were treated with either H_2O or $CoCl_2$ according to the aforementioned procedure. 100% ice-cold methanol (Fisher) was used to fix cells for 5 mins at RT followed by PBS washes. Cells were then permeabilized with 0.1% Triton-X-100 (Sigma), 10 mins at RT then washed at least 5x with PBS, blocked with 5% normal goat serum (NGS, Sigma), 1 h at RT, and finally incubated with 1:100 dilution of anti-Glut1 in blocking buffer overnight at 4°C. Subsequent multiple washes with 5% NGS, 5 mins at RT were carried out. Cells were then incubated with

Alexa 488-coupled secondary antibodies directed against rabbit IgG diluted (Life Technologies) at 1:2000, RT in the dark for 1 h. Following washing off of secondary antibody, anti-calreticulin (Abcam) conjugated with Alexa 647 and diluted in blocking buffer (1:100) was applied to the cells at 4°C, overnight. The coverslips were mounted on microscope slides using fluoroshield with DAPI (Sigma). Confocal images were acquired by LEICA SP8 microscope and analyzed using LEICA application suite X (LAS X).

2.6.7 Rhbdl4-deficient mice

The generation of these mice were done in Pr. Matthew Freeman's lab (University of Oxford, UK). Rhbdl4-deficient mice were generated by deleting the second exon of *Rhbdl4* (first coding exon) in embryonic stem cells which were then injected into C57Bl/6J blastocysts. This resulted in the generation of chimeric rodents from which the first heterozygous mice were obtained. Subsequent in-breeding of another heterozygous yielded mice homozygous for *Rhbdl4*. Rhbdl4-deficient strain was maintained by setting up homozygous breeders. Mice were maintained under specified pathogen free conditions in accredited facility at the Genome Cancer Research Centre, McGill University, Canada. They were kept at constant temperature and humidity, on a 12h/12h light/dark cycle and had ad libitum access to food and water.

2.6.8 Glucose tolerance test (GTT)

Rhbdl4-deficient and wild type (C57BL/6J) mice of both sexes, ranging between 2 to 4 months of age (fed with normal diet) undergone GTT. Animals were initially starved for 5 h starting at 7 AM (scheduled onset of daytime in the animal housing facility). Baseline blood glucose concentration was measured prior to an intraperitoneal injection of 2 g/kg of D-(+)-glucose (Sigma) dissolved in sterile saline solution (9.0 g/L NaCl, Quality Biological) according to the animal's body weight. Measurement of blood glucose levels were conducted in the span of 2 h in either 15- or 30-mins increments following glucose bolus intake using Aviva Accu-Chek glucose monitor (Roche).

Blood was taken by cutting a small tip of the animal's tail and directly applied to the glucose strips that came with the glucometer.

2.6.9 Cerebral microvessel isolation/endothelial preparation

Each cerebral hemisphere of mice aged 3 to 5 months old were collected, snap frozen and stored at -80 °C for long-term use. Only the left hemisphere was used for all endothelial preparations. On ice, 1 hemisphere was homogenized in 1.5 mL cold PBS mixed with protease inhibitor cocktail using a loosely fitting Dounce homogenizer then centrifuged at 2000xg, 5 mins, 4°C. The supernatant was collected while remaining pellet was resuspended in PBS and spun down at 2000xg, 5 mins, 4°C. The resulting supernatant was collected and combined with the first one, then again centrifuged at 3000xg, 10 mins, 4°C. The pellet constitutes the parenchymal fraction. The acquired pellet after the second centrifugation, which contains the vascular fraction, was further resuspended in 1.5 mL PBS and spun down 3,500 rpm, 5 mins, 4°C. The resuspension and centrifugation of the vascular fraction was repeated thrice or until a clear supernatant was observed. A final resuspension with 500 μL PBS was performed after discarding the supernatant. The resuspended pellet was subsequently passed through 50 μm pore size nylon mesh (CellMicroSieve) using vacuum. Retained microvessels in the mesh were eluted with cold PBS by simultaneous washing and scraping the mesh with an automatic pipette, and subsequently collected in eppendorf tubes. Lastly, samples were centrifuged at 4,500 rpm, 10 mins, 4°C. The supernatant was discarded, and the resulting pellet contains the purified microvessels.

2.6.10 Tissue protein extraction

Subsequent to microvessel isolation, both parenchymal and vascular fractions were incubated with lysis buffer containing 150 mM NaCl, 20% glycerol, 2 mM EDTA, 1% NP40, 0.1% deoxycholate, 20 mM HEPES, pH 7.4 for 30 mins, 4°C followed by centrifugation at 14,000 rpm, 10 mins, 4°C.

Protein extracts in the supernatant were collected, and protein concentration were measured by BCA. A concentration of 1 μg/uL proteins were prepared for SDS-PAGE. Subsequently, 4x LDS sample buffer with 10% β-mercaptoethanol was added to the samples for a final concentration of 1 x. For protein extraction from the different mouse organs, similar lysis buffer was used, but with extra detergent (NP40) added for muscular tissues (liver, heart and kidney). Snap frozen tissues were first weighed and cut to obtain about 30 mg of tissue. Each tissue lysed with 5 x its weight then homogenized using sterilized pistils. Samples were rotated for 1 h, at 4°C followed by centrifugation at 15 000 rpm, 1 h, 4°C. Protein extracts were collected and prepared for SDS-PAGE in a similar manner as the microvessel proteins.

2.6.11 Data Analysis and Statistics

Western blot images were quantified with ImageJ. Statistical data analysis was performed with GraphPad Prism 7 and applied tests are indicated in the figure legends.

2.7 Acknowledgements

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III. GENERAL DISCUSSION

Various non-canonical processes for APP have been elucidated over the last few years. Our knowledge on the physiological functions of APP is yet nonetheless poorly understood. Not only many of the APP cleaving proteases necessitate *in vivo* evidence, the distinct, novel APP processing pathways identified thus far are likely regulated in a cell-type specific and context-dependent manner. The most recent processing enzyme discovered belongs to a clade of mammalian proteases conserved down to the least common ancestors. Their recent implication in various diseases, including Alzheimer's disease and cancer, also evince that they must possess important cellular roles. However, progress in unravelling their biological significance is also stagnant. In this manuscript-based thesis, I presented two independent lines of research that touch upon the underlying mechanisms for how human rhomboid RHBDL4 acts: 1) as an APP cleaving enzyme and 2) a modulator of cellular glucose metabolism. Ultimately, this thesis was formulated with an overarching goal to expand our knowledge on the physiology of mammalian rhomboid proteases, in particular RHBDL4.

1. RHBDL4 implication in Alzheimer's disease

The multitude of data from early onset familial cases firmly reinforces the disease-contributing nature of Aβ [reviewed in (Cacace et al., 2016)] (Jonsson et al., 2012). Imbalance in Aβ production and/or clearance have therefore been a robust indication of the disease pathology in both pre- and clinical settings. Strikingly, we observed alterations in Aβ levels consequent to differences in RHBDL4 expression; however, our *in vitro* and *in vivo* evidence do not align. Based on our RHBDL4 overexpressing cell culture experiments, RHBDL4-mediated APP processing precludes amyloidogenic processing of APP, thereby led to diminished levels of secreted Aβ peptides (Paschkowsky et al., 2016). Conversely, loss of Rhbdl4 in mice brains yielded less soluble Aβ levels than wild type. Furthermore, analyses of post-mortem brains from AD versus healthy control cases revealed significant positive correlation between *RHBDL4* mRNA expression and AD

pathology. Accordingly, higher RHBDL4 protein levels was shown in AD as compared to healthy controls brains (Paschkowsky, 2018). Considering reduced cerebral glucose metabolic rate is also manifested in sporadic AD, it is tempting to speculate an interplay between APP processing and glucose metabolism mediated by RHBDL4 activity.

Hamilton *et al.* postulated a modulation in glucose uptake and oxidation in cultured rodent myotubes as a result of altered amyloidogenic APP processing. Upon BACE-1 inhibition engendering decreased Aβ levels, GLUT1 expression was enhanced accompanied by upregulation in glucose consumption (Hamilton et al., 2014). Conversely, elevated Aβ depositions in AD brains likely reduce cerebral glucose metabolism. We thus pose the intriguing notion that RHBDL4 is upregulated in AD as a compensatory mechanism to promote GLUT1 trafficking. It will be interesting to detect whether Aβ accumulation and cerebral hypometabolism indeed precede RHBDL4 overexpression in pre-symptomatic patients. In light of various studies highlighting hypercholesterolemia as a risk factor to AD (Helzner et al., 2009; Kivipelto et al., 2002) and recent finding of high cellular cholesterol impeding RHBDL4 activity (Paschkowsky, Recinto, et al., 2018), the enzyme is perhaps inactive in diseased brains. Consequently, increased RHBDL4 expression in AD would fail to circumvent amyloidogenic processing. It also raises the likelihood that the guidance function of RHBDL4 necessitates its catalytic activity. Wunderle *et al.* previously insinuated the occurrence of an unknown retention signal cleaved by RHBDL4 to mediate the exit of pro-TGFα out of the ER (Wunderle et al., 2016). A non-biased microarray study could avail to unveil this retention signal although it is tempting to investigate the possibility that APP may be the missing piece of the puzzle.

To fully comprehend the physiological role of RHBDL4 in non-canonical APP processing, a mouse model for AD with deficiency in Rhbdl4 would be necessary. We anticipate that this double transgenic animal would display GLUT1 paucity in endothelial cells lining cerebral

microvasculature and thereby reduced cerebral glucose uptake. Meanwhile, another study demonstrated glucose as an upstream regulator of Aβ generation; high glucose environment accentuated β-CTF and Aβ levels in neuroblastoma cell lines (Yang, Wu, Zhang, & Song, 2013). We therefore also expect a downregulation in Aβ levels in the double transgenic animal as compared to APP transgenic alone if the former does manifest a deficit in intraneuronal glucose levels consequent to Rhbdl4 abrogation. Collectively, we proposed a potential role of Aβ in modulating cellular glucose uptake. These enthralling ideas currently remain speculative and thus insufficient to draw a well-founded conclusion as to a beneficial or detrimental effect of RHBDL4 on AD. Our data are nonetheless suggestive of its critical implication in AD pathology.

2. RHBDL4 as part of the ER quality control and exit machinery

The endoplasmic reticulum (ER) directs proper folding and maturation of polypeptides owing to the abundance of residing molecular chaperones and folding sensors. These comprise the ER quality control and exit machinery, which work together to ensure a tight control of the systems governing cargo retention, selective uptake and retrieval [reviewed in (Ellgaard & Helenius, 2003)]. Only properly folded proteins are moved to ER exit sites (ERES) where they assemble into a pre-budding complex along with the subunits of coat proteins complex II (COPII) vesicles. Subsequent recruitment of the heterodimers Sec13/31 drive membrane curvature by forming a cage-like outer coat layer in the nascent vesicle. Upon completion of COPII assembly, GTP hydrolysis is effectuated for the ultimate vesicle release. COPII protein, Sec 24, mediates the selection of cargo through direct interaction upon recognizing specific ER export signals (e.g. di-acidic, di-aromatic residues) [reviewed in (Barlowe, 2003)]. Considering there exist merely four isoforms of Sec24 in mammalian cells while a wide array of proteins passes through the secretory pathway, the use of membrane-spanning proteins aiding in the exit of luminal cargoes from the ER does not come as a surprise. These are termed cargo receptors whereby many remain largely

unknown. Through a proximity-dependent biotin identification (BioID) method, we mapped possible RHBDL4 interacting partners (Jacqueline Hsiao, personal communication, data not shown); one being COPII protein subunit Sec24. Hence, it raises the likelihood of RHBDL4 serving as a cargo receptor to facilitate the translocation of GLUT1. This speculation was also postulated by another group who claimed RHBDL4 could be analogous to the cargo receptor, Star, which controls ER exit of EGF ligand in *Drosophila melanogaster* (J. R. Lee et al., 2001; Wunderle et al., 2016).

If RHBDL4 is indeed critical in ER quality control and exit machinery, deciphering other cargo proteins affected could be of importance. Strikingly, knockdown of RHBDL4 in HEK cells led to lower endogenous APP levels at the cell surface (data not shown), suggesting that reduced endogenous Aβ levels in Rhbdl4-deficient mice could rather be a result of APP retention in the ER and thus fewer proteins available in plasma membrane and endocytic compartment where β- and γ-secretases are present. Based on the evidence presented in the first manuscript (see experimental section) wherein the enzyme acts as both a sheddase and intramembrane protease, a likely primordial function of RHBDL4 is to scan along the ER membranes and preferentially bind to unfolded regions of proteins whether these are in the ectodomain or transmembrane sequence. As such, when the cargo proteins assemble into a proper folded tertiary structure, they are docked into COPII vesicles potentially via interaction with RHBDL4 while if unsuccessful, RHBDL4 possibly cleaves and/or promotes ERAD of these proteins. Together, RHBDL4 possibly serves as a pivotal checkpoint to select for properly folded, mature COPII cargoes (such as GLUT1 and APP) while trigger the degradation of misfolded ones.

3. RHBDL4 as a target for breast and colorectal cancer therapy

Presently, the most compelling evidence of RHBDL4 partaking in tumorigenesis is its substantial high expression in human colorectal and breast cancer samples along with its high prognostic

potential (Miao et al., 2017; W. Song et al., 2015; Stangeland et al., 2015; M. Zhang et al., 2018; X. Zhang et al., 2018). The enzyme has been linked to various cellular processes, often dysregulated in cancer cells. The underpinning mechanism for how RHBDL4 engenders tumorigenesis rests nonetheless debatable. Several lines of evidence highlight its anti-apoptotic feature: RHBDL4 was shown to cleave pro-apoptotic factors, BIK and tumor suppressor-activated pathway 6 (TSAP6), leading to their inactivation while overexpression stimulates the anti-apoptotic factor, Bcl-3 (Ren et al., 2013; Wan et al., 2012; Y. Wang et al., 2008). RHBDL4 is also suggested to play a key role in cell cycle regulation wherein its loss in breast cancer cell lines halted tumor progression through downregulation of Akt-CDK2 (X. Zhang et al., 2018). Additionally, the involvement of elevated RHBDL4 expression in colorectal cancer metastasis was prompted by upregulation of the Wnt/β-catenin signaling pathway, which predisposes cells to tumorigenesis as a result of excessive stem cell renewal (Miki, Yasuda, & Kahn, 2011; M. Zhang et al., 2018). Lastly, knockdown of RHBDL4 in colorectal tumors repressed EGF receptor (EGFR) mRNA and protein expression. Notably, advanced colorectal cancer displayed the highest positive correlation between RHBDL4 and EGFR levels (Miao et al., 2017). The findings laid out in the second manuscript (see experimental section) add another layer of complexity in unraveling the dominant role of the enzyme in cancer.

Exacerbation of GLUT1 expression has been a long-standing attribute of tumorigenesis although the extent of its contribution between cancer types is not entirely lucid. Indeed, immunohistochemical analyses revealed variability in GLUT1 expression amongst tumor types; for instance, a mere 5% out of 254 breast cancer samples exhibited Glut1-positive staining (Carvalho et al., 2011). Tumorigenesis can be prevented upon deletion of one or both *Glut1* alleles in mammary epithelial cells harboring the active human epidermal growth factor receptor 2 (HER2) oncogene (Wellberg et al., 2016). Glut1 expression however declines as breast tumor

grows (Wellberg et al., 2016), insinuating the reduced efficacy of Glut1-targeting therapies in advanced stages. In essence, RHBDL4 may not only display differential modes of action depending on tumor types, but its influence may also fluctuate at varying stages. The much-awaited cross breed of Rhbdl4-deficient mice with either HER2-induced or colorectal cancer-induced animal models will surely shed further insight into the carcinogenic property of RHBDL4. If the catalytic activity of the enzyme is deemed essential in regulating the trafficking of GLUT1, a search for RHBDL4 inhibitors could be another milestone in developing cancer treatments. As demonstrated by APP mutants bearing positively-charged amino acid residue in their transmembrane sequence, the higher propensity of RHBDL4 to recognize intrinsically unstable regions does not only corroborate its potential chaperoning function but could also constitute as a framework to design more efficient competitive, substrate-mimicking agents. Understanding the proteolytic mechanism of the enzyme could thus greatly aid in drug design and development, especially considering that all existing mammalian rhomboid inhibitors have either low selectivity or low potency (Hameed, Aslam, & Ying, 1998; Strisovsky, 2017; Verhelst, 2017).

4. Conclusion

In summary, we aimed to delve deeper into the biological significance of mammalian rhomboid proteases by investigating the proteolytic mechanism of RHBDL4-mediated APP processing and the chaperoning function of RHBDL4 on GLUT1. Various structural and kinetics studies have been conducted for bacterial rhomboids due to accessible recombinant expression systems. Meanwhile, the lack of known enzyme-substrate complexes for mammalian rhomboids limited the investigation of their proteolytic mechanism. Our discovery of APP as a prime substrate for RHBDL4 has not only shed light into its potential role in AD, but also provided us a tool to further comprehend the gating strategies and substrate specificity of the enzyme. A detailed understanding of how RHBDL4 cleaves its substrates could be of benefit for its functional analyses as well.

Whether the catalytic activity of RHBDL4 is indispensable to traffic GLUT1 out of the ER remains to be addressed. In essence, an imbalance in RHBDL4 expression in particular cell types engenders both a hypo- and hyper-glucose metabolic profile displayed in patients with AD and cancer, respectively. Targeting RHBDL4 expression and/or activity could thus ignite novel lines of research in the treatment of such diseases.

ABBREVIATIONS

Aβ	Amyloid-β
α/β - CTF	C-terminal fragment α/β
AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
APP	Amyloid precursor protein
ApoE ε4	Apolipoprotein E ε4
ATP	Adenosine triphosphate
βACE-1	β-site cleaving enzyme 1
BBB	Blood-brain-barrier
BGC	Blood glucose concentration
BIK	Bcl-2 interacting killer
CDK2	Cyclin-dependent kinase 2
CTE	Chronic traumatic encephalopathy
CTD	C-terminal domain
CuBD	Copper-binding domain
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERAD	ER-associated degradation

FTD	Frontal temporal dementia
G1D	GLUT1 deficiency syndrome
GAIP	G-α interacting protein
GFLD	Growth factor-like domain
GLUT	Glucose transporter
HER2	Human epidermal growth factor receptor 2
HIF1α	Hypoxia inducible factor 1α
INPP4B	Inositol polyphosphate-4-phosphatase
IMP	Intramembrane protease
LDH	Lactate dehydrogenase
MPZ	Myelin-protein-zero
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFT	Neurofibrillary tangles
NTD	N-terminal domain
PARL	Presenilin-associated rhomboid-like protein
PD	Parkinson's disease

PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PPP	Pentose phosphate pathway
PSEN1/2	Presenilin 1 or 2
PTEN	Phosphatase and tensin homolog
RACK1	Receptor activated protein C kinase-1
RB	Retinoblastoma
RHBDL4	Rhomboid-related protein 4
S2P	Site-2-protease
sAPPα/β	Soluble APPα/β
SGLT	Sodium glucose transporter
SLC2A	Solute carrier 2A
SNP	Single nucleotide polymorphism
SPPL	Signal peptide peptidase like
TACE	Tumor necrosis factor α converting enzyme
TGFα	Transforming growth factor α
TSAP6	Tumor suppressor-activated pathway 6

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APPENDIX A

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An alternative processing pathway of APP reveals two distinct cleavage modes for rhomboid protease RHBDL4

Sherilyn Junelle Recinto, Sandra Paschkowsky, Lisa Marie Munter

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