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Stem Cells and Metabolism Research Program
Faculty of Medicine
Doctoral Program in Integrative Life Science
University of Helsinki
Minerva Foundation Institute for Biomedical Research

CROSSTALK BETWEEN

SPHINGOLIPID METABOLISM AND

NUTRIENT SIGNALING: STUDIES

ON LAPT4B

Kecheng Zhou

DOCTORAL DISSERTATION

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<th>Description</th>
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<tbody>
<tr>
<td>4F2hc</td>
<td>CD98 heavy chain</td>
</tr>
<tr>
<td>A4GATL</td>
<td>Alpha-1,4-galactosyltransferase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ASAH1</td>
<td>Acid ceramidase</td>
</tr>
<tr>
<td>B4GALT6</td>
<td>Beta-1,4-galactosyltransferase</td>
</tr>
<tr>
<td>bSMase</td>
<td>Bacterial sphingomyelinase</td>
</tr>
<tr>
<td>C1P</td>
<td>Ceramide-1-phosphate</td>
</tr>
<tr>
<td>Cer</td>
<td>Ceramide</td>
</tr>
<tr>
<td>CERK</td>
<td>Ceramide kinase</td>
</tr>
<tr>
<td>CerS1-6</td>
<td>Ceramide synthases 1-6</td>
</tr>
<tr>
<td>CERT</td>
<td>Ceramide transfer protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPTP</td>
<td>Ceramide-1-phosphate transfer protein</td>
</tr>
<tr>
<td>EE</td>
<td>Early endosomes</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosomal antigen-1</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicle</td>
</tr>
<tr>
<td>FAPP2</td>
<td>Four-phosphate adaptor protein</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GALC</td>
<td>Beta-galactocerebrosidase</td>
</tr>
<tr>
<td>GalCer</td>
<td>Galactosylceramide</td>
</tr>
<tr>
<td>Gb3</td>
<td>Globotriaosylceramide</td>
</tr>
<tr>
<td>GBA</td>
<td>Glucosylceramidase beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>GCS</td>
<td>Glucosylceramide synthase</td>
</tr>
<tr>
<td>GlucCer</td>
<td>Glucosylceramide</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipids</td>
</tr>
<tr>
<td>HexCer</td>
<td>Hexosylceramide</td>
</tr>
<tr>
<td>ILV</td>
<td>Intraluminal vesicle</td>
</tr>
<tr>
<td>KDSR</td>
<td>3-ketosphinganine reductase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LacCer</td>
<td>Lactosylceramide</td>
</tr>
<tr>
<td>LAPTM4B</td>
<td>Lysosome associated protein transmembrane 4B</td>
</tr>
<tr>
<td>LAT1</td>
<td>CD98 light chain</td>
</tr>
<tr>
<td>LBPA</td>
<td>Lysobisphosphatidic acid</td>
</tr>
<tr>
<td>LE</td>
<td>Late endosomes</td>
</tr>
<tr>
<td>LMP</td>
<td>Lysosome membrane proteins</td>
</tr>
<tr>
<td>Lo/Ld</td>
<td>Liquid ordered/disordered</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>TORC1</td>
<td>The target of rapamycin complex 1</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>RE</td>
<td>Recycling endosomes</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SMPD1</td>
<td>Acid sphingomyelinase</td>
</tr>
<tr>
<td>SphK</td>
<td>Sphingosine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TFEB</td>
<td>Transcriptional factor EB</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TSC1/2</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>UGCG</td>
<td>Ceramide glucosyltransferase</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
ABSTRACT

Sphingolipids, a major component of the plasma membrane, regulate various cellular activities and display essential physiological functions. The cellular processes that control sphingolipid homeostasis are complex and incompletely understood. The lysosomes are responsible for digesting intracellular and extracellular materials, including sphingolipids, and these organelles play a role as a hub in metabolic signaling, coordinating cellular anabolism and catabolism. Lysosome-associated protein transmembrane 4 B (LAPTM4B) is highly expressed in many types of cancers and associates with poor prognosis. On the cellular level, LAPTM4B affects cellular processes including nutrient sensing, autophagy, proliferation, cell migration, drug resistance, and sphingolipid homeostasis. Less is known regarding the function of LAPTM4B at the molecular level, because studies have been hampered by a lack of molecular tools and of specific LAPTM4B antibodies. In this thesis, we sought to elucidate the lipid and protein environment of LAPTM4B, and how these are coordinated to regulate downstream signaling events.

In our first study, we combined approaches from biochemistry, computer simulations, and lipid biology to show that the third transmembrane domain of LAPTM4B contains a ceramide-regulated element consisting of a sphingolipid interaction motif and an adjacent aspartic acid residue (D202). This feature is essential
for regulating the interaction between LAPTM4B and 4F2hc, a subunit of the amino acid transporter CD98. The ceramide induced an interaction between LAPTM4B and 4F2hc, and this interaction promotes downstream mTORC1 signaling.

In our second study, we characterized a novel monoclonal antibody, which we used to visualize the subcellular distribution of endogenous LAPTM4B for the first time. By site-directed mutagenesis of putative start codons, we identified LAPTM4B-24 as the major expressed and physiologically important isoform in a majority of human cells and tissues. We further showed that endogenous LAPTM4B has a fast turnover, and is regulated by nutrient availability and ceramide, pointing to a role at the crossing-point of sphingolipid metabolism and nutrient signaling.

In our third study, we investigated the role of LAPTM4B in regulating the lipidomes of cells and the secreted extracellular vesicles. We found LAPTM4B to be sorted into intraluminal vesicles of multivesicular bodies and subsequently to be released from cells in small extracellular vesicles (sEVs). The secretion dynamics of LAPTM4B was dependent on the ceramide-regulated motif. Shotgun lipidomics revealed that LAPTM4B regulates the sphingolipid- and ether lipid content in cells and in sEVs.

This thesis provides evidence that LAPTM4B is a dynamically regulated protein that functions at the intersection of sphingolipid
metabolism and nutrient signaling. Crosstalk between LAPT4B and ceramide facilitates nutrient signaling, and these processes may in part underlie the association observed between LAPT4B and cancer progression. Future work is warranted to elucidate the molecular function of LAPT4B in the endosomal-lysosomal system, and to elucidate the LAPT4B isoform-specific functions in different organs and diseases, as well as to solve the three-dimensional structure of LAPT4B. Such knowledge will deepen our understanding of LAPT4B- and ceramide-related pathophysiology in cancers and in sphingolipid-metabolism disorders.
1. REVIEW OF THE LITERATURE

1.1 SPHINGOLIPIDS

The sphingolipids were discovered by J.L.W Thudichum in 1884 (Thudichum et al., 1884), and this class of lipids constitutes about 10~20% of the total membrane lipids in eukaryotic cells (Holthuis et al., 2001; Jafurulla et al., 2017). However, the complexity and functional significance of the sphingolipids has only started to be uncovered in the past few decades (Hannun and Obeid, 2018).

The finding that sphingolipids function as “second messengers” in signal transduction led to a revived interest in sphingolipid research in the 1980’s. It was then shown that sphingosine is an inhibitor of protein kinase C (Hannun et al., 1986) and slows cell growth (Bode et al., 2014). The identification of G protein-coupled receptors activated by sphingosine-1-phosphate (S1P) (Lee et al., 1998) was a breakthrough finding and revealed that sphingolipids are not merely structural components of membranes, but actively participate in regulating cell functions as messenger molecules. The sphingosine-1-phosphate receptors were found to regulate cell migration and promote cell survival, and have been explored as targets for cancer therapies (Cartier and Hla, 2019). Several sphingolipid modulating drugs have been developed, and fingolimod, a sphingosine-1-phosphate mimic, is in clinical use for treating multiple sclerosis (Tintore et al., 2019). Functional roles of other sphingolipids have also emerged in recent decades. Ceramide
is generally exerting the opposite effects of S1P in cells, meaning the ceramide is pro-apoptotic, whereas S1P promotes cell survival (Hannun and Obeid, 2008).

The mechanisms that mediate ceramide signaling are not well understood, but may result in downregulation of nutrient-transporter proteins (Guenther et al., 2008), or induction of caspase-dependent apoptosis (Chipuk et al., 2012; Siskind et al., 2006; Teichgräber et al., 2007). Apart from their function as messenger molecules, sphingolipids play a fundamental role in forming membrane nanodomains, which serve as platforms for signaling transduction (Bieberich, 2018). In this regard, the correct subcellular localization of sphingolipid types is critical for cellular function. Studying the correlation between sphingolipid subcellular distribution and function is technically challenging, but the recent advances in the fields of model membranes and lipid probes (Blom et al., 2015; Colom et al., 2018; Mouritsen and Bagatolli, 2015), super-resolution microscopy (Adhikari et al., 2019), genetic manipulation technologies (Jiang and Doudna, 2017; Pickar-Oliver and Gersbach, 2019), and simulations of membrane dynamics (Marrink et al., 2019) have opened up new means for unraveling the functions of an increasing number of sphingolipid species.

1.1.1 Sphingolipid synthesis pathways

One central building block of sphingolipids is ceramide. Its
*novo* synthesis takes place in the endoplasmic reticulum (ER), starting with the condensation of L-serine and palmitoyl-CoA by the serine palmitoyltransferase, generating the reaction-intermediate 3-ketosphinganine. The reduction of 3-ketosphinganine by a reductase yields sphinganine, which is in turn acylated by ceramide synthase (CerS) to generate dihydroceramide. Finally, dihydroceramide desaturase reduces dihydroceramide to form ceramide (Hannun and Obeid, 2018).

In mammals, six CerS forms (CerS1–CerS6) have been identified, and these show differences in acyl-chain preference, in tissue distribution, and in involvement in biological functions (Table 1) (Levy and Futerman, 2010; Mullen et al., 2012).
### Table 1: Summary of mammalian Ceramide Synthases (CerS)

<table>
<thead>
<tr>
<th></th>
<th>CerS1</th>
<th>CerS2</th>
<th>CerS3</th>
<th>CerS4</th>
<th>CerS5</th>
<th>CerS6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acyl-chain preference</strong></td>
<td>C18</td>
<td>C20-C26</td>
<td>C22-C26</td>
<td>C18-C20</td>
<td>C16</td>
<td>C14, C16</td>
</tr>
<tr>
<td><strong>Tissue-specific distribution</strong></td>
<td>High in brain, skeletal muscle</td>
<td>High in liver, kidney, thymus</td>
<td>High in testis, prostate</td>
<td>High in heart, skin, liver</td>
<td>High in skeletal muscle, prostate</td>
<td>High in intestine, spleen</td>
</tr>
<tr>
<td><strong>Related cellular functions</strong></td>
<td>PCD; Chemotherapeutic sensitivity</td>
<td>PCD</td>
<td>Keratinocyte differentiation</td>
<td>Stem cell homeostasis; proliferation</td>
<td>P38/MAPK signaling; Bax activation; PCD</td>
<td>ER stress; PCD</td>
</tr>
<tr>
<td><strong>Related biological disease</strong></td>
<td>Cerebellar function; HNSCC</td>
<td>Breast cancer</td>
<td>Ichthyosis; congenital</td>
<td>Fetal Akinesia Deformation Sequence 1</td>
<td>Hereditary sensory; autonomic neuropathy Type 1</td>
<td>HNSCC</td>
</tr>
<tr>
<td><strong>KO mice phenotypes</strong></td>
<td>Protects from insulin resistance; improves glucose metabolism</td>
<td>Myelin-sheath defects; cerebellar degeneration</td>
<td>Loss of ultra-long chain-ceramides (≥C26)</td>
<td>Alopecia; decrease in C20-containing sphingolipids</td>
<td>Decreased palmitoyl (C16:0) ceramide pools</td>
<td>Behavioral abnormalities; decrease in C16-containing sphingolipids</td>
</tr>
</tbody>
</table>

PCD: programmed cell death; CNS: central nervous system; HNSCC: head and neck squamous cell carcinoma

Ceramide, one of the functionally best characterized sphingolipids, displays proapoptotic properties. Various mechanisms may lead to the occurrence of ceramide induced cell death. Ceramide causes mitochondrial membrane permeabilization, facilitates cytochrome-c release and BAX/BAK activation, and induces mitochondrial...
apoptosis (Chipuk et al., 2012; Siskind et al., 2006). Ceramide, moreover, activates protein phosphatase-1 (PP1) and protein phosphatase 2A (PP2A), leading to AKT dephosphorylation and sensitization to apoptosis (Ghosh et al., 2007; Van Hoof and Goris, 2003). Moreover, ceramide participates in CD95-mediated apoptosis (Herr et al., 1997), and can drive cell death by downregulating nutrient-transporter proteins (Guenther et al., 2008).

Following its de novo synthesis in the ER, ceramide can be further metabolized to more complex sphingolipids in the Golgi apparatus to which ceramide can be transported either by non-vesicular trafficking mediated by ceramide transfer protein (CERT), or via vesicular trafficking. The ceramide that is transferred by CERT is primarily utilized for sphingomyelin synthesis in the luminal leaflet of the trans-Golgi, catalyzed by sphingomyelin synthases (SMS1/2) (Hanada et al., 2003; Hannun and Obeid, 2018). After its synthesis, sphingomyelin undergoes very limited transmembrane movement: one estimate is that >85% of sphingomyelin is located in the extracellular leaflet of the plasma membrane (Kobayashi and Menon, 2018). ATP-binding cassette (ABC) transporters may facilitate the transbilayer movement of sphingomyelin, to maintain its asymmetrical membrane distribution (Aye et al., 2009; Kobayashi et al., 2006). In addition to its role as a structural component in the plasma membrane, sphingomyelin may, together with cholesterol, pack tightly into ordered membrane domains,
which may function as platforms for signal transduction (Lingwood and Simons, 2010). Sphingomyelin can be rapidly mobilized to form ceramide for metabolism into other sphingolipid species, and therefore plays an essential role in maintaining cellular sphingolipid homeostasis (Hannun and Obeid, 2018).

Ceramide that is transferred to the cis-Golgi by vesicular transport can be utilized by ceramide glucosyltransferase (UGCG), which transfers glucose to it from UDP-glucose to generate glucosylceramide (GluCer). GluCer serves as a precursor for generating more complex glycosphingolipids (GSL) (Hannun and Obeid, 2018). Alternatively, in the ER, ceramide can undergo conversion to galactosylceramide (GalCer), a primary lipid component of the myelin sheath (Aggarwal et al., 2011; Hill et al., 2018).

GluCer, the simplest of the glycosphingolipids, can undergo modification into hundreds of different GSL species by addition of various glycan moieties to the lipid head group. On the Golgi lumen, GluCer undergoes a galactose addition and conversion by LacCer synthase to lactosylceramide (LacCer) (Lannert et al., 1994). After its synthesis, LacCer constitutes the base for complex GSLs including gangliosides, globo sides, and lactosides according to the internal core carbohydrate sequence (Russo et al., 2016). The highly diverse group of GSLs are involved in signal transduction, in cell-cell recognition, and in communication, and carry out...
fundamental roles in regulating growth, migration, and differentiation (Wennekes et al., 2009).

The metabolic fate of GluCer is in part determined by whether it is transported through the Golgi apparatus via vesicular or non-vesicular routes. The vesicular transport route across the Golgi complex feeds GluCer into a metabolic pathway that generates the ganglio series of GSLs (D’Angelo et al., 2013a). Ganglio series of GSLs display vital cellular function: for instance, GM3 strongly inhibits EGF-dependent cell growth and EGFR tyrosine kinase (Yoon et al., 2006).

Instead, a non-vesicular route mediated by the four-phosphate adaptor protein (FAPP2) delivers GluCer to the trans-Golgi, where it is utilized for synthesis of the Globo series of GSLs (D’Angelo et al., 2007, 2013a). Globotriaosylceramide (known as Gb3) is the simplest globoside and is formed by Gb3 synthase (encoded by the A4GALT gene in humans), linking galactose to LacCer in the trans-Golgi (D’Angelo et al., 2013b, 2013a). Gb3 is the target of Shiga toxin produced by Escherichia coli, which can cause in gut cells inflammatory responses, lesions, and bloody diarrhea, as well as hemolytic-uremic syndrome (HUS) (Johannes and Römer, 2010).

After their synthesis and maturation in the Golgi network, glycosphingolipids and sphingomyelin are transported in
membrane-bound transport carriers, to the plasma membrane (PM) (Van Meer et al., 2008). In the PM, glycosphingolipids and sphingomyelin are involved in membrane remodeling and in cellular signaling transduction (Van Meer et al., 2008).

Ceramide is not only a basic building block for synthesis of sphingomyelin and complex sphingolipids, it is also a source of bioactive sphingolipids. Ceramide can undergo direct phosphorylation by ceramide kinase (CERK) to form ceramide-1-phosphate (C1P) in the trans-Golgi, which is further transferred to the plasma membrane by ceramide-1-phosphate transfer protein (CPTP) (Simanshu et al., 2013). In mammalian cells, C1P stimulates cell growth, migration, and inflammation (Gangoiti et al., 2010; Gomez-Muñoz et al., 2013).

Hydrolysis of ceramide yields sphingosine, which can be phosphorylated by one of two sphingosine kinases (SphK1 and SphK2) to form sphingosine-1-phosphate (S1P). (Hannun and Obeid, 2018; Pyne et al., 2016). S1P regulates cellular functions both via intra- and extracellular mechanisms. Cellular S1P concentrations are kept at low levels, and are subject to rapid degradation in the ER. S1P that is exported out of the cells can act on a set of G-protein-coupled receptors (S1PR1-S1PR5) in an autocrine or paracrine manner, regulating cell processes including cell survival and migration (Cartier and Hla, 2019; Pyne et al., 2016). Alternatively, intracellular S1P may participate in signal
transduction (Cartier and Hla, 2019), and may regulate gene transcription by binding and inhibiting histone deacetylases (HDAC) (Hait et al., 2009).

In addition to de novo synthesis, much of the cellular ceramide is generated by recycling of degradation products of sphingomyelin and complex sphingolipids. In this salvage pathway, ceramide is formed by direct N-acylation of sphingosine (Hannun and Obeid, 2018; Zhou and Blom, 2015). The main sphingolipid synthesis pathways are summarized in Figure 1.
Figure 1. The main sphingolipid synthesis pathways and their subcellular localization.

Cer: Ceramide; GalCer: Galactosylceramide; GluCer: Glucosylceramide; LacCer: Lactosylceramide; Gb3: Globotriaosylceramide; SM: Sphingomyelin; C1P: Ceramide-1-phosphate; GSL: Glycosphingolipid;
CERT: Ceramide transport protein; FAPP2: Four-phosphate adaptor protein; CPTP: Ceramide-1-phosphate transfer protein. Black arrows indicate sphingolipid metabolic enzyme reactions. Blue arrows indicate lipid transfer by transporter proteins, and red dashed arrows indicate vesicular trafficking.

### 1.1.2 Sphingolipid degradation pathways

Cells display a dynamic turnover of sphingolipids. A major pathway for glycosphingolipid and sphingomyelin catabolism is via degradation in the endosomal-lysosomal system (Breiden and Sandhoff, 2019; Hannun and Obeid, 2018).

Glycosphingolipids are degraded by a series of glycoside hydrolases that remove terminal sugar moieties from GSLs in a stepwise manner. Little redundancy exists between the lysosomal sphingolipid degrading enzymes, and loss of function in these enzymes often associates with lysosomal storage disease. For instance, alpha-galactosidase hydrolyzes the terminal alpha linkage of Gb3 and yields LacCer. Lack of alpha-galactosidase leads to cellular Gb3 accumulation, causing Fabry’s disease, which is characterized by painful crisis, angiokeratomas, and gastrointestinal disturbances (Clarke, 2007). Currently, a pharmaceutical drug, “Migalastat” has been developed to specifically enhance alpha-galactosidase enzyme activity; it is effective in the treatment of Fabry’s disease (McCafferty and Scott,
LacCer is further hydrolyzed by lactosylceramidase to generate GluCer, which is eventually broken down by beta-glucocerebrosidase (GBA) to generate ceramide (Breiden and Sandhoff, 2019; Hannun and Obeid, 2018). GalCer, mainly expressed at high levels in myelin, the membranous sheath around nerve axons (Boggs et al., 2008), is degraded by the hydrolase beta-galactocerebrosidase (GALC) to generate ceramide (Breiden and Sandhoff, 2019) (Figure 2).

Sphingomyelinases (SMase) catalyze the removal of the phosphorylcholine headgroup of sphingomyelin (Figure 2). SMase can be divided into three categories depending on each one’s pH optimum: alkaline SMase, acid SMase, and neutral SMase (Hannun and Obeid, 2018). Alkaline SMase is highly expressed in the intestine and liver and is responsible for digestion of dietary sphingomyelin, whereas the other SMases are ubiquitously expressed. Inside the cells, acid SMase localizes to the lysosomes, but neutral SMase mainly localizes on the plasma membrane (Goi and Alonso, 2002). The differing subcellular distribution of the enzymes suggests that compartmentalization of SM hydrolysis is crucial for maintaining proper cellular functions (Herz et al., 2009; Stoffel et al., 2005).

Ceramide can further be catabolized to sphingosine by acid ceramidase (ASAHI), the only enzyme to degrade lysosomal ceramide (Jameson et al., 1987; Sugita et al., 1972). Ceramide
degradation yields a free fatty acid and sphingosine, which can exit the lysosome and be reutilized by the cell. At the acidic pH of the lysosome, sphingosine exists in its protonated form for most of the time, and this positively charged sphingosine cannot freely diffuse out of the lysosome. In combination with the finding that sphingosine often accumulates in lysosomal storage disorders, this has led to the suggestion that cells may have a system for active sphingosine transport (Carreira et al., 2017; Lloyd-Evans and Platt, 2010; Lloyd-Evans et al., 2008). However, it is unclear whether the sphingosine accumulation in lysosomal storage diseases is due to impaired export, or is simply a reflection of an enlarged acidic compartment (Blom et al., 2012). Sphingosine was initially shown to be an efficient inhibitor of protein kinase C (Hannun et al., 1986), a known apoptosis-related molecule (Isakov, 2018). Sphingosine itself and its metabolites are involved in the activation of the mitochondrial apoptosis pathway and in cell senescence (Hernández-Corbacho et al., 2017). Abnormal levels of sphingosine cause defects in cellular functions, and are observable in several diseases such as cystic fibrosis (Becker et al., 2018).
Figure 2. The main sphingolipid degradation pathways in late endosomes/lysosomes.

Cer: Ceramide; Sph: Sphingosine; GalCer: Galactosylceramide; GluCer: Glucosylceramide; LacCer: Lactosylceramide; Gb3: Globotriaosylceramide; GM3: monosialodihexosylganglioside; SM: Sphingomyelin; GALK: Beta-galactocerebrosidase; GBA: Beta-glucocerebrosidase; SMPD1: Acid Sphingomyelinase; ASAHI: Acid Ceramidase. Ceramide generating and degrading enzymes are indicated with italics. Black arrows indicate enzyme-mediated sphingolipid-metabolism reactions. The dashed arrow indicates the unknown mechanism of sphingosine-exporting lysosome.

1.1.3 Sphingolipid metabolism disorders
Sphingolipid homeostasis regulates cellular functions under physiological and pathological conditions, and aberrant sphingolipid metabolism is evident in various diseases, such as atherosclerosis (Vorkas et al., 2015), diabetes (Sokolowska and Blachnio-Zabielska, 2019), Farber’s disease (acid ceramidase deficiency) (Sands, 2013), and in cancers (Morad and Cabot, 2013).

For any organism, sphingolipids are essential, because sphingolipid metabolic defects give rise to characteristic disorders. Moreover, one sphingolipid type cannot fully compensate for the lack of another. Sphingolipidoses are a class of lipid storage diseases caused by sphingolipid metabolism dysfunction. In such diseases, certain key enzymes are defective, ones which induce accumulation of intermediate metabolites and clinical symptoms (Kolter and Sandhoff, 2006; Platt, 2014). Many efforts have been made to pinpoint the molecular mechanisms of these diseases and to develop targeted drugs (Table 2).
<table>
<thead>
<tr>
<th>Disease</th>
<th>Defected enzyme</th>
<th>Disordered lipid</th>
<th>Syndrome</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaucher disease</td>
<td>Beta-glucosidase</td>
<td>Glucocerebroside</td>
<td>Enlarged spleen and liver, liver malfunction</td>
<td>Alglucerase, Miglustat, Eliglustat</td>
</tr>
<tr>
<td>GM1-Gangliosidosis</td>
<td>Beta-galactosidase</td>
<td>GM1 ganglioside</td>
<td>Neurodegeneration</td>
<td>No known cure</td>
</tr>
<tr>
<td>GM2-Gangliosidosis</td>
<td>Beta-hexosaminidase</td>
<td>Gangliosides</td>
<td>Slowing of growth, plateau of motor development</td>
<td>No known cure</td>
</tr>
<tr>
<td>Krabbe Disease</td>
<td>Galactosyleramidase</td>
<td>Galactosyleramide, Psychosine</td>
<td>Irritability, fevers, limb stiffness, seizures</td>
<td>No known cure</td>
</tr>
<tr>
<td>Metachromatic</td>
<td>Arylsulfatase A</td>
<td>Sulfatides</td>
<td>Demyelination of nervous system</td>
<td>No specific treatment</td>
</tr>
<tr>
<td>Leukodystrophy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fabry Disease</td>
<td>Alpha-galactosidase A</td>
<td>Gb3</td>
<td>Chronic pain, kidney damage</td>
<td>Migalastat</td>
</tr>
<tr>
<td>Faber Disease</td>
<td>ASAH1</td>
<td>Ceramide</td>
<td>Abnormalities in the joints, liver</td>
<td>No known cure</td>
</tr>
</tbody>
</table>
1.2 THE ENDOSONAL-LYSOSOMAL SYSTEM

The endosomal-lysosomal system, including early endosomes (EE), recycling endosomes (RE), multivesicular bodies (MVB), late endosomes (LE), and lysosomes, controls cargo sorting, trafficking, and degradation, and it participates in cellular physiological activities such as endocytosis and exocytosis (Naslavsky and Caplan, 2018).

Endocytosis refers to the cellular activity taking up extracellular substances; the whole process includes 1) Plasma membrane (PM) folding inward, forming a cavity surrounding the cargos, the PM then folds back until the ends of the in-folded membrane contact, and the vesicle is pinched off as the ends of the in-folded membrane fuse together. 2) The endocytic vesicles transport cargos to EE, under the regulation of small GTPases such as Rab5 (Langemeyer et al., 2018). 3) The cargos are further sorted to MVB and LE. 4) The contents of MVBs or LEs are transferred to lysosomes to become degraded.

Membrane fusion and vesicular-sorting events are key steps during the whole process, regulated by various pathways or molecules, such as clathrin-mediated endocytosis (Kaksonen and Roux, 2018), caveolae, and phagocytosis (Baranov et al., 2019; Sandvig et al., 2018). The endosomal cargo (carried in EE, RE, MVB) can alternatively be recycled back to the PM. Some cargos in MVBs are contained in membrane-enclosed intraluminal vesicles (ILVs),
which can be secreted in the form of extracellular vesicles (EVs) (Eitan et al., 2016).

1.2.1 Endosome maturation and trafficking

1.2.1.1 Early Endosomes
Material internalized from plasma membrane enters EE, which function as key sorting stations, for instance of internalized membrane proteins and lipid (Naslavsky and Caplan, 2018). EE, which are dynamic and undergo multiple fusion and fission events during this sorting process, have certain characteristics that distinguish these organelles from later endosomal compartments. Phosphatidylinositol 3-phosphate (PI3P) is enriched in their limiting membranes (Gillooly et al., 2000), and this lipid is central in regulating the sorting of receptor in EE (Petiot et al., 2003). EEs contain vacuolar ATPases (V-ATPase), a family of proton pumps that couple ATP hydrolysis to proton transport across the EE membrane, thus generating a slightly acidic EE lumen with a pH about 6.5 (Hu et al., 2015; Jovic et al., 2010). The small GTPase Rab5 is a central player in EE maturation and regulates cargo entry from PM to EE, as well as EE homotypic fusion and motility (Langemeyer et al., 2018). Early endosomal antigen-1 (EEA1) is a frequent marker for EE; this protein coordinates EE fusion together with proteins of the SNARE family (Jovic et al., 2010).
1.2.1.2 Recycling Endosomes

Cargos that are not destined for degradation in late endosomes can instead be targeted back to the plasma membrane via recycling endosomes (RE), located in the perinuclear region of the cell. RE typically display a tubular shape and form a network with tubule-vesicular endosomes. Rab proteins are key factors in regulating RE activities; for instance, Rab11A/B regulates the transport of RE to PM, and Rab8A/B and Rab15 modulate endocytic recycling (Goldenring, 2015; Wandinger-Ness and Zerial, 2014).

1.2.1.3 Multivesicular Bodies and Extracellular Vesicles

Endocytosed material can be further sorted into MVB, a special category of endosomes that contain membrane-bound intraluminal vesicles (ILVs). The ILVs are formed by budding into the MVB lumen portion by means of the concerted action of ESCRT (Endosomal Sorting Complex Required for Transport) machinery (Juan and Fürthauer, 2018). ILVs may, however, also be formed independently of ESCRT proteins, for example by neutral sphingomyelinase acting on the endosome surface, generating ceramide that induces a negative membrane curvature and inward budding (Trajkovic et al., 2008). Phosphatidic acid may similarly induce a negative membrane curvature due to their biophysical properties, and directly interact with essential proteins in the ILVs budding process, and as a consequence, regulate ILV formation (Egea-Jimenez and Zimmermann, 2018).
The cargos in MVB can either be targeted for lysosomal degradation, or alternatively be released into extracellular space by MVB fusion with the PM. The ILVs released in this pathway will form a subtype of extracellular vesicles (EVs) termed exosomes (van Niel et al., 2018). The term “extracellular vesicle” initially appeared in a scientific publication in 1970 (Bonucci, 1970), although studies utilizing ultracentrifugation and electron microscopy suggested the existence of extracellular vesicles as early as in the 1940s (Chargaff and West, 1946; Wolf, 1967). EVs are lipid bilayer-delimited particles released from cells and play a role in mediating intercellular communication. According to size and origin, EVs are usually classified into three categories: exosomes, microvesicles, and apoptotic bodies (Table 3).

<table>
<thead>
<tr>
<th></th>
<th>Exosomes</th>
<th>Microvesicles</th>
<th>Apoptotic bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td>40-120 nm</td>
<td>50-1,000 nm</td>
<td>500-2,000 nm</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td>Endocytic path</td>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td><strong>Physiological function</strong></td>
<td>Cell-cell communication</td>
<td>Cell-cell communication</td>
<td>Phagocytosis induction</td>
</tr>
<tr>
<td><strong>Marker proteins</strong></td>
<td>CD81, CD9, CD63</td>
<td>CD40, integrins</td>
<td>Annexin V</td>
</tr>
</tbody>
</table>
Exosomes and microvesicles represent EVs in a normal physiological state. Exosomes are formed by inward budding of the delimiting endosomal membrane to generate MVBs containing ILVs. Some MVBs eventually fuse with the plasma membrane to release their vesicular content as exosomes into the extracellular space. Microvesicles, on the other hand, are the particles directly or indirectly formed by shedding of plasma membrane (Mathieu et al., 2019).

1.2.1.4 Late Endosome
Cargos in LE are preferably targeted for lysosomal degradation. The properties of LE are closer to those of lysosomes than of other endosomes. LE have a low pH (about 5.0-6.0), and are enriched in PI(3,5)P2 and 2’2-dioleoyl lysobisphosphatidic acid (LBPA) (Naslavsky and Caplan, 2018). PI(3,5)P2 regulates the sorting of degradative cargos into MVB (Odorizzi et al., 1998), LBPA regulates endosome structure and function (Kobayashi et al., 1998), and as a negatively-charged lipids, LBPA may serve as a calcium buffer in the lumen of LE (Gruenberg, 2020).

1.2.1.5 Lysosome
Lysosomes are capable of digesting intercellular and extracellular materials such as damaged organelles, malformed proteins, and lipids into simpler compounds that can be recycled by the cell as building blocks for more complex biomolecules, and for energy production (Luzio et al., 2007). The degradative capacity of lysosomes depends on V-ATPase-mediated acidification of this
organelle (~pH 5), a process dependent on the ATP hydrolysis activity of this proton pump (Mindell, 2012). The lysosomal hydrolases work efficiently at low pH (Mellman et al., 1986), and more than 60 hydrolases with acidic pH optima have been identified; these are, in combination, able to degrade all cellular macromolecules. The lysosomal acid hydrolases include glycosidases, lysosomal proteases, lipases, phospholipases, and others (Feher, 2017). Some ion channel transporters such as anion-transporter channel 7 (CLC7), the cation-transporter mucolipin 1 (MCOLN1) and the two-pore calcium channel 1 (TPC1 and TPC2) are capable of releasing protons/cations from the lysosome lumen into cytosol (Mindell, 2012; Xu and Ren, 2015).

Characteristics of the endosomal-lysosomal system compartments are summarized in Table 4.

<table>
<thead>
<tr>
<th>Function</th>
<th>EE</th>
<th>RE</th>
<th>MVB</th>
<th>LE</th>
<th>Lysosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal pH</td>
<td>6.5</td>
<td>6.4-6.5</td>
<td>5.5</td>
<td>5.0-6.0</td>
<td>5.0-5.5</td>
</tr>
<tr>
<td>Protein marker</td>
<td>Rab5, EEA1</td>
<td>Rab11</td>
<td>Rab7</td>
<td>Rab7</td>
<td>LAMP1</td>
</tr>
</tbody>
</table>
1.2.2 Physiological functions of the endosomal-lysosomal system

1.2.2.1 The lysosome as a hub for metabolic signaling

Advances in the past decades have shown that the physiological significance of lysosomes goes beyond just degradation of biomolecules. The target of rapamycin complex 1 (mTORC1) is a driving force in many cellular anabolic processes and is active at the lysosomal membrane (Sancak et al., 2008, 2010; Zoncu et al., 2011). The discovery of the mTORC1 machinery sparked an interest in research on lysosomes as a metabolic hub, mediating the crosstalk between cellular catabolic and anabolic processes.

mTORC1 is evolutionarily conserved machinery regulating cellular growth and autophagy, as well as cellular nutrients and energy (Saxton and Sabatini, 2017). When cellular amino acid or other nutrients levels are high, mTORC1 is recruited to the lysosomal surface in a translocation process that requires active Rag GTPases, further sense amino acid by a lysosome-centric inside-out mechanism dependent on V-ATPase (Sancak et al., 2008, 2010; Zoncu et al., 2011).

mTORC1 is not only a master controller of cell growth but also regulates lysosome biogenesis and positioning (Poüs and Codogno, 2011; Settembre et al., 2012). Gene expression of lysosomal proteins has long been thought to be constitutive. This was,
however, challenged by the discovery that the transcriptional factor EB (TFEB), a member of the basic helix-loop-helix leucine zipper family of transcriptional factors, controls the expression of lysosomal proteins (Sardiello et al., 2009). The activity of TFEB is regulated by mTORC1, which phosphorylates several serine residues in TFEB. Phospho-serine 211 in TFEB promotes its interaction with the protein 14-3-3, resulting in the retention of TFEB in the cytosol. Dephosphorylation of TFEB Serine 211 releases TFEB from 14-3-3, allowing its translocation to the nucleus to regulate gene expression (Martina et al., 2012). TFEB binds directly to the 10-base-pair motif (GTCACGTGAC), called the coordinated lysosomal expression and regulation (CLEAR) element, which is present in the promoter region of many lysosomal genes (Napolitano and Ballabio, 2016). As such, the mTORC1-TFEB pathway is a bridge between lysosome biogenesis and the sensing of cellular nutrients.

mTORC1 also regulates lysosomal positioning within the cell. Active mTORC1 on the lysosome, when there is high nutrient availability, leads to lysosomes being located closer to the plasma membrane, whereas starvation-induced low mTORC1 activity is linked to perinuclear lysosome distribution (Korolchuk et al., 2011). The detailed molecular mechanisms underlying this observation currently remain unsolved, but several proteins are critical in this process, including KIF2 (Kinesis heavy chain member 2) and ARL8B (ADP-ribosylation factor-like 8B) (Korolchuk et al., 2011).
This suggests that mTORC1 controls lysosome positioning by regulating cytoskeleton-dependent motors (Korolchuk et al., 2011). In summary, the lysosome emerges as a hub of cellular metabolism, regulating cellular growth, autophagy, and metabolism (Lawrence and Zoncu, 2019).

1.2.2.2 EV function in physiological and pathological conditions
EVs display vital functions in cell-to-cell communication. Cargos in EVs consist of a range of molecules, including nucleic acids, proteins, and lipids, all of which can modulate the function of the recipient cells (van Niel et al., 2018). Various known mechanisms exist by which acceptor cells take up EVs, broadly divided into two categories: clathrin-dependent endocytosis and clathrin-independent mechanisms. The clathrin-independent pathways include caveolin-mediated uptake, macropinocytosis, phagocytosis, and lipid raft-mediated internalization (Maas et al., 2017; Mathieu et al., 2019). The manner in which the cellular uptake of EVs occurs depends on the protein and lipid signature of the EV membrane surface, as well as on the recipient cell’s plasma membrane structure and content. Studies have documented key protein-protein interactions taking place between EV membranes and the recipient cell’s plasma membrane (Maas et al., 2017; Mathieu et al., 2019). Proteinase K treatment of the EVs markedly reduces the uptake activity by ovarian cancer cell line SKOV3 (Escrevente et al., 2011), and antibodies against either key ligands (CD81, CD9) or receptors (like Tim4) block EV uptake. These findings argue for
the key role of these proteins in the EV-uptake process (Mulcahy et al., 2014). Other studies have shown the critical roles of lipids in mediating EV uptake; in particular, glycosphingolipids and cholesterol, which regulate membrane order, can control EV uptake by modulating lipid raft-mediated endocytosis (Mulcahy et al., 2014). Key proteins and lipids regulating the EV uptake process (Mulcahy et al., 2014) are listed in Table 5.

Table 5: Factors regulating cellular EV uptake

<table>
<thead>
<tr>
<th>Category</th>
<th>Key molecule</th>
<th>Key pathway</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>CD91</td>
<td>Endocytosis</td>
<td>Human receptor associated protein (RAP)</td>
</tr>
<tr>
<td></td>
<td>Galectin-5</td>
<td>Endocytosis</td>
<td>Asialofetuin</td>
</tr>
<tr>
<td></td>
<td>Dynamin</td>
<td>Clathrin- and caveolin-dependent endocytosis</td>
<td>NSC23766</td>
</tr>
<tr>
<td></td>
<td>Dynamin-2</td>
<td>Clathrin- and caveolin-dependent endocytosis</td>
<td>Dynasore</td>
</tr>
<tr>
<td></td>
<td>Phosphoinositide 3-kinases (PI3Ks)</td>
<td>Phagocytosis</td>
<td>Wortmannin, LY294002</td>
</tr>
<tr>
<td><strong>Lipid</strong></td>
<td>Glycosphingolipid</td>
<td>Lipid raft-mediated endocytosis</td>
<td>Fumonisin B1, N-butyldideoxyojirimycin hydrochloride</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Lipid raft-mediated endocytosis</td>
<td>Filipin, Simvastatin, Methyl-b-cyclodextrin</td>
</tr>
</tbody>
</table>

Studies have documented several physiological functions of EVs, especially in the immune system and central nervous system (CNS). For example, EVs inhibit the immune response by mediating
immunosuppressive effects on T lymphocytes and natural killer (NK) cells (Robbins and Morelli, 2014). Microglia-derived EVs stimulate synaptic activity by promoting ceramide and sphingosine production (Antonucci et al., 2012). Moreover, studies have indicated an association between EVs and disease progression, including viral spread (Urbanelli et al., 2019), tumorigenesis (Becker et al., 2016), and in neurodegenerative diseases (Pegtel et al., 2014).
1.3 THE LYSOSONE-ASSOCIATED PROTEIN TRANSMEMBRANE (LAPTM) FAMILY

The biological processes of the endosomal-lysosomal system (such as endocytosis, exocytosis, and secretion) are dependent on proteins that mediate membrane fusion and vesicular trafficking, as well as molecular pumps to maintain the optimal pH. Lysosome membrane proteins (LMPs) are essential in maintaining these activities and in preserving organelle function (Schwake et al., 2013). To date, more than 100 LMPs have been identified (Schröder et al., 2010; Schwake et al., 2013). The lysosome-associated protein transmembrane (LAPTM) family, one of the LMP families, consists of three members: the four-membrane-spanning LAPTM4A and LAPTM4B, and the five-membrane-spanning LAPTM5 (Figure 3, Table 6). LAPTM4A and -4B are ubiquitously expressed, whereas the expression of LAPTM5 is restricted to cells of lymphoid origin (Adra et al., 1996; Hogue et al., 1999; Shao et al., 2003). The LAPTM4A and -4B amino acid sequences display ~46% identity, and ~25% and 23% sequence conservation to LAPTM5, respectively.
Figure 3. Structural models of LAPTM family proteins.

LAPTM4A and LAPTM4B are four-transmembrane proteins, with both their N-terminus and C-terminus facing cytosol, whereas LAPTM5 is a five-transmembrane protein, with its N-terminus facing the lysosome lumen and its C-terminus facing cytosol. LE/LY: late endosome/lysosome.
### Table 6. Properties of the LAPT M protein family

<table>
<thead>
<tr>
<th></th>
<th>LAPT M4A</th>
<th>LAPT M4B</th>
<th>LAPT M5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expression</strong></td>
<td>Ubiquitous</td>
<td>Ubiquitous</td>
<td>Restricted to cells of lymphoid origin</td>
</tr>
<tr>
<td><strong>Studied in cancer</strong></td>
<td>None</td>
<td>Highly expressed in many cancers; Promotes cancer-related processes</td>
<td>Low expression in many cancers; Sensitizes cells to death</td>
</tr>
<tr>
<td><strong>Studied in Sphingolipids</strong></td>
<td>Regulates Gb3 synthesis</td>
<td>Facilitates ceramide export from lysosome</td>
<td>None</td>
</tr>
<tr>
<td><strong>Studied in Immunity</strong></td>
<td>Regulates Shiga-toxin-binding activity</td>
<td>Reduces human regulatory T cell immunosuppression</td>
<td>Promotes proinflammatory signaling</td>
</tr>
<tr>
<td><strong>Functional motifs</strong></td>
<td>Tyr-based motifs (YxxΦ); PY motifs; lysosomal targeting signals</td>
<td>SH3-domain-binding motif; Arginine-rich polybasic stretch; PY motifs; lysosomal targeting signals; sphingolipid interaction motif</td>
<td>PY motif (L/PPxY); ubiquitin-interacting motif</td>
</tr>
<tr>
<td>KO mice phenotypes</td>
<td>Increased glucose tolerance; Abnormal prepulse inhibition; Abnormal tibia morphology</td>
<td>Subviable</td>
<td>Decreased leukocyte cell number; Increased mean corpuscular hemoglobin</td>
</tr>
</tbody>
</table>

#### 1.3.1 LAPT M4A

In 1996, Hogue et al. discovered the first member of the LAPT M family (Hogue et al., 1996). In their seminal paper, a mouse cDNA was identified that was able to complement a thymidine-transport deficiency in *S. cerevisiae*. This protein they predicted to consist of four transmembrane domains, a short N-terminus, and a C-terminal
domain containing lysosomal-targeting motifs (Hogue et al., 1996). The authors provisionally named their newly discovered protein MTP (mouse transport protein). In a follow-up study by the same laboratory, MTP showed expression in yeast and reportedly caused hypersensitivity to some drugs and resistance to others, presumably by affecting their subcellular compartmentalization and accessibility to target proteins (Hogue et al., 1999). The authors speculated that MTP may localize to lysosomes based on its C-terminally located tyrosine-sorting motifs. This was later confirmed in a study demonstrating that overexpressed MTP co-localizes with the late endosomal markers M6PR and LAMP-2 (Cabrita et al., 1999). The MTP protein was renamed LAPT4M4A (lysosome associated protein transmembrane 4A) due to its subcellular localization and its high similarity to the then-discovered LAPT5 (Adra et al., 1996).

Although evidence is ample that LAPT family proteins regulate the subcellular distribution of small solutes, what is currently unclear is whether these proteins are bona fide transporters or whether they are regulators of other proteins that mediate transmembrane transport. Some evidence exists in support of both models. In favor of a direct transporter/unipporter activity is the observation that LAPT4M4A can complement a thymidine transport defect in *S. cerevisiae* (Hogue et al., 1996). Since yeast has no known endogenous LAPT4M4A homolog, it is unlikely that complementation would take place by ectopically expressed mouse
LAPT M4A specifically regulating an endogenous yeast transporter protein. However, recent studies have shown that LAPT M4A can interact with the organic cation transporter hOCT2 and can regulate its function and localization (Grabner et al., 2011). Similarly, LAPT M4B has been shown to interact with amino acid transporter 4F2hc/LAT1 and cause increased lysosomal leucine uptake (Milkereit et al., 2015; Zhou et al., 2018), suggesting that the LAPT M protein family may function in the form of complex mediating transmembrane transport processes. A recent study shows that LAPT M4A-knockout cells express low levels of the glycosphingolipid Gb3, thereby providing resistance to EHEC infection (Pacheco et al., 2018; Tian et al., 2018; Yamaji et al., 2019). The mechanism by which LAPT M4A affects Gb3 levels remains to be established, but it might depend on Golgi localization of substrates required for Gb3 synthesis.

1.3.2 LAPT M5

LAPT M5 contains five predicted membrane-spanning domains with a cytosolic C-terminus and an N-terminus facing the endosomal lumen, thus contrasting with the other members of the protein family, which have four TM domains with both termini facing the cytosol (Adra et al., 1996). Similar to the other family members, LAPT M5 was designated a lysosomal protein based on its colocalization with LAMP1 (Adra et al., 1996). Targeting of LAPT M5 to the late endosomal compartment requires interaction with Nedd4 but is independent of LAPT M5 ubiquitination (Pak et
LAPMT5 is mainly expressed in cells of lymphoid origin, and studies have documented roles for LAPMT5 in the immune response.

LAPMT5 has been identified as one of the nine genes highly upregulated in Sjögren’s syndrome (Azuma et al., 2002), an autoimmune disease characterized by lymphocytic infiltration and atrophy of exocrine glands (Brito-Zerón et al., 2016). LAPMT5 has also been reported to cause downregulation of the pre-B-cell receptor (Kawano et al., 2012), which could prove a contributing factor in its association with Sjögren’s syndrome (Zouali, 2014) and Waldenstrom macroglobulinemia (Roccaro et al., 2016).

LAPMT5 mRNA is abundant in resting B cells and is rapidly downregulated in response to B cell activation (Seimiya et al., 2003). Mature B cells from LAPMT5−/− mice display increased amounts of cell-surface B-cell receptors, and develop high amounts of autoantibodies in their serum during aging (Ouchida et al., 2010). In dendritic cells, LAPMT5 interacts with CD1e (Angénieux et al., 2012), a glycoprotein that mediates presentation of lipid antigens of microbial origin to T cells. LAPMT5 has been further shown to control surface T cell-antigen receptors (Ouchida et al., 2008). Low LAPMT5 expression has also been observable in certain types of cancer and associated with a poor prognosis; examples are non-small cell lung cancer and esophageal squamous cell carcinoma (Nuylan et al., 2016). In support of this finding, high levels of
LAPTM5 have shown themselves able to sensitize cells to cell death (Ishihara et al., 2011).

1.3.3 LAPTM4B

1.3.3.1 LAPTM4B gene and transcript
Due to its association with poor prognosis in several types of cancer, LAPTM4B, despite being the last one to be identified, has become the most studied of the LAPTM family of proteins. LAPTM4B, originally discovered from a transcript upregulated in hepatocellular cancer, is controlled by the LAPTM4B gene, which in humans is located on chromosome 8q22.1 (Shao et al., 2003), and its promoter region contains cis-regulatory elements including cAMP-responsive element binding protein-1 (CREB1) (Zhang et al., 2013) and transcription factor AP-4 (TFAP4), both of which serve to elevate LAPTM4B expression. A common polymorphism in the human LAPTM4B gene is a 19bp duplication in the 5’-UTR of the gene, resulting in an allele called LAPTM4B*2 (Wang et al., 2013; Xu et al., 2012). The *2 allele contains two functional binding sites for TFAP4 instead of the one found in LAPTM4B*1. The TFAP mediated regulation of LAPTM4B expression may prove of importance, since the *2 allele is associated with risk for hepatocellular carcinoma (Yang et al., 2012; Zhai et al., 2012) and gastric cancer (Liu et al., 2007). The LAPTM4B transcript has been targeted by miR-188-5p, which, in a mouse xenograft model of prostate cancer, downregulates LAPTM4B expression and thus
reduces tumor growth and metastasis (Zhang et al., 2015). The LAPT4B mRNA contains alternative start codons (ATG), and can be translated into either a 35-kDa (LAPT4B-35) or a 24-kDa isoform (LAPT4B-24) lacking amino acids 1-91 (Shao et al., 2003).

1.3.3.2 LAPT4B protein and functional motifs
Currently, studies of LAPT4B mostly rely on protein overexpression studies in which either LAPT4B-35 or LAPT4B-24 is expressed. LAPT4B-35 represents the longest open reading frame (ORF) of the transcript and has been the focus of most investigations. Several functional amino acid motifs have emerged in LAPT4B (Figure 4). The LAPT4B-35-specific N-terminus contains two functional amino acid motifs: an SH3 domain binding motif (PPRP; position: aa 12-15) which interacts with PI3K subunit p85α to promote efflux of chemotherapeutic drugs (Li et al., 2010a) and cell migration (Liu et al., 2009), and an arginine-rich polybasic stretch (8R; position: aa 52-67) which interacts with PIPKIγi5 and regulates autophagy (Tan et al., 2015a). Other motifs are shared by both LAPT4B isoforms, including PY motifs (L/PPXY, position: aa 295-298, and aa 311-314), which are necessary for binding the ubiquitin ligase Nedd4 (Milkereit and Rotin, 2011), lysosomal targeting signals (Milkereit and Rotin, 2011; Shao et al., 2003), and a sphingolipid interaction motif in the third transmembrane segment ([V/I/T/L]XX[V/I/T/L][V/I/T/L]XX[V/I/T/L][F/W/Y]; position:
aa 208-216) which was identified in this study (Zhou et al., 2018).

**Figure 4: Known functional motifs in LAPT4B protein.**

The SH3-domain-binding motif (PPRP) and arginine-rich polybasic stretch (8R) are located in the LAPT4B-35 N-terminus. The SPL interaction motif is found in the third transmembrane domain, whereas the PY motif is in the C-terminus of LAPT4B.

Overexpressed LAPT4B localizes to endosomal/lysosomal compartments, as well as to the plasma membrane (Blom et al., 2015; Milkereit and Rotin, 2011). In this thesis, we have shown that endogenous LAPT4B co-localizes mainly with the MVB marker lipid LBPA, and to a lesser extent with LAMP-1 and CD63 (Dichlberger et al., 2021).

### 1.3.3.3 Functional properties of LAPT4B

Most studies of LAPT4B have focused on cancer-related processes. At the cellular level, LAPT4B regulates various cellular functions, including autophagy (Li et al., 2011), drug
resistance (Blom et al., 2015; Li et al., 2010a, 2010b), and cell migration and invasion (Xiao et al., 2017). Li et al initially made the observation that LAPTM4B depletion induces loss of lysosomal integrity and blocks autophagic flux (Li et al., 2011). Research later showed that a PI(4,5)P2-binding polybasic motif in LAPTM4B-35 is required to stabilize the epidermal growth factor receptor (EGFR) at endosomes to initiate autophagy (Tan et al., 2015a, 2015b). Milkereit et al reported that LAPTM4B can alternatively suppress autophagy and promote mTORC1 activation by recruiting the leucine transporter LAT1/4F2hc to lysosomes (Milkereit et al., 2015). We similarly showed that LAPTM4B promotes mTORC1 activity and that the interaction with 4F2hc is regulated by ceramide together with a ceramide-regulated element in the third transmembrane domain of LAPTM4B (Zhou et al., 2018).

Despite being the topic of many studies, the role of LAPTM4B in cancer progression is unclear. On the one hand, LAPTM4B has been described as promoting survival signaling and as desensitizing cells to chemotherapy by facilitating drug efflux (Li et al., 2010a), or facilitating their sequestration in lysosomes (Li et al., 2010b). On the other hand, others have instead suggested a negative correlation between LAPTM4B and chemotherapy resistance. For instance, a low level of LAPTM4B has been identified as part of an expression signature that predicts poor response to neoadjuvant chemotherapy in HER2-negative breast
cancers (de Ronde et al., 2013). In agreement with this, we have reported that LAPTMB depleted cells are protected from anthracycline-induced apoptosis (Blom et al., 2015). LAPTMB has also been associated with epithelial-mesenchymal transition (Wang et al., 2018; Xiao et al., 2017), with cell migration, with invasion (Liu et al., 2009), and with metastasis (Yang et al., 2010). Overexpression of LAPTMB expression can lead to an increase in actin-based plasma-membrane protrusions (Milkereit and Rotin, 2011), structures that are known to be critical in directed cell migration (Mattila and Lappalainen, 2008). Part of the migratory phenotype may be mediated by the SH3-domain-binding motif in the N-terminus of LAPTMB-35 (Liu et al., 2009).

1.3.3.4 LAPTMB mediated regulation of sphingolipid metabolism
The four-membrane-spanning LAPT proteins are emerging as regulators of sphingolipid metabolism. Ceramide was generally considered to be unable to exit the lysosome, instead requiring degradation by acid ceramidase (ASAH1), generating a free fatty acid and sphingosine that can subsequently leave the lysosome (Lloyd-Evans et al., 2008; Parveen et al., 2019). We recently reported, however, that ceramide is not inevitably degraded in late endosomes, and it may exit this compartment without being subject to hydrolysis (Blom et al., 2015). In an unbiased screen of multimembrane-spanning lysosomal proteins, we noted that
LAPTM4B silencing causes a severe ceramide accumulation in the lysosome, similar to that observable in ASAH1-depleted cells.

Even more severe ceramide accumulation occurred with LAPTM4B/ASAH1 double depletion. By taking advantage of a cross-linkable ceramide probe, we established that LAPTM4B directly interacts with ceramide, facilitates its export from the endosomal compartment, and consequently sensitizes cells to chemotherapeutic treatment (Figure 5) (Blom et al., 2015). This is in agreement with reports that ceramide outside the lysosomes, in particular in ER-mitochondrial contact sites, promotes cell death and chemotherapy sensitivity (Chipuk et al., 2012; Tafesse et al., 2014). Whether LAPTM4B itself transports lysosomal ceramide or modulates other export mechanisms is unknown.

Figure 5: A model of LAPTM4B in ceramide metabolism.
LAPTM4B facilitates ceramide export from the endosome/lysosome compartment. Modified from (Blom et al., 2015). The detailed mechanism as to whether LAPTM4B is a direct transporter or regulates other export machineries remains unresolved.
1.3.3.5 LAPTM4B study in organs and subjects

LAPTM4B research in human subjects comes chiefly from cancer cohort studies. High expression of LAPTM4B mRNA emerged in several types of solid cancer, including of the prostate (Zhang et al., 2014), colon (Kang et al., 2012; Kasper et al., 2005), pancreas (Zhang et al., 2012), gallbladder (Zhou et al., 2007), lung (Kasper et al., 2005; Tang et al., 2014), breast (Kasper et al., 2005; Li et al., 2010b; de Ronde et al., 2013; Xiao et al., 2017), uterus (Kasper et al., 2005), and ovary (Kasper et al., 2005; Yang et al., 2008). A recent study in acute myeloid leukemia (AML) identified LAPTM4B as one of the 17 genes which predict leukemia stemness, helping us to assess whether patients can benefit from standard therapy options (Ng et al., 2016). Interestingly, a study of autophagy-related gene networks revealed that some cancers display increased (e.g. lung adenocarcinoma), and others decreased (kidney clear renal cell carcinoma) levels of LAPTM4B mRNA, attributable to the specific clinical condition of the patient (Lebovitz et al., 2015).

LAPTM4B is ubiquitously expressed in healthy human beings, with the highest mRNA levels detectable in the heart, cervix, testis, and kidney (https://gtexportal.org/home/). LAPTM4B studies in cancer patients and in normal tissue are based mainly on transcript levels, however, and functional studies rely mainly on protein-overexpression models. Due to the lack of advanced tools, study of endogenous LAPTM4B is still in its infancy. The cellular
expression and organ distribution of the endogenous LAPT4B isoforms, as well as isoform-specific physiological functions all demand further investigation.
2 AIMS OF THIS STUDY

The aim of this thesis was to provide insights into the function and regulation of LAPTM4B, with a focus on its role in sphingolipid metabolism.

In the first study, we investigated the significance of the interaction between LAPTM4B and ceramide. We took a cross-disciplinary approach including biochemistry, computer simulations, and lipid biology to identify an amino acid motif necessary for the LAPTM4B-ceramide interaction. Protein interaction studies, combined with lysosomal leucine uptake and measurements of downstream mTORC1 signaling, allowed us to assess the physiological significance of this protein-lipid interaction.

In the second study, we sought to gain a deeper understanding of the expression and regulation of endogenous LAPTM4B isoforms. We validated a novel monoclonal antibody developed for this purpose. We selectively disrupted start codons of the individual LAPTM4B isoforms using CRISPR/Cas9-mediated gene editing, and assessed isoform expression in cells and tissues of various origin, and we further assessed the regulation of endogenous LAPTM4B, as well as the differential functions between LAPTM4B isoforms.

In the third study, we extended our previous investigations of the role of LAPTM4B in lipid metabolism. Shotgun lipidomics
allowed us to quantify the lipidomes of cells and secreted extracellular vesicles from WT and LAPTMB4B knockout cells, as well as from knockout cells rescued by expression of either WT or mutant LAPTMB4B. The overarching goal was to provide a comprehensive dataset describing the lipid phenotypes regulated by LAPTMB4B.
3 MATERIALS AND METHODS

The main methods used in this thesis are summarized in Table 7. More detailed descriptions are in the “Materials and Methods” sections of the original publications (I-III).

Table 7. Main methods used in this thesis.

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### 3.1 MODEL SELECTION

#### 3.1.1 System selection

We utilized multiple models to identify a ceramide-regulated element in LAPTM4B. First, we employed biochemical methods to study the direct association between LAPTM4B and ceramide, including the use of cross-linkable and fluorescent ceramide derivatives in artificial vesicles and in cells. Second, we performed computer simulations to predict the motifs and amino acid residues required for the LAPTM4B-ceramide interaction. Third, we generated cell lines expressing LAPTM4B with mutations in the ceramide interaction motifs identified *in silico*. These cell lines we then used to verify the functionality of the predicted motifs and their functional impact on downstream signaling pathways.

By combining these approaches, we were able to identify a ceramide-regulated element in LAPTM4B, and show its importance for nutrient signaling.
3.1.2 Cell lines and tissues

In this work, we utilized multiple immortalized cell lines, including human epidermoid carcinoma cells (A431), human breast cancer cells (KPL-7, BT474, ZR-75-1, MCF-7, MDA-MB-231), human cervical cancer cells (Hela), human prostate cancer cells (PC-3), and human bone osteosarcoma epithelial cells (U2OS), as well as non-transformed human fibroblast cells (AG08498, GM00321).

A431 cells were our favored model in most experiments, since these cells are fast-proliferating due to the abnormal high expression of EGFR, and they contain no functional p53, which makes them easy to manipulate by use of CRISPR/Cas9-based techniques ( Ihry et al., 2018). In this work, CRISPR/Cas9 allowed us to generate knockout (KO) cells (Studies I-III), and knock-in cells (in Studies II-III). Additionally, we generated a set of mutant cell lines expressing ceramide binding-deficient LAPTM4B (Studies I and III). All the KO and knock-in cell lines (in Studies I-III) are derived from single cell clone.

However, because drawing wide-reaching conclusions from only one cell line is insufficient, we performed key experiments in additional cell lines. In Study I, Hela cells were used to confirm the effect of ceramide and LAPTM4B on mTORC1 activity. In Study II, LAPTM4B-24 was identifiable as the major expressed isoform, and this finding was confirmed in other transformed cancer cells (Hela, PC-3, and breast cancer cells), non-transformed fibroblasts
(AG08498, GM00321), and in healthy and tumor-derived tissues of various origins. In Study III, we showed that LAPTM4B is enriched in the extracellular vesicles (EVs) secreted from A431 cells, and these data were further validated in PC-3 cells and in urine samples from four healthy subjects.

3.2 STRATEGIES TO STUDY LAPTM4B FUNCTIONS

We mainly utilized loss-of-function and gain-of-function strategies to address LAPTM4B function. For loss-of-function, we used either small interfering RNA (siRNA)-mediated gene knockdown (Studies I-III) or CRISPR/Cas9-mediated gene knockout (Studies I-III). For gain-of-function studies, we used pEFRES-P to generate cells stably expressing intact or mutated LAPTM4B proteins (Studies I-III). The cell lines were established on a knockout background to eliminate any possibility of the endogenous protein interfering with the experimental observations. Additionally, we used CRISPR/Cas9 to selectively disrupt isoform-specific LAPTM4B start codons (Study II) and to generate a knock-in cell line expressing LAPTM4B with C-terminally fused superfold GFP (Study III). These methods have strengths and limitations, and this requires caution in selection of a suitable method to address the question at hand.
An easily adoptable method for studying protein functions is siRNA-mediated knockdown. For most proteins of interest, siRNAs and transfection reagents are commercially available at a reasonable cost. However, the transfection efficiency and gene-depleting percentage is seldom 100%, and the knockdown may be inconsistent between experiments. Moreover, siRNA-mediated gene silencing is transient, and the time needed to achieve the highest effect depends on many factors, such as the efficiency of transfection, cell state and cell confluence during the transfection, transcript level of the target mRNA, and the stability of the protein under study. Therefore, siRNA-mediated gene knockdown is a convenient way to conduct pilot experiments or screening, but in order to conduct massive numbers of protein functional studies, siRNA alone may not be the optimal option, especially considering the increasing use of the CRISPR/Cas9 techniques which have become widespread in the past few years.

CRISPR/Cas9-mediated gene knockout (KO), refers to disrupting a gene by modification of its genomic DNA. This method makes the cell permanently incapable of producing the protein of interest. However, it should be noted that depending on the gene modification, truncated proteins may still be expressed and can retain some functions of the original protein. The downside of CRISPR/Cas9 is that establishing a genetically modified cell line may be time consuming. To establish a KO cell line using this technique requires molecular cloning, transfection, selection,
single-cell clone expansion, and multiple validation experiments. Another potential disadvantage is that during the time period of single-clone expansion (1-2 months), modified cells may compensate for the effects of gene KO. Thus, phenotypes observed in the final KO cells may result from compensatory mechanisms, rather than directly from depleted protein. For these reasons, we used in our studies both siRNA-mediated gene knockdown and CRISPR/Cas9-mediated gene KO methods. More importantly, we additionally employed a gain-of-function approach to rescue the phenotypes of gene knockdown or KO, in order to confirm that the phenotypes observed are dependent on the protein of interest.

The pEFIRES-P plasmid served to generate a pool of cells with stable expression of the proteins of interest. Such stable cell pools are relatively easy to obtain, but need continuous monitoring to confirm that the level of protein expression is maintained, in order to achieve reliable results. In Study I, we used this vector for generating mutant cell lines expressing WT or mutant variants of LAPTM4B, in Study II, for assessing the functional differences between LAPTM4B-35 and LAPTM4B-24, and in Study III, for investigating EV properties.

Generating knock-in cell lines is a process similar to CRISPR/Cas9-mediated gene KO, and is similarly laborious and time-consuming. By this knock-in process, we transfected cells with plasmids containing Cas9 and sgRNAs, together with a
plasmid that contains homology arms flanking the targeted genomic locus where the knocked-in sequence is to be inserted, such as mutated start codon in Study II or sfGFP in Study III. This technique can overcome some limitations of existing methods to address the question of endogenous expression, for instance, lack of antibodies to detect endogenous protein. In Study II, we used this method to confirm the existence of LAPTM4B isoforms, and in Study III, to examine LAPTM4B subcellular localization at the endogenous level.

### 3.3 PROTEIN INTERACTION STUDIES

We have here used mass spectrometry (MS), immunoprecipitation (IP), and fluorescence resonance energy transfer (FRET) techniques to study protein-protein interaction. MS allowed us to screen for potential LAPTM4B-interacting proteins, and selection of candidates of interest was based on criteria such as total number of identified peptides (PSM) in the experimental group and in the control group, protein function, and also the current level of knowledge in the literature. Afterwards, we utilized IP to confirm the protein-protein interactions. To address the subcellular localization where the interaction takes place, we utilized the FRET efficiency between donor and acceptor proteins. The FRET experiments also provide additional means of validating the MS and IP results.
4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF A CERAMIDE-REGULATED ELEMENT IN LAPTM4B

We have earlier shown that LAPTM4B interacts with a cross-linkable ceramide and regulates the subcellular distribution of ceramide by facilitating its export from the endosomal/lysosomal compartment (Blom et al., 2015). In this study, we set out to gain mechanistic insights into the LAPTM4B-ceramide interaction, and to elucidate the domains or amino acid motifs required for this interaction. Contreras et al had postulated the existence of a relaxed sphingolipid interaction motif (Contreras et al., 2012), and analysis of the LAPTM4B amino acid sequence revealed matching putative motifs in transmembrane domains 1 and 3 (I, Fig. 1A).

4.1.1 Biochemical evidence: LUV lipid transfer assay

To assess whether the putative sphingolipid binding motifs in LAPTM4B take part in the ceramide interaction, we first performed a lipid transfer assay between large unilamellar vesicle (LUVs). In this assay, LUVs containing the FRET pair DPH-PC and N-palmitoyl-D-erythro-sphingosine-BODIPY were mixed with LUVs containing synthetic LAPTM4B transmembrane peptides. The changes in the fluorescence lifetime of DPH-PC after mixing it with the LUV populations allowed us to calculate the association between ceramide and LUVs containing transmembrane peptides (I, Fig. 1B). Our data showed that LAPTM4B-derived TM3, but
not TM1, had significant affinity for ceramide compared to vesicles containing the control peptide WALP23 (I, Fig. 1C).

4.1.2 Computer modeling: atomistic simulations
To gain further insight into how LAPTM4B-TM3 causes an increased membrane affinity for ceramide, we employed atomistic simulations. We used simulation boxes of a POPC-acceptor membrane with or without transmembrane peptides (LAPTM4B-TM3 or control peptides WALP23), and C16-ceramides (N-palmitoyl-D-erythro-sphingosine) were present as monomers in the aqueous phase at the start of the simulations. Our data showed that C16-ceramides quickly formed micelles in the aqueous phase and did not insert into the membrane bilayers (POPC-only or POPC-WALP23) during the 5000- to 5500-ns simulation time. Conversely, when the membranes contained LAPTM4B-TM3, ceramide micelles became incorporated, inserted into bilayers within 190-1415 ns (I, Fig. 1F), suggesting that TM3 facilitates ceramide uptake. Detailed analysis of the simulations revealed potential factors that guided ceramide insertion into the lipid bilayer. First, a central aspartate residue (D202) in TM3 induced peptide bending and the exposure of hydrophobic amino acid residues towards the aqueous phase. The contacts between the hydrophobic region and ceramide promoted the incorporation of ceramide into the lipid bilayer (I, Fig. 3A). Second, after being inserted into the bilayers, ceramides interacted preferentially with amino acid residues of the putative sphingolipid binding motif (I, Fig. 2A-B). These two
findings suggest that LAPTM4B-TM3 may mediate both ceramide uptake and promote its interaction with LAPTM4B within the plane of the membrane.

4.1.3 Cell biology: ceramide-binding deficient mutant proteins

Based on the results from biochemical experiments in vitro and computer simulation in silico, we next measured whether TM3 is essential for ceramide interaction also in the cellular context. Is the central aspartate residue (D202) and the sphingolipid-binding motif each the mechanism of ceramide interaction? To this end, we generated stable cell lines expressing mutated LAPTM4B proteins on the KO background, and took advantage of the previously characterized cross-linkable BP-BPY-ceramide as a reporter molecule to study ceramide interaction (Blom et al., 2015). The cross-linkable BP-BPY-ceramide could be displaced from LAPTM4B by an excess of C6-ceramide, but not by C6-sphingomyelin, C6-gluocosylceramide, or sphingosine, indicating that the cross-linkable probe reveals a specific interaction between ceramide and LAPTM4B (I, Fig. 1D).

We then generated cells expressing LAPTM4B variants with single transmembrane domains exchanged for the corresponding domain from the control protein CD63, another four-membrane-spanning endosomal protein. The cell lines were designated CTM1-CTM4, where the number denotes which LAPTM4B TM domain has been
exchanged for a TM domain from CD63. The cross-linkable ceramide probe revealed a reduced interaction with all the mutant LAPTM4B proteins, suggesting that each transmembrane segment is required for efficient ceramide binding. However, CTM3 cells showed the lowest interaction with the ceramide probe (I, Fig. 1E), which supports the data from in vitro and in silico experiments, reinforcing the notion that TM3 is essential and is at the center of the ceramide interaction.

We next generated cell lines with a D202A point mutation. The D202A protein had a similar subcellular localization as the wild type protein (I, Fig. 3D) but displayed a remarkably reduced interaction with BP-BPY-ceramide (I, Fig. 3E), suggesting that the central aspartate is vital for the ceramide interaction.

We further dissected the amino acid motifs in TM3 responsible for the ceramide interaction. To this end, we generated cells expressing LAPTM4B with mutations in either the cytosolic or luminal membrane leaflet-spanning parts of TM3. These were named CTM190-201 (luminal leaflet-spanning TM3 mutation) and CTM203-216 (cytosolic leaflet-spanning TM3 mutation, I, Fig. 2C). Our data showed that the CTM190-201 mutant interacts with the cross-linkable ceramide, but CTM203-216 does not. These data support the argument that the predicted sphingolipid interaction motif in the TM3 luminal membrane leaflet is functional (I, Fig. 2D). Both CTM190-201 and CTM203-216 distribute on the
endoplasmic reticulum, however, which is not the typical localization of LAPTMB4B (I, Fig. S3). We therefore generated an additional mutant with a subcellular distribution similar to that of WT LAPTMB4B, to be able to draw a more reliable conclusion. The TM3 domains of LAPTMB4A and LAPTMB4B display 89% similarity, but LAPTMB4A does not contain a putative sphingolipid interaction motif (I, Fig. S2) and displays no interaction with ceramide (I, Fig. 2E). We therefore generated a mutant cell line expressing LAPTMB4B with TM3 exchanged with the corresponding transmembrane segment from LAPTMB4A. This construct was designated ATM3. Our data showed that ATM3 was expressed and localized similarly to LAPTMB4B, and importantly, ATM3 displayed a significantly reduced interaction with ceramide compared to that of WT LAPTMB4B (I, Fig. 2F-G).

In short, the in vitro, in silico, and cell biological assay data agree that TM3 is necessary for LAPTMB4B ceramide interaction. Mechanistically, the central aspartate (D202) and the sphingolipid interaction motif are essential for maintaining the ceramide interaction and potentially for regulating the downstream signaling pathways (Figure 6).
Figure 6: Model of LAPTM4B ceramide-regulated element.

Inside LAPTM4B, the central aspartate (D202) and putative sphingolipid interaction motif are essential for efficient interaction with ceramide. Reproduced from (Zhou et al., 2018). The figure is reproduced with permission from the copyright holder.
4.2 SIGNIFICANCE OF LAPTM4B-CERAMIDE INTERACTION

Specific lipid interactions are functionally important for regulating transmembrane protein function (Corradi et al., 2019). We therefore explored the outcome of the LAPTM4B-ceramide interaction for downstream signaling events. To obtain information regarding which cellular functions and signaling events may be regulated by LAPTM4B, we performed a proteomic analysis to elucidate LAPTM4B protein interaction partners. This was followed by investigations of how ceramide impacts LAPTM4B protein interactions, as well as downstream signaling events.

4.2.1 Ceramide promotes LAPTM4B and 4F2hc interaction

4.2.1.1 LAPTM4B-interacting proteins identified by mass spectrometry

A proteomics screen allowed us to identify potential LAPTM4B-interacting proteins. Cells that stably express either LAPTM4B or control protein CD63 with 3xFLAG tags were subjected to reversible cross-link immunoprecipitation. The proteins precipitated with the anti-FLAG antibody were then quantified by protein mass spectrometry (MS). The criteria for a specific LAPTM4B-interacting protein was that a minimum of five peptides should be detectable in the immuno-precipitated LAPTM4B samples and also be enriched by more than three-fold compared to
the CD63 control sample. Our MS data suggested that LAPTM4B interacts mainly with nutrient transporters and proteins of the solute carrier family. In particular, the leucine transporter heavy-chain 4F2hc (CD98hc) was the top LAPTM4B-interacting protein after threshold selection, and leucine-transporter light-chain LAT1 (CD98lc) was also among the specific interactors (I, Table. S10). This agrees with an earlier findings showing that LAPTM4B interacts with 4F2hc and is involved in mTORC1/autophagy signaling (Milkereit et al., 2015). LAPTM4B was also recently shown to interact with EGFR and to regulate its sorting and degradation, as well as to initiate EGFR-dependent autophagy (Tan et al., 2015a). EGFR was highly associated with LAPTM4B in our proteomic screen, but was not on the list of specific interactors, since a substantial amount of EGFR was also visible in the CD63 control sample. This may have been due to the high expression of EGFR in the A431 cell line that we used in the experiments. Nevertheless, the results from the screen were in good agreement with previous results in other cell models (I, Table S10), and we therefore focused on further exploring the LAPTM4B-4F2hc interaction.

4.2.1.2 Validation of the LAPTM4B-4F2hc interaction
Co-immunoprecipitation experiments verified that 4F2hc interacts with LAPTM4B, but not with the control protein CD63 or LAPTM4A, suggesting the interaction is specific (I, Fig. 4A, Fig. S4). Interestingly, a short (30-min) treatment with bacterial
sphingomyelinase (bSMase) raised cellular ceramide levels and enhanced the interaction between 4F2hc and LAPT4B. The same treatment did not induce interaction between LAPT4B and the control proteins (I, Fig. 4A, Fig. S4), suggesting that ceramide specifically promotes the interaction between 4F2hc and LAPT4B.

LAPT4B has a mainly endosomal/lysosomal distribution, and partially localizes on the plasma membrane (Milkereit and Rotin, 2011). Conversely, the identified interaction partner 4F2hc localizes mainly on the plasma membrane and partially on endosome/lysosome (Mastroberardino et al., 1998; Wagner et al., 2000). We therefore asked what might be the compartment where LAPT4B and 4F2hc interact. To address this, we expressed LAPT4B-Venus and 4F2hc-Cerulean in A431 cells, and utilized the fluorescence resonance energy transfer (FRET) technique to obtain spatial information regarding the LAPT4B-4F2hc interaction. As a positive control, we expressed Cerulean fused by a short linker to Venus, and cells co-expressing CD63-Venus and 4F2hc-Cerulean as a negative control. Our FRET experiments showed that LAPT4B and 4F2hc interact mainly in endosomal compartments, and bSMase treatment further enhanced this interaction (I, Fig. 4B).

To further address whether the lysosomal ceramide specifically regulates LAPT4B-4F2hc interaction, we depleted cells of acid
ceramidase (ASAH1), the lysosomal ceramide-degrading enzyme (Parveen et al., 2019). As expected, our immunoprecipitation experiments showed that ASAH1 silencing increased the LAPTM4B-4F2hc interaction (I, Fig. 4C), and significantly increased the FRET signal between LAPTM4B-Venus and 4F2hc-Cerulean in endosomal compartments (I, Fig. 4D). Added together, these data show that lysosomal ceramide enhanced the LAPTM4B-4F2hc interaction.

4.2.2 LAPTM4B regulates lysosomal leucine uptake and mTORC1

4.2.2.1 The LAPTM4B-ceramide interaction regulates lysosomal leucine

The transporter CD98 (4F2hc and LAT1) imports essential amino acids (EAA), i.e leucine, isoleucine, and arginine, in exchange for glutamine (Mastroberardino et al., 1998; Milkereit et al., 2015; Wagner et al., 2000). A recent study showed that LAPTM4B recruits CD98 to lysosomes, and thereby regulates leucine uptake and downstream pathways (Milkereit et al., 2015). We thus further explored whether ceramide would display a modulating effect on this signaling pathway.

To this end, we measured [3H]-leucine uptake into cells and into lysosomes. Cellular uptake was measured from the A431 wild type,
LAPTM4B KO, and LAPTM4B stably expressing cells that we treated with or without 4F2hc siRNA. Our data showed that approximately 50% of leucine uptake from the medium was 4F2hc-dependent in A431 cells. However, the leucine uptake was unaffected by LAPTM4B (I, Fig. S5A). In support of this, the immunofluorescence staining indicated that the majority of the 4F2hc was still distributed on the plasma membrane with and without LAPTM4B depletion (I, Fig. S5B).

We next took advantage of the D202A- and ATM3-expressing cell lines, which display reduced ceramide interaction but display similar subcellular distribution as that of the WT protein. We adopted methods from Milkereit et al and Zoncu et al to study lysosomal leucine uptake (Milkereit et al., 2015; Zoncu et al., 2011). Briefly, LAMP1-mGFP expressing cells were incubated with $[^{3}H]$-leucine, and LAMP1-mGFP-positive lysosomes were immunoprecipitated, followed by radioactivity measurements in those organelles by scintillation counting. We found that lysosomes derived from either LAPTM4B-ATM3 or LAPTM4B-D202A cells displayed a substantially reduced $[^{3}H]$-leucine uptake compared to that of lysosomes from cells expressing wild type LAPTM4B (I, Fig. 6C).

4.2.2.2 LAPTM4B promotes mTORC1 activity in a ceramide-dependent manner
Since LAPT4B could affect lysosomal leucine transport, which in turn has been shown to regulate mTORC1 activation (Jewell et al., 2015), we next investigated how ceramide fits into this model.

We first assessed the effect of LAPT4B on mTORC1 signaling, to confirm that what has been observed in HeLa cells (Milkereit et al., 2015) is reproducible in our A431 cell model. We utilized WT and KO cells, as well as cells overexpressing LAPT4B or CD63 on a KO background. The cells were starved in EBSS for one hour to suppress phosphorylation of the mTORC1 substrate S6K. Then the induction of phospho-S6K was assessed by Western blotting upon refeeding with stimulation medium for 20 minutes and 40 minutes. In these experiments, knockout of LAPT4B dramatically reduced pS6K. This phenotype was rescued by re-expressing LAPT4B but not CD63 on the knockout background (I, Fig. S6A-B). Moreover, reduction in pS6K level was evident upon silencing of either LAPT4B or 4F2hc, whereas no additional reduction of pS6K level was evident in double-depleted cells (I, Fig. 5A). These results confirm that LAPT4B and 4F2hc act in concert to promote mTORC1 activity, and we thus next examined whether lysosomal ceramide would regulate this signaling pathway. We found that raising the amount of lysosomal ceramide by ASAH1 depletion promoted pS6K phosphorylation. Conversely, reducing lysosomal ceramide by SMPD1 depletion led to decreased pS6K levels. Moreover, silencing of LAPT4B rendered the cells unresponsive to ceramide modulations (I, Fig.
5B-C, Fig. S6C). We further measured mTORC1 activity in ceramide-binding-deficient mutant cells. As expected, ATM3 and D202A cells displayed a reduced pS6K level upon the simulation (I, Fig. 6D). This is in line with these cells’ reduced lysosomal leucine uptake (I, Fig. 6C). We propose that lysosomal ceramide promotes mTORC1 signaling by enhancing LAPTMC4B-4F2hc interaction.

In summary, combining biochemistry, computer simulation, and lipid biology, Study 1 identified a ceramide-regulated element in LAPTMC4B, and further revealed the physiological significance of the LAPTMC4B-ceramide interaction in promoting lysosomal leucine uptake and mTORC1 signaling (Figure 7).
Figure 7: The physiological significance of LAPTM4B ceramide interaction.

Lysosomal ceramide enhances the interaction between LAPTM4B and 4F2hc; the ceramide-regulated element in the third transmembrane regulates this interaction, which promotes lysosomal leucine uptake and the downstream mTORC1 signaling. (From Zhou et al, 2018).
4.3 LAPTM4B ISOFORM EXPRESSION AND REGULATION

The main LAPTM4B transcript (ENST00000619747.1) contains alternative start codons for two protein isoforms: LAPTM4B-24 and LAPTM4B-35. The longest open reading frame (ORF) of the transcript encodes LAPTM4B-35, and the general assumption has been that this is the major isoform (Meng et al., 2016). This fact, however, had remained unconfirmed at the protein level due to a lack of validated antibodies that detect endogenous LAPTM4B. Moreover, most LAPTM4B functional studies are based on overexpression of either LAPTM4B-35 or LAPTM4B-24, and the expression, regulation, and functions of the individual LAPTM4B isoforms in human cells and tissues were unknown. In Study II, we explored the endogenous expression of LAPTM4B, as well as the regulation and divergent functions of the isoforms.

4.3.1 Characterization of a monoclonal anti-LAPTM4B antibody

To assess the endogenous expression of the protein isoforms, we evaluated a novel monoclonal anti-LAPTM4B antibody. The antigenic epitope is localized in the loop between transmembrane domains 3 and 4, which is present in both LAPTM4B-24 and LAPTM4B-35, and the antibody is capable of detecting both isoforms (II, Fig. 1A).
4.3.1.1 Antibody validation by gene knockdown and gene knockout
In Wild Type (WT) A431 cells, the antibody detected 35 kDa and 24 kDa proteins that were absent after LAPTM4B knockdown or knockout. Surprisingly, the 35 kDa band was expressed at low levels compared to the 24 kDa protein isoform levels in A431 cells (II, Fig. 1D). The antibody was further validated for immunofluorescence, which showed that LAPTM4B mainly distributes to the endosomal compartment based on co-localization with endocytosed dextran (II, Fig. 1B-C).

4.3.1.2 Antibody validation by CRISPR/Cas9-mediated knock-in
We next tested whether the bands detected at 35 kDa and 24 kDa were different protein isoforms, or whether they represented one isoform with differing post-translational modifications. We took advantage of the CRISPR/Cas9-mediated homology-directed repair technique to generate cell lines with targeted inactivating mutations in the predicted LAPTM4B-35 or LAPTM4B-24 start codons. For each mutation, we established two clones to reduce the risk of observing off-target effects (II, Fig. 1E). The 35 kDa protein was not expressed in the LAPTM4B-35 mutant cell lines, whereas the 24 kDa band was still present, indicating that LAPTM4B-35 is indeed translated from the predicted start codon. Interestingly, mutating the LAPTM4B-24 start codon led to loss of both protein isoforms (II, Fig. 1F). This is likely due to reduced LAPTM4B
mRNA levels in the mutant cells, as assessed by qPCR (II, Fig. S1A-B). Thus, the start-codon-edited mRNA is likely subjected to nonsense-mediated decay, similar to what occurs in GAP1 (Neymotin et al., 2016).

These data provide the first validation of a high-quality LAPTM4B antibody capable of detecting endogenous LAPTM4B both by Western blotting and immunofluorescence. Interestingly, in A431 cells, the major isoform was not LAPTM4B-35, despite its being encoded by the longest ORF of the transcript. As this may be a cell-specific expression pattern, we continued by using the same antibody to assess LAPTM4B expression in additional systems.

4.3.2 LAPTM4B-24 is the major isoform in most cells and tissues

4.3.2.1 LAPTM4B isoform expression in cultured human cells
Since the LAPTM4B expression pattern observed in A431 cells differed significantly from what has been reported (Meng et al., 2016), we assessed LAPTM4B expression in HeLa, PC-3, and a panel of breast-cancer-derived cell lines. LAPTM4B-24 was abundant in all of these, whereas LAPTM4B-35 expression was marginal. Moreover, in healthy human primary fibroblasts, only LAPTM4B-24 expression was at detectable levels, suggesting that this is the major isoform expressed in human cells (II, Fig. 2A). Moreover, transformed malignant cells express high levels of
LAPTM4B-24 compared to levels of healthy fibroblasts, in line with earlier findings that the LAPTM4B transcript is upregulated in cancers (Meng et al., 2016).

### 4.3.2.2 LAPTM4B isoform expression in human tissue

To obtain information regarding LAPTM4B expression in a more physiologically relevant setting, we measured the isoform expression in human cancer tissues and matching healthy tissues. Surprisingly, LAPTM4B-24 was the major isoform in all samples tested, and was upregulated in two out of four breast-tumor samples (**II, Fig. 2B**), and in two out of three lung-tumor samples (**II, Fig. 2C**), when compared with the paired adjunct normal tissue from the same donor.

In addition, we measured LAPTM4B isoform expression in 14 tumor tissues of differing origins and in 11 normal tissues. Intriguingly, 24 kDa protein was the predominant or the only detected band in almost all the tissues, whereas 35 kDa was absent or rarely expressed (**II, Fig. 3A-B**). One exception is that in one sample of brain tissue, LAPTM4B-35 was exclusively expressed (**II, Fig. 3B**). Currently, the mechanism of LAPTM4B-35 selective expression in brain and whether it associates with central nervous system (CNS) functions remains unknown.
4.3.2.3 Data mining suggests LAPTM4B-24 is the physiologically relevant isoform

Interestingly, the exome- and genome-sequencing database gnomAD (Karczewski et al., 2020) reports a frame-shift mutation (S75Afs) that specifically disrupts the open reading frame (ORF) of LAPTM4B-35 without affecting the expression of LAPTM4B-24 (II, Fig. S2A). The S75Afs allele occurs at a frequency of 7.9% worldwide, and 473 persons in the database were identifiable as homozygous for the mutation. Importantly, no clinical manifestations are reportedly associated with the S75Afs variant. Moreover, 22.9% of the S75Afs homozygotes are over 70 years old, compared to 12.2% of the S75Afs heterozygotes and 14.1% of the wild types in the exome-sequenced subjects (II, Fig. S2B). These data indicate that the LAPTM4B-35 isoform plays no critical role in normal human physiology. In support of this, the genomic sequence encoding the LAPTM4B-35-specific N-terminus is poorly conserved between species, further supporting the notion that, in mammals, LAPTM4B-35 is not critical. In fact, common laboratory animals, including the mouse, rat, and zebrafish, do not encode the N-terminal of LAPTM4B-35, whereas the LAPTM4B-24 isoform ORF is highly conserved between species, suggesting the importance of the LAPTM4B-24 isoform from the evolutonal perspective (II, Fig. S3A-B). In support of this, the International Mouse Phenotyping Consortium (IMPC) (Dickinson et al., 2016) has documented that LAPTM4B-24-knockout mice display severe developmental defects and die in the preweaning stage. Taking the
LAPTM4B isoform expression and data mining results together, we propose that LAPTM4B-24 is the major isoform expressed and the most physiologically important isoform in human cells and tissues.

### 4.3.3 LAPTM4B-24 expression is regulated by nutrients and ceramide

Most studies of LAPTM4B have focused on the 35 kDa isoform (Blom et al., 2015; Meng et al., 2016; Tan et al., 2015a), while our current knowledge regarding LAPTM4B-24 is very limited. We therefore took advantage of the newly characterized LAPTM4B monoclonal antibody to further investigate the regulation and functions of LAPTM4B-24.

#### 4.3.3.1 LAPTM4B-24, a fast-turnover protein

To gain some initial idea regarding LAPTM4B regulation, we first measured its half-life in cells. We treated cells with cycloheximide (CHX) to block their protein synthesis and assessed cellular LAPTM4B levels at various time points. We found that LAPTM4B-24 has a half-life of approximately one hour (**Fig. 4A**). A similar turnover time was observable in LAPTMB-24 stable expressing cells (**Fig. S6A**), suggesting that post-translational mechanisms are mainly responsible for the fast protein turnover. Interestingly, LAPTM5 also has a short half-life, suggesting that this may be a common property of the LAPTM-family proteins (Glowacka et al., 2012).
The lysosome and the proteasome are two major sites at which protein degradation occurs (Pohl and Dikic, 2019). To further clarify the reason for the fast turnover of LAPT4B-24, we used Bafilomycin-A1 (a V-ATPase inhibitor) to block lysosomal degradation function, and MG-132 to specifically impair proteasome function. Our data show that each individual inhibitor reduced LAPT4B-24 degradation, and a more intense effect was observable in cell combined treated with both inhibitors (II, Fig. 4B-C). These data indicate that lysosomal and proteasomal pathways both contributed to LAPT4B-24 turnover, and agree with previous data utilizing LAPT4B overexpression to show that the lysosome is involved in LAPT4B degradation (Vergarajauregui et al., 2011).

4.3.3.2 Nutrients and ceramide regulate LAPT4B-24 expression

LAPT4B is emerging as a factor regulating vital parts of cell metabolism. This includes regulating subcellular ceramide distribution (Blom et al., 2015), promoting lysosomal leucine uptake, and regulating mTORC1 signaling (Milkereit et al., 2015; Zhou et al., 2018). Moreover, we (II, Fig. 2B-C) and others (Meng et al., 2016) observed LAPT4B levels undergoing upregulation upon malignant transformation. Considering our earlier publications showing a link between LAPT4B, sphingolipid metabolism, and mTORC1 (Blom et al., 2015; Zhou et al., 2018), we wondered whether nutrients and ceramide affect LAPT4B
turnover and expression. To test this, we starved WT A431 cells with EBSS, with or without inhibitors of protein degradation. Our data showed that LAPTM4B-24 decreases rapidly during EBSS starvation, and this degradation was blocked by treatment with Balfilomycin-A1 and MG-132 (II, Fig. 5A). Interestingly, in starved cells, LAPTM4B-24 expression was quickly restored to normal levels after refeeding of cells with stimulation medium (II, Fig. 5B). These data indicate that LAPTM4B probably undergoes a rapid synthesis-degradation cycle which is regulated by cellular nutrient status.

We further tested whether ceramide regulates LAPTM4B expression. A cell-permeable short-chain C6-ceramide raised endogenous LAPTM4B-24 levels in a concentration-dependent manner (II, Fig. 5C). The ceramide effect on LAPTM4B expression was also observable in stable LAPTM4B-expressing cells, suggesting that the regulation takes place at the post-translational level (II, Fig. S6B-C). Treating cells with C6 sphingomyelin, with cholesterol, or with oleic acid, did not have a visible effect on LAPTM4B expression (II, Fig. S7), implying that ceramide is a specific modulator of this protein. The role of ceramide we further investigated in cells in which lysosomal ceramide generation was impaired by the silencing of either acid sphingomyelinase (SMPD1) that degrades SM to ceramide, or of glucocerebrosidase (GBA) that degrades glucoceramide to ceramide (II, Fig. 5D). Depleting either of these proteins caused a
small but significant reduction in cellular LAPTM4B-24 (II, Fig. 5E). On the other hand, inhibiting lysosomal ceramide degradation by knocking out acid ceramidase (ASAH1 KO), led to increased LAPTM4B levels (II, Fig. 5F).

Taken together with previous studies by us and others (Blom et al., 2015; Milkereit et al., 2015; Zhou et al., 2018), it is becoming apparent that LAPTM4B functions at the crossroad of sphingolipid metabolism and mTORC1 activity. Sphingolipids have been shown to regulate mTORC1 activity (Jęśko et al., 2019; Young et al., 2013), and here we have shown LAPTM4B to be a key protein in this respect.

4.3.4 Subcellular distribution and functions of LAPTM4B-24 and -35

Functional studies of LAPTM4B have been based mostly on overexpression of LAPTM4B-35 (Blom et al., 2015; Tan et al., 2015a, 2015b), or occasionally on LAPTM4B-24 (Milkereit and Rotin, 2011). Moreover, in earlier studies, the proteins were overexpressed on the wild type background, which adds to the risk that interplay between the overexpressed isoform and the endogenous protein may affect the outcome of the experiments and not necessarily reflect a specific effect of the overexpressed isoform. Since no study had yet systematically compared these two isoforms, we next set out to investigate whether between them are any dissimilar functional properties.
4.3.4.1 LAPTM4B-24 and LAPTM4B-35 have different subcellular distributions

First, we generated cell lines that stably express Flag tagged LAPTM4B-35 or LAPTM4B-24 on the LAPTM4B KO background (II, Fig. S4A). Immunofluorescence stainings revealed that both isoforms mainly localize to intracellular punctate endosome/lysosome structures, and that LAPTM4B-35 is, additionally, in part distributed in the cell edges (II, Fig. S4C). The differences between distribution of LAPTM4B isoforms was further confirmed in a cell line co-expressing LAPTM4B-35-mCherry and LAPTM4B-24-Flag (II, Fig. S4B). As an extra confirmation that these isoforms have partially differing localizations, we further validated these results by a cell-surface biotinylation assay. This approach confirmed that, when compared to LAPTM4B-24, LAPTM4B-35 was more enriched on the cell surface, whereas the total cellular expression of the two isoforms was similar (II, Fig. S4D).

4.3.4.2 Comparison of functional properties of LAPTM4B-24 and -35

Since the LAPTM4B-35 isoform contains a SH3 domain-binding motif (Li et al., 2010a; Liu et al., 2009) and a phosphoinositide binding arginine-rich polybasic stretch (Tan et al., 2015b), neither of which was present in the major LAPTM4B-24 isoform, we next studied whether there are functional differences between two
isoforms.

We took advantage of the xCELLigence method to measure cell migration in a label-free and real-time manner (Bird and Kirstein, 2009). Our data showed that LAPTMB4B-35 expression promoted cell migration, whereas LAPTMB4B-24 did not (II, Fig. S4E). This is in line with what occurs in cells overexpressing LAPTMB4B-35 on the WT background (Liu et al., 2009; Yang et al., 2010).

We next measured whether the isoforms, upon starvation and refeeding, would differentially affect mTORC1. We found that the phosphorylation of the mTORC1 substrates S6K, 4EBP, and ULK1 dropped to a very low level (almost zero in the case of pS6K) after 1 hour of EBSS starvation. Upon refeeding, LAPTMB4B-24- and LAPTMB4B-35-expressing cells displayed a very rapid and similar increase in the key mTORC1 substrate phosphorylation, but LAPTMB4B KO cells displayed only a minor increase, suggesting that both LAPTMB4B isoforms promote mTORC1 signaling similarly (II, Fig. S5A-C). Indeed, this is plausible for three reasons: 1) LAPTMB4B-35 and LAPTMB4B-24 both mainly localize on the lysosome (II, Fig. S4B-C), 2) Lysosomes function as centers for mTORC1 activation (Lamming and Bar-Peled, 2019; Zoncu et al., 2011), 3) In both isoforms, the ceramide-regulated element vital in mTORC1 activation is identical (Zhou et al., 2018).
In summary, Study II established LAPT4B-24 as the major and physiologically relevant isoform in human cells and tissues, even though it is not encoded by the longest ORF and has been understudied compared to the minor LAPT4B-35 isoform. We further show LAPT4B to be under dynamic and rapid regulation by cell-nutrient status. We also show the LAPT4B isoforms to have shared and discrepant effects on cell functions. A central finding of this study was that LAPT4B-35 is undetectable in a wide range of tumor tissues (II, Fig. 3A), even though it is encoded by the longest ORF of the transcript. What is thus unclear is whether this isoform plays any role in cancer progression *in vivo*. Considering the dominant expression and physiological significance of LAPT4B-24, we suggest that future studies should focus on this isoform. On a general level, this study shows that caution is necessary when assuming which ORF of a transcript is made into a protein. Moreover, the identity of the endogenously expressed protein must be verified by several independent methods.
4.4 LAPTM4B CONTROLS THE SPHINGOLIPID AND ETHER LIPID SIGNATURE OF EVS AND CELLS

Three recent studies show that LAPTM4A is a critical factor for synthesis of the lipid Gb3, a receptor of Shiga toxin (Pacheco et al., 2018; Tian et al., 2018; Yamaji et al., 2019). Considering that LAPTM4B and -4A are homologous proteins with high sequence similarity, they potentially both regulate lipid metabolism by similar mechanisms. To achieve a more comprehensive view of how LAPTM4B regulates the composition of cellular membranes, we turned to shotgun lipidomics. In Study III, we set out to investigate the role of the ceramide-regulated element (CRE) in regulating cellular lipid homeostasis.

We first investigated the colocalization of LAPTM4B with endogenous markers of endosomal compartments. Endogenous LAPTM4B was tagged with a superfold green fluorescent protein (sfGFP) by CRISPR/Cas9-mediated homology-directed repair. The tagged cell line was used, rather than the antibody validated in Study II, for detecting endogenous LAPTM4B. This was because the antibodies marking endosomal compartments were all raised in mice and thus were not compatible with the LAPTM4B antibody. These experiments showed that LAPTM4B-sfGFP-positive endosomes co-localize partially with LAMP1 (Pearson coefficient 0.23 ± 0.03, Mean ± SEM) and CD63 (Pearson coefficient 0.39 ± 0.04, Mean ± SEM). Interestingly, the best colocalization we
observed was between endogenous LAPTM4B and Lysobisphosphatidic acid (LBPA) (Pearson coefficient 0.45 ± 0.05, mean ± SEM) (III, Fig. 1A). LBPA is a late endosome-specific lipid involved in the formation and release of exosomes (Larios et al., 2020; Piper and Katzmann, 2007). It is noteworthy that LAPTM4B knockout cells displayed no visible changes in the structures of CD63-, LAMP1-, or LBPA-positive endosome, compared with wild-type (WT) cells (III, Fig. S1A). Additionally, the colocalizations between LAPTM4B and endosomal markers were further observable in stably expressing cells (III, Fig. S1B), in agreement with previous studies (Blom et al., 2015; Milkereit et al., 2015; Tan et al., 2015b).
4.4.1 LAPTМ4B is secreted in sEV

4.4.1.1 The ceramide-regulated element facilities LAPTМ4B sorting into MVEs

Electron microscopy has shown that LAPTМ4B localizes to multivesicular endosomes (Blom et al., 2015). We hypothesized that the ceramide-regulated element (Zhou et al., 2018) may influence the subcellular distribution of LAPTМ4B. To address this, we stably expressed the 3xFLAG-tagged LAPTМ4B-24 (termed “L4B”) or ceramide-binding-deficient mutant (termed “ATM3”) on the LAPTМ4B knockout background (III, Fig. 1B, S1C-D).

Immuno electron microscopy (Immuno EM) revealed that LAPTМ4B was enriched in the intraluminal vesicles (ILV) of multivesicular bodies (MVB) (III, Fig. 1C). Since ceramide can promote ILV budding into the endosomal lumen (Trajkovic et al., 2008), we asked whether the CRE affects LAPTМ4B sorting into ILVs. Quantification of the ILV position within multivesicular bodies revealed that ATM3-positive ILVs were more frequently located in close proximity to the delimiting membrane compared to the position of L4B-positive ILVs (III, Fig. 1D, S2A).

To further investigate whether CRE regulates sorting of LAPTМ4B to ILVs, we treated L4B and ATM3 cells with 1 μM Vacuolin-1 to enlarge endosomal compartments, in order to allow assessment of LAPTМ4B distribution in endosomes by confocal microscopy. In
line with the electron microscopy data, the number of ATM3-positive structures inside the enlarged endosomes was 75% less than with L4B-positive fluorescence (III, Fig. 1E-G). These data suggest that the CRE plays a vital role in LAPTM4B protein sorting in the endosome-lysosome system.

4.4.1.2 The ceramide-regulated element affects LAPTM4B secretion
ILV-sorting and -budding associate with extracellular vesicle (EVs) biogenesis and protein secretion (Colombo et al., 2014). Considering that LAPTM4B showed good co-localization with the exosome-associated lipid LBPA, we wondered whether LAPTM4B is secreted by cells. Indeed, LAPTM4B was detectable in conditioned cell-culture medium, and in 24-hour conditioned media, ATM3 secretion was 60% lower than L4B secretion. Importantly, the exosome markers CD63 and CD81 were similarly secreted from ATM3 and L4B cells (III, Fig. 1H-I), suggesting that the CRE does not affect exosome release as such.

4.4.1.3 LAPTM4B is found in EVs isolated from cells and human urine samples
We next asked whether LAPTM4B is present in a certain EV population. EVs were isolated from the conditioned medium of A431 cells by sequential ultracentrifugation. We separated two EV fractions, the exosome-enriched fraction (here called “small EVs” or “sEVs”), and microvesicles (“large EVs” or “lEVs). We found
LAPTM4B to be markedly enriched in the sEVs population, as indicated by the well-established markers CD63 and CD81 (III, Fig. 2A-B). We obtained similar results from the conditioned medium of PC-3 cells (III, Fig. S3A-B), suggesting that LAPTM4B enrichment in sEVs is not a cell-specific effect.

In humans, EVs can be secreted and can be found in, for example, blood and urine (Yáñez-Mó et al., 2015). To test whether LAPTM4B release also occurs in physiological settings, we measured LAPTM4B from EVs isolated from urine samples of four healthy donors. LAPTM4B was detectable in the sEVs fraction of the urine from all subjects (III, Fig. 2C), suggesting that LAPTM4B release in sEVs may be part of a physiologically relevant process. These data are the first to show LAPTM4B as being secreted in sEVs isolated from cancer cell lines and to be found in healthy human urine samples.

4.4.2 Effects of LAPTM4B on EV properties

4.4.2.1 LAPTM4B does not affect EV morphology or size
We next studied whether LAPTM4B regulates certain EV properties. Transmission-EM allowed us to study EV morphology, and a nanoparticle tracking technique to measure EV size distribution/particle number. Both wild-type and LAPTM4B KO A431 cells secreted intact EVs with similar and morphology, and there existed no significant difference regarding either the number
or the size distribution of secreted EVs (III, Fig. 2E-F). Similar results emerged in regard to cells stably expressing L4B and ATM3 (III, Fig. S3D-E), suggesting that neither LAPTM4B nor CRE regulates EV morphology, size distribution, or EV particle number.

4.4.2.2 EV-associated LAPTM4B affects uptake by recipient cells
EV uptake by recipient cells is a vital step for EV-mediated cell-to-cell communication. We therefore asked whether LAPTM4B affects EV properties related to their uptake. Since EVs are a heterogenous group of nanoparticles, a large number of measurements are necessary to draw a reliable and unbiased conclusion. We therefore used a high-content imaging technique to study the overall uptake of sEVs secreted from WT and KO cells by acceptor cells.

Freshly isolated sEVs we labeled with the fluorescent lipophilic membrane dye FAST DiOTM. A431 cells we incubated overnight with equal amounts of sEVs from either WT or KO cells. We then used Opera Phenix high-content microscopy to image the fluorescent EV label inside the cells. These images underwent automated analysis with the “CellProfiler” software, using similar segmentation protocol as described previously (Hollandi et al., 2020; Salo et al., 2019). Intriguingly, sEV from KO cells was taken up less efficiently than was sEV from WT cells (Figure 8A-B, unpublished data). Similar differences in EV uptake occurred
when we used U2OS cells as recipients, suggesting that LAPTM4B exerts a general impact on EV uptake (Figure 8C-D, unpublished data).

Figure 8. LAPTM4B facilitates EV uptake into recipient cells.
sEV isolated from WT and KO A431 cells were incubated with A431 or U2OS cells overnight. (A) Representative images of sEV uptake by A431 cells and (B) Quantification from three independent experiments. (C) Representative images of overnight uptake of sEV by U2OS cells and (D) Quantification from three independent experiments. Mean ± SEM, *P<0.05.

4.4.3 LAPTM4B affects lipid composition of sEVs and parental cells

4.4.3.1 The effect of LAPTM4B on lipid profiles in sEVs and parental cells

We hypothesized that one property that may explain the differences
observed occurring in sEV uptake is alteration in EV lipid composition. We therefore performed a shotgun lipidomics analysis of isolated sEVs and their parental cells: WT vs KO cells and L4B rescue vs ATM3 ‘mock rescue’ cells.

In cells, we could identify 22 lipid classes in WT and 24 lipid classes in KO (III, Table. S1, S2). Principal Component Analysis (PCA) revealed a clear separation of the lipidomes in WT cells from KO cells. KO cells rescued by stable expression of wild-type LAPTM4B were distributed largely similar to the distribution of WT cells on the PCA plot. This partial rescue was not evident in cells with ATM3 “mock rescue”. This suggests that the data contain lipids that are regulated by the ceramide-regulated element (III, Fig. 3A-B).

In the case of sEV, we observed a similar distribution of the samples in the PCA plot, although the number of lipid classes detected was slightly reduced compared to numbers in cells (III, Fig. 3C-D). In sum, the PCA indicates that LAPTM4B can regulate certain lipids in sEVs and their parental cells, and that this regulation requires a functional ceramide-regulated element.

Further analysis of the lipidomic data showed that different lipid classes preferably associated with either sEVs or with parental cells (III, Fig. 4A-B), which is in agreement with reports concerning PC-3 cells and Oli-neu cells (Skotland et al., 2017). Certain lipids
were enriched in sEV compared to their levels in parental cells, ones such as phosphatidylserine (PS; 2.95x), ceramide (Cer; 1.85x), cholesterol (Chol; 1.34x), and sphingomyelin (SM; 1.3x) (III, Table. S1). Conversely, compared with their levels in parental cells, some lipid classes were significantly reduced in sEV, for instance, all lysophospholipids and phospholipid classes, phosphatidylethanolamine (PE; 0.77x), phosphatidylglycerol (PG; 0.54x), and phosphatidylinositol (PI; 0.36x). In addition, some storage lipids such as triacylglycerol (TAG; 0.02x) and cholesterol esters (CE; 0.04x) were nearly absent from sEV (III, Table. S1), a finding which serves as confirmation of the purity of the isolated vesicles.

Moreover, LAPTM4B KO cells showed a minor decrease in total ether-PE and SM, whereas ether-LPE and LPE were elevated in EVs from KO cells. Interestingly, in sEV samples, dramatically increased total HexCer levels were detectable in sEVs from KO cells (III, Fig. 4B). The significant CRE dependent regulation of ether-linked phospholipids and sphingolipids is particularly interesting due to recent developments showing that these classes of lipids are coregulated (Jiménez-Rojo et al., 2020). It is currently unclear what part of this pathway is LAPTM4B-regulated.

4.4.3.2 LAPTM4B regulates glycosphingolipid content of sEVs and cells
We next performed a detailed analysis of sphingolipid species.
Sphingolipids with C16 and C24 fatty acids are the ones most abundant in A431 cells (III, Fig. 5, S5). We found that LAPTM4B knockout causes a significant increase in C16- and C24 ceramide species in cells, whereas their SM class appears not to be visibly altered; this is in agreement with findings from our earlier study using siRNA-mediated gene knockdown (Blom et al., 2015). However, the impact of LAPTM4B KO on C16 and C24 ceramide in the sEVs fraction was minor (III, Fig. 5, S5).

Intriguingly, the most dramatic change in sEVs secreted by LAPTM4B KO cells was the increase in HexCer species. This HexCer phenotype was rescued by expression of L4B, but not by ATM3, suggesting that CRE has a regulatory effect on HexCer metabolism (III, Fig. 5, S5). Recently, studies from three independent groups using CRISPR/Cas9-mediated screening have identified LAPTM4A (another LAPTM family member) to be a critical factor for cellular Gb3 synthesis (Pacheco et al., 2018; Tian et al., 2018; Yamaji et al., 2019). Due to the LAPTM4B-mediated effects on HexCer, we wondered whether other sphingolipid metabolites including Gb3 were also affected. Gb3 is undetectable by the shotgun lipidomics approach, so we instead performed experiments measuring [³H]-sphingosine incorporation into more complex sphingolipids. Interestingly, Gb3 levels were significantly increased in LAPTM4B KO cells and were suppressed by re-expressing LAPTM4B (III, Fig. S6). Furthermore, LAPTM4B silencing enhanced cellular staining with the Gb3-binding Shiga-
toxin, and LAPTM4A silencing reduced the staining (III, Fig. S6). These data indicate that LAPTM4A and LAPTM4B display opposite effects at the cellular Gb3 level. LAPTM4A and LAPTM4B have both been shown to interact with Gb3 synthase, but only LAPTM4A has demonstrated a stimulatory effect on Gb3 synthesis (Tian et al., 2018). We thus suspected that LAPTM4B may function as a competing paralog of LAPTM4A, thus suppressing Gb3 synthesis. Because of the low yield of radiolabeled sphingolipids in the sEVs fraction, we unfortunately were unable to reliably detect Gb3 in sEVs samples. The detailed mechanism of LAPTM4B regulation at the cellular Gb3 level, and whether LAPTM4B regulates Gb3 in sEVs are interesting issues to be answered.

4.4.3.3 LAPTM4B controls the membrane order of sEVs
Since ceramide and glycosphingolipids may drive the formation of lipid nanodomains in biological membranes (Cebecauer et al., 2018), we wondered whether the LAPTM4B-associated lipid phenotype has an effect on membrane order. To address this, we took advantage of a highly sensitive probe by the name of trans-parinaric acid (tPA) to measure membrane order in the sEV membrane. tPA has been used in the membrane-order study of liposomes (Al Sazzad et al., 2017), but, to our knowledge, not in biological specimens. Our data showed that tPA can measure order in biological membranes and the average tPA lifetimes for
WT and KO sEVs membranes are similar, but the longest tPA lifetime is higher in KO- than in WT-derived sEVs membranes. These data suggest that LAPTM4B displays a regulatory effect on membrane nanodomain formation (III, Fig. 7).

In summary, Study III revealed that LAPTM4B is sorted into intraluminal vesicles of multivesicular endosomes and is subsequently released from cells in sEVs. Our data further show that LAPTM4B regulates the functional properties of EVs by altering their lipid composition. Combining this with the role of LAPTM4A in Gb3 synthesis, as well as the cellular and physiological functions of sphingolipids, we anticipate that the LAPTM protein family are modulators of sphingolipid metabolism.
5. CONCLUSIONS AND FUTURE PROSPECTS

Many studies have suggested a link between LAPTM4B and poor prognosis in cancers, but few have aimed at elucidating its molecular function. In this thesis, we investigated the expression and regulation of LAPTM4B, as well as its role in regulating sphingolipid metabolism and downstream signaling events. Together, the findings suggest that LAPTM4B functions at a crossroad between sphingolipid metabolism and nutrient signaling.

The lipid environment is critical for maintaining the proper functions of membrane proteins. Therefore, elucidating protein-lipid interactions is an important aspect of understanding membrane protein function. In Study 1, we identified how LAPTM4B senses and responds to the lipid environment. In particular, we found LAPTM4B to have a ceramide-regulated element consisting of a relaxed sphingolipid binding motif in tandem with an acidic aspartate residue inside the third membrane-spanning region. The aspartate residue provides structural flexibility to the transmembrane domain, which is central for the ceramide sensing of LAPTM4B and for downstream mTORC1 activation. Earlier studies have indicated a role for LAPTM4B in regulating cellular response to nutrient availability (Li et al., 2011; Milkereit et al., 2015). Our data have now provided evidence that an important part of this regulation involves lipid metabolism.
(Zhou et al., 2018, 2020).

The longest ORF of a transcript is often assumed to encode the physiologically relevant protein, and thus typically serves as the cDNA for overexpression studies. In **Study II**, we established that LAPTM4B-24, although not encoded by the longest transcript ORF, is the major and most physiologically relevant isoform in most human cells and tissues. Our data further show that LAPTM4B-24 displays a short half-life and is rapidly regulated by nutrient (e.g. amino acid) and ceramide, suggesting that it may sense cellular nutrient status. Interestingly, we noted that, in cancers, LAPTM4B-35 expression is negligible, despite the long-held preconception that high expression of LAPTM4B-35 relates to poor cancer prognosis. These data suggest that caution is essential when interpreting the relation between mRNA and protein isoforms. Future studies are needed to clarify the functional differences and tissue distribution of the isoforms.

Whereas LAPTM4A has been found to be important for the conversion of LacCer to Gb3, the effect of LAPTM4B on lipid metabolism is less well understood. In **Study III**, we utilized shotgun lipidomics to investigate how LAPTM4B regulates lipid composition of sEVs and of their parental cells. We found that LAPTM4B regulates glycosphingolipids and ether lipids in a ceramide-interacting motif dependent manner. We further revealed that LAPTM4B is present in sEVs, and affects their membrane
properties. An interesting finding was that LAPTMB was detectable at different levels in the urine of healthy subjects, showing that LAPTMB secretion is a physiological process and is not restricted to cells in culture. Considering that LAPTMB has a fast turnover and is dynamically regulated by nutrient status, it would be interesting to assess LAPTMB in urine under various conditions such as fasting, overeating, or exercise, or in disease states such as cancers and lipid metabolic disorders.

Another intriguing finding in Study III was that LAPTMB and LAPTMA exhibit opposing effects on cellular Gb3 levels. The regulation of Gb3 synthesis is particularly interesting, since Gb3 synthase acts in the Golgi apparatus, whereas the LAPTM proteins are mainly lysosomal. Both LAPTMA and LAPTMB can interact with Gb3 synthase in immunoprecipitation experiments (Tian et al., 2018), and it would be interesting to address whether the paralogs compete with each other and regulate Gb3 synthase by direct interaction in cells, as well. It is possible that the LAPTM proteins perform some of their functions in compartments other than the late endosomes and lysosomes, where these proteins are most abundant.

Overall, this thesis provides molecular insights into LAPTMB function and regulation. LAPTMB is emerging as a modulator of nutrient sensing, and may prove to be a link between metabolic stress and cancer progression. Several aspects of LAPTMB study remain uncovered, however, with advancements in techniques such
as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy, it should become feasible to solve the molecular structure of LAPT4B in the near future, in order to provide vital information regarding its function and regulation at the atomistic level. Moreover, considering the selective expression of LAPT4B-35 in brain tissue, and the roles of sphingolipids in maintaining neuronal properties, the potential role of LAPT4B-35 in the brain and whether it associates with neurological disorders will need further investigation. LAPT4B-24 knockout mice are sub-viable, and no human beings homozygous for loss of function mutations in LAPT4B have yet been identified. This suggests that LAPT4B-24 plays a critical but unknown physiological role. The further molecular investigation of LAPT4B may throw light upon its pathophysiological function.
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