Lysosomal Targeting and Functions of the LAPTM4 Family of Proteins

by

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Abstract

The Lysosome Associated Protein Transmembrane (LAPTM) family is comprised of LAPTM4 and 5 proteins. LAPTM5 regulates B- and T-cell receptor internalization and pro-inflammatory signaling and its lysosomal sorting was previously characterized. This thesis elucidates the lysosomal sorting determinants of LAPTM4s (LAPTM4a and LAPTM4b) and LAPTM4b function.

Here I show that LAPTM4s bind the E3 ubiquitin ligase Nedd4 via their PY motifs and that both Nedd4 and PY motifs are required for their lysosomal sorting. In the absence of PY motifs, LAPTM4 lysosomal localization is reduced and LAPTM4b expression at the plasma membrane is enhanced. Next, PY motifs and Nedd4 are explored as conserved determinants of lysosomal membrane protein (LMP) targeting. Putative PY-motif containing proteins are identified among previously published LMPs. TMEM55B was shown to bind Nedd4 via its PY motif and to utilize this motif for lysosomal targeting, and binding of Nedd4 family members WWP2 and Smurf1 to another PY motif containing LMP (ABCB6) is demonstrated. Lastly, a role for LAPTM4b in mTORC1 signaling is established. mTORC1 activation takes place at the lysosomal membrane, and involves V-ATPase stimulation by intra-lysosomal essential amino acids (EAA, such as Leu)
by an inside-out mechanism. How Leu enters the lysosomes is unknown. Here it is shown that LAPTM4b binds the plasma membrane Leu Transporter LAT1-4F2hc and recruits it to lysosomes, leading to uptake of Leu into lysosomes and mTORC1 activation via v-ATPase upon EAA stimulation. These results provide the first demonstration of localization and function of the Leu transporter at the lysosome, and help explain the inside-out activation of mTORC1 by EAA in this organelle.
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4F2hc – 4F2 Cell-Surface Antigen Heavy Chain or CD98
4E-BP – Eukaryotic translation initiation factor 4E binding protein
ABC – ATP-binding Cassette
AP – Adaptor Protein
APF1 – ATP-dependent proteolytic factor 1
ADP – Adenosine diphosphate
ARF – ADP-ribosylation factor
ATP – Adenosine Triphosphate
BCR – B Cell Receptor
β(2)AR – β(2)-adrenergic receptor
C2 domain – Conserved region 2 of Ca2+-dependent isoforms of PKC
Cbl – Casitas B-lineage lymphoma
CCV – Clathrin Coated Vesicle
CD98 – 4F2 Cell-Surface Antigen Heavy Chain
CLEAR – Coordinated lysosomal expression and regulation
CLN3 – Ceroid-lipofuscinosis, neuronal 3
CMA – Chaperone mediated autophagy
CNrasGEF – Cyclic nucleotide Ras guanine nucleotide exchange factor
Comm – Commissureless
ConA – Concanavalin A
Co-IP – Co-immunoprecipitation
CNS – Central Nervous system
CPS – Carboxypeptidase S
CS – Catalytically-inactive
DMT – Divalent metal ion transporter
DUB – Deubiquitinating enzyme
E1 – Ubiquitin activating enzyme
E2 – Ubiquitin conjugating Enzyme
E3 – Ubiquitin Ligase Enzyme
E4 – Ubiquitin editing Enzyme
EAA – Essential Amino Acids
EE – Early Endosome
eIF–4E – Eukaryotic initiation factor 4E
EGF – Epidermal Growth Factor
ENaC – Amiloride-sensitive epithelial sodium channel
Eps15 – EGFR–pathway substrate 15
Eps15r – Eps15related
ER– Endoplasmic Reticulum
ESCRT – Endosomal Sorting Complex Required for Transport
FGF – Fibroblast Growth Factor
GAE – γ–adaptin ear domain
Gap – General amino acid permease
GAP – GTPase-Activating Proteins
GAT – GGA and TOM domain
GDP – guanosine diphosphate
GGA – Golgi-localizing γ-adaptin ear domain homology, ADP-ribosylation factor binding protein
GEF – Guanine nucleotide exchange factor
GGA – Golgi-localized, γ-adaptin ear domain homology, ARF–binding
GHR – Growth Hormone Receptor
GPCR – G-protein Coupled Receptor
Grb – Growth factor receptor–bound
GST – Glutathione S-Transferase
GTP – Guanosine triphosphate
FLNC – Folliculin
HA – Hemaglutinin
HECT – Homologous to E6–AP–C–terminus
HEK293T – Human Embryonic Kidney cells expressing the large T–antigen of simian virus 40
I-CLIP – intra-membrane cleaving proteases
IGF-1R – Insulin–like growth factor 1 Receptor
IPTG – isopropyl-1-thio-β-D-galactopyranoside
IL – Interleukin
ILV – Intraluminal Vesicle
IP – Immunoprecipitation
Itch – Itchy E3 Ubiquitin Ligase
KO – Knockout
LE – Late Endosome
LLnL – N-acetyl-Leu-Leu-norleucinal
Lamp – Lysosome Associated Membrane Protein
LAPTM4 – Lysosome associated protein transmembrane
LAT1 – L–Type Amino Acid Transporter 1
Leu – Leucine
Limp – Lysosome Integral Membrane Protein
LMP – Lysosomal Membrane Protein
LYNUS – Lysosome nutrient sensing
mCh – mCherry fluorescent tag
MCLN1 – Mucolipin 1
MEFs – Mouse Embryonic Fibroblasts
MHC – Major Histocompatibility Complex Class II
MPR – Mannose Phosphate Receptor
MS – Mass Spectroscopy
MVB – Multivesicular body
mTORC1 – mammalian Target of Rapamycin Complex 1
NDFIP – Nedd4 family interacting proteins
Nedd4 – Neuronal Precursor Cell-Expressed Developmentally Downregulated
NPC – Niemann-Pick C
PAGE – Polyacrylamide Gel Electrophoresis
PBS – Phosphate Buffered Saline
PDGFR – Platelet-derived Growth Factor Receptor
PH – Pleckstrin homology
PI3K – Phosphatidylinositol 3-kinase
PMSF – phenyl methyl sulfonyl fluoride
PTEN – Phosphatase and tensin homolog
PTK – Protein tyrosine kinase
Rag – Ras-Related GTP Binding
Raptor – Regulatory associated protein of mTOR
Rheb – Ras homolog enriched in Brain
RING – Really Interesting New Gene
RIP – Regulated Intramembrane Proteolysis
RNA – Ribonucleic acid
RTK – Receptor Tyrosine Kinase
S6K – p70, Ribosomal protein S6 kinase
SDS – Sodium Dodecyl Sulfate
sh – small hairpin
SH3B4 – Src homology 3 (SH3) binding protein 4
SLC – Solute Carrier
Smurf – Smad ubiquitin regulatory factor
TFEB – Transcription Factor EB
TGFβ – Transforming Growth Factor β
tGN – Trans Golgi Network
TSC – Tuberous sclerosis complex
Ub – Ubiquitin
UBA – Ubiquitin Association domain
UBD – Ubiquitin Binding domain
UIM – Ubiquitin Interacting Motif
v-ATPase – vacuolar ATPase
VHS – Vps27, Hrs, Stam
VEGF – Vascular Endothelial Growth Factor
Vps – Vacuolar Protein Sorting
WT – Wild-type
WW domain – protein-protein interaction domain containing two conserved tryptophans
WWP – WW domain containing E3 Ubiquitin Protein Ligase
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Chapter 1
Introduction
1 Thesis Overview

This doctoral thesis primarily examines the lysosomal targeting determinants and functions of the LAPTM4 proteins: LAPTM4a and LAPTM4b (24kDa isoform). I demonstrate that LAPTM4s share a varying dependence on their PY (L/PPXY) motifs and Nedd4 (which binds PY motifs) for lysosomal sorting. I investigate the potential for a conserved PY motif/Nedd4 dependent targeting mechanism for other lysosomal transmembrane proteins beyond LAPTMs through the study of a candidate protein, TMEM55B. Furthermore I demonstrate that LAPTM4b plays a key role in amino acid mediated activation of mTORC1, through recruitment of the leucine transporter (LAT1-4F2hc) to lysosomes.

Chapter 1 serves as a literature review divided into four sections. The first introduces the ubiquitination pathway and its key players, including the Nedd4 family of E3 ligases and the fates of ubiquitinated proteins. The second provides an overview of the lysosome including: its biogenesis, physiological roles and the determinants of lysosomal membrane protein sorting. The third outlines the emerging functions of lysosomal membrane proteins, with a particular emphasis on LAPTM proteins. Finally, I address the role of the lysosome as a major player in nutrient sensing and signaling via mTORC1.

Chapter 2 investigates whether LAPTM4s share conserved lysosomal targeting determinants with LAPTM5. The role of PY motifs and Nedd4 is investigated in LAPTM4 trafficking to the lysosome. I demonstrate that although LAPTM4s, like LAPTM5, require PY motifs and Nedd4 for proper lysosomal targeting, the degree upon which they depend on PY motifs and Nedd4 varies between family members. This work was published in PLoS One (PLoS One 6(11):e27478). I expand on these results in Appendix A and demonstrate that LAPTM4b sorting is dependent on additional lysosomal targeting motifs (dileucine and tyrosine-based).

In Chapter 3 I examine the possibility that additional lysosomal transmembrane proteins might utilize PY motifs and Nedd4 proteins as determinants for their lysosomal sorting. I present my preliminary data that identifies putative PY motif containing proteins within the cohort of proteins previously isolated by others in a proteomics screen for resident lysosomal transmembrane proteins. I demonstrate that the candidate protein TMEM55B is a novel Nedd4 binding partner and that TMEM55B’s PY motif plays a role in its lysosomal localization. In addition, I demonstrate the candidacy of ABCB6 as a binding partner for additional Nedd4
family members Smurf1 and WWP2. Together, the work presented in this chapter provides a basis to explore the role of PY motifs and Nedd4 family members as conserved determinants of lysosomal membrane targeting beyond the LAPTM family.

**Chapter 4** is a collaborative effort that demonstrates a role for LAPTM4b as a positive regulator of amino acid dependent mTORC1 activation in lysosomes. I identify novel putative binding partners of LAPTM4b through mass spectrometric analysis and confirm the interaction of LAPTM4b with key players of the nutrient sensing machinery (LAT1 and 4F2hc). I generate stable LAPTM4b knock-down HeLa cell lines to investigate LAPTM4b’s role in nutrient sensing at the lysosome. Through knock-down and reconstitution experiments, we demonstrate a dose-dependent activation of mTORC1 in HeLa cells by LAPTM4b, through recruitment of the leucine transporter LAT1-4F2hc to lysosomes. This work is currently being prepared as a manuscript for publication.

Finally, **Chapter 5** discusses how the body of work contained in this thesis expands our understanding of how LAPTM4 proteins are targeted to and function at the lysosome. I conclude by proposing additional experiments to address questions that remained or arose subsequent to my analysis in chapters 2, 3 and 4.
2 Ubiquitin and the Ubiquitination Pathway

Ubiquitin is small, conserved 76 amino acid protein that is covalently linked to target proteins in an enzymatic cascade termed ubiquitination (or ubiquitylation) (Figure 1.1). Target proteins are differentially ubiquitinated (monoubiquitinated, multi-monoubiquitinated or polyubiquitinated), whereby the modification dictates its cellular fate. Ubiquitination was originally characterized in the context of 26S proteasome degradation. Ubiquitination also plays key roles in cellular processes such as vesicular trafficking (endocytic and biosynthetic), signaling, cell cycle control and DNA repair. This section provides an overview of the key players of ubiquitination. Importantly for this doctoral thesis, the role of ubiquitin and ubiquitin ligases in vesicular traffic (including endocytosis, Golgi to endosome trafficking and sorting to luminal vesicles) are reviewed.

2.1 Ubiquitination – The Ubiquitin-Protein Ligase Cascade

At the time of its discovery ubiquitin was originally called ATP-dependent proteolytic factor 1 (APF1) [1] and shown to form covalent conjugates with protein substrates in an ATP-dependent process [2]. Later APF-1 was designated ubiquitin [3] and found to generate a multitude of covalently modified conjugates [4]. This process requires the enzymatic activation of ubiquitin [5] as part of what has become known as the ubiquitin-protein ligase system [6].

Ubiquitination is a multistep process, requiring the hierarchical action of the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase enzyme (E3) (C). In this process, the E1 enzyme activates ubiquitin in an ATP-dependent fashion through the formation of a high-energy thioester bond between its catalytic cysteine residue and ubiquitin. A thiol group of the E2’s active site cysteine then accepts the activated ubiquitin. Finally, substrate ubiquitination proceeds through the activity of the E3 ligase.

In eukaryotes E3 ligases fall into two major classes: the RING (Really Interesting Novel Gene) and HECT (Homologous to E6-AP Carboxyl Terminus) domain containing ligases [7]. In the case of HECT-type E3 ligases, the E3 ligase accepts the activated Ub to transfer directly onto a substrate. RING E3s, on the other hand, recruit their substrates in proximity to E2s, such that the E2 ubiquitinates the substrate. Typically substrate ubiquitination proceeds through the formation
of an isopeptide bond between ubiquitin’s carboxy-terminal (C-terminal) glycine residue (Figure 1.1A) and the ε-amino group of the target substrate’s lysine residue. Additionally, thiol- and hydroxyl- groups in cysteine and serine/threonine residues can also be ubiquitinated [8,9]. While there are only estimated to be 2 E1s and ~40 E2s in eukaryotes, there are over 600 E3 Ligases [10]. Consequently, it is the E3 that dictates ubiquitination specificity through its substrate recognition.

Ubiquitin contains 7 lysine residues (K6, K11, K27, K29, K33, K48, K63,Figure 1.1 B), to which other ubiquitin can be covalently linked[11]. Substrate ubiquitination typically results in monoubiquitination, multi-monoubiquitination or polyubiquitination (Figure 1.1C) and dictates target substrate fate. The most abundant modifications occur at K48 and K63 [12]. K48 polyubiquitination results in proteosomal degradation[13], while K63-linkages have been implicated in DNA repair, signal transduction and endocytosis[14]. Interestingly head-to-tail linear ubiquitination between Ubiquitin Glycine 76 and Methionine 1 of ubiquitin moieties has been observed [15]. NFkB is modified in such a manner [16].

Ubiquitination is an editable and reversible process: ubiquitin chains can be elongated through the activity of E4 enzymes [17] or ubiquitin moieties can be removed through the activity of deubiquitination enzymes (DUBs)[18].
Figure 1.1: **Structural features of ubiquitin and the ubiquitin pathway.**

(A) Ribbon and surface representation of ubiquitin with its c-terminal Gly^{76} \[19\]. (B) Space filling schematic view of the seven-lysine residues (blue) used in ubiquitin chain formation. The hydrophobic ubiquitin binding domain (UBD) binding patch (Ile^{44}) is indicated in yellow \[20\]. (C) The ubiquitin pathway as adapted from \[21\]. An E1 (ubiquitin activating enzyme) activates free Ub in an ATP-dependent manner through the formation of a thiolester bond between the E1 and the carboxyterminus of ubiquitin. The E1 transfers the Ub to an ubiquitin conjugating enzyme (E2), which associates with an E3 Ubiquitin Ligase (RING or HECT type). The Ub is then transferred either directly to the substrate by the E2 through close association with the E3 RING ligase or to the catalytic cysteine of the HECT E3 active site and subsequently to the substrate(s). The type and level of substrate ubiquitination determine its cellular fate (some examples indicated).
Figure 1.1: Structural features of ubiquitin and the ubiquitin pathway.

Note: (Figure 1A) Reproduced with permission, from James H. Hurley, Sangho Lee, Gali Prag, (2006), *Biochemical Journal*, (399 (3)), (361-72). © the Biochemical Society.
2.2 Ubiquitin Binding Domains (UBDs)

Ubiquitination is a post-translational modification that must first be recognized by cellular machinery to facilitate a downstream effect on the target substrate. Ubiquitin is recognized through ubiquitin-binding domains (UBDs) (20-150 amino acid sequences) in ubiquitin binding proteins [22]. The proteasome subunit S5A/RPN contains the first characterized UBD known as the Ubiquitin Interacting Motif (UIM) [23]. To date, there are approximately 20 different families of UBDs which all bind non-covalently to ubiquitin. Most UBDs bind the same hydrophobic patch surrounding Isoleucine 44 in ubiquitin [24] (Figure 1.1B). UBDs can be classified into sub-families based on shared structural features, including the presence of conserved single or multiple α-helices, zinc fingers, pleckstrin-homology folds, ubiquitin-conjugating-like structures or being unique. A list of a variety of these domains is listed in Table 1. UBDs generally have a weak binding affinity for mono-ubiquitin (Kd>100 µM), but can be found clustered in tandem within a single protein or through association with multiple proteins to result in high-avidity interactions [25].

Table 1 Ubiquitin Binding Domains. Adapted from [22]

<table>
<thead>
<tr>
<th>UBD Sub-Family (Structural Similarity)</th>
<th>Examples of Ubiquitin Binding Domain (UBD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single or Multiple α-helices</td>
<td>Ubiquitin associated (UBA) domain, ubiquitin interacting motif (UIM), double-sided ubiquitin-interacting motif (DUIM), motif interacting with ubiquitin (MIU), coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE), GGA and Tom1 (GAT), Vps27/Hrs/STAM (VHS), ubiquitin binding in ABIN and NEMO (UBAN)</td>
</tr>
<tr>
<td>Zinc Fingers</td>
<td>Nuclear protein localization 4 zinc finger (NZF), zinc-finger ubiquitin-binding protein (ZnF UBP), zinc finger in A20 protein (ZnF A20), ubiquitin-binding zinc finger (UBZ)</td>
</tr>
<tr>
<td>Pleckstrin-homology (PH) Folds</td>
<td>Gramlike ubiquitin-binding in Eap45 (GLUE), pleckstrin-like receptor for ubiquitin (PRU)</td>
</tr>
<tr>
<td>Ubiquitin-conjugating-like Structures</td>
<td>Ubiquitin E2 variant (UEV) and UBC</td>
</tr>
<tr>
<td>Unique Structures</td>
<td>Ubiquitin-binding motif (UBM), PLAA family ubiquitin binding domain (PFU), Jab1/MPN</td>
</tr>
</tbody>
</table>
2.3 The Fates of Ubiquitinated Proteins.

Ubiquitinated proteins are recognized by UBD containing proteins, which either leads to their degradation or affects their cellular trafficking.

2.3.1 Proteosomal Degradation

The 26S proteasome is composed of two major sub-complexes: the 20S core and 19S regulatory particle. The 26S proteasome degrades ubiquitinated proteins in a multi-step process that involves polyubiquitin chain recognition, substrate unfolding, translocation into the core particle where the substrate is degraded into oligopeptides via hydrolysis [26]. K48 ubiquitination was recognized early on as a signal for protein degradation [27]. Specifically it is the UIM of the S5A/RPN subunit that recognizes the K48 polyubiquitin chain [28]. Subsequently other ubiquitin linkages, termed “atypical” linkages through lysines K11, K29 and K33 have also been linked to proteasome degradation [11].

2.3.2 Ubiquitin in Cellular Trafficking of Proteins

The role of ubiquitination in cellular trafficking is complex. Ubiquitination affects endocytosis of receptors and membrane proteins from the plasma membrane, sorting of cargo proteins into intraluminal vesicles (ILV) of Multivesicular Bodies (MVBs) for lysosomal degradation, as well as sorting proteins directly from the trans-Golgi Network (TGN) to the lysosome [20]. Most studies that shed light into these processes were first conducted in yeast and later confirmed in mammalian systems. The lysosome is the mammalian counterpart of the yeast vacuole.

i. Ubiquitin in Endocytosis

Ubiquitin mediates receptor/membrane protein endocytosis through direct or indirect processes. Proteins (typically receptors and transporters) are endocytosed subsequent to their direct ubiquitination or through their association with ubiquitinated adaptor proteins and/or members of the endocytic machinery. Ubiquitination in the context of endocytosis drives receptor down-regulation and/or recycling, degradation and/or sorting [29].

In yeast, a variety of plasma membrane transporters and receptors were shown to utilize ubiquitin for their endocytosis. For instance, the ATP-binding Cassette (ABC) transporter Ste6 (required for mating pheromone a-factor secretion) accumulates as an ubiquitinated protein at the
plasma membrane in endocytosis deficient cells [30]. The general amino acid permease Gap1 requires the presence and activity of the HECT domain containing E3 ligase Rsp5 (yeast orthologue of Nedd4) for its internalization [31, 32]. Additionally, the uracil permease Fur4 requires Rsp5 mediated K63 ubiquitination for its endocytosis and down-regulation [33, 34]. Similarly, the G-protein Coupled Receptor (GPCR) Ste2p (required for binding the alpha-factor mating pheromone) is mono-ubiquitinated upon ligand binding, leading to its internalization and signal down-regulation[35]. A single ubiquitin moiety is sufficient to promote the internalization of cell surface proteins like Ste2p [36]. In fact, fusion of a single ubiquitin moiety in frame with the c-terminus of proteins can enhance their endocytosis and degradation as was observed with Pma1p (plasma membrane proton ATPase) [36]. Furthermore, while mono-ubiquitination is sufficient to induce endocytosis, chain elongation can enhance it. An example of this type of regulation is the K63 mono- vs. polyubiquitination of Fur4 [33].

The first example of ubiquitin-mediated endocytosis of a mammalian plasma membrane protein was our lab’s work on the epithelial Na⁺ channel (ENaC) and Nedd4, where it was demonstrated that ENaC function was regulated by ubiquitination [37, 38]. The degree of receptor ubiquitination can dictate whether or not the receptor is internalized and recycled back to the plasma membrane, or ubiquitinated, internalized and degraded via the lysosome. This is the case for the Epidermal Growth Factor Receptor (EGFR). Most papers suggest that ubiquitination of EGFR plays a key role in its sorting and less so in its internalization. Upon ligand-binding the Epidermal Growth Factor Receptor (EGFR) is internalized and recycled back to the plasma membrane, unless it is ubiquitinated leading to its degradation [39]. Specifically, mono-ubiquitination is sufficient for EGFR internalization, but multi-monoubiquitination (not polyubiquitination) by c-Cbl leads to its degradation [40]. In the case of GPCR signaling, direct ubiquitination of the receptor or adaptors of the receptor have been shown to regulate their endocytosis and/or sorting. For instance ubiquitination of adaptor proteins (β-arrestins) mediates the ubiquitination of the GPCR β2-adrenergic receptor [41]. Arrestin-related trafficking adaptors (ARTs) are a family of proteins in yeast that target specific plasma membrane proteins (like amino acid transporters) to the endocytic system. Similar to arrestins, ARTs contain PY motifs, which recruit Rsp5 to the plasma membrane leading to ART and cargo ubiquitination. Upon ubiquitination cargo is internalized and targeted for vacuolar degradation [42].
On the other hand, sometimes ubiquitination of the receptor itself is not the signal for internalization, but instead is responsible for the post-endocytic sorting of the protein. For instance, mono-ubiquitination of the GPCR CXCR4 is required for sorting into MVB for lysosomal degradation, but not for internalization [43, 44]. Additionally, recruitment and ubiquitination of the MVB sorting machinery itself can be a requirement of membrane protein internalization and sorting. This is the case for the Growth Hormone Receptor (GHR). GHR requires the recruitment of the ubiquitin conjugation machinery, rather than ubiquitination of the GHR itself for its endocytosis[45]. Similarly, the yeast GPCR for the a-factor mating pheromone Ste3 has ubiquitin independent internalization, but ubiquitin dependent entry into MVB for degradation[46].

Interestingly, ubiquitination can dictate the mode of endocytosis of some proteins. For instance, ubiquitination mediates clathrin-dependent and independent pathways of EGFR [47] and TGF-βR (Transforming Growth Factor β Receptor) signaling [48, 49].

Upon endocytosis, the cell must decide whether the internalized protein is destined for degradation or should recycle back to the plasma membrane. Mono (or multi-mono-ubiquitination) acts as a sorting signal for cargo proteins along the endocytic trafficking pathway [50]. Various UBD containing proteins such as Eps15 (EGFR-pathway substrate 15), Eps15r (Eps15related) and epsin guide this process. These proteins bind ubiquitinated proteins through their UIMs [36]. They also bind additional adaptor proteins (like AP2) and clathrin, which collectively facilitates endocytosis [51]. The endocytic machinery is ubiquitinated itself and can stimulate cargo ubiquitination [52]. This leads to the accumulation of both ubiquitinated cargo and UIM containing proteins on the endosomal structures.

i. Multivesicular Body (MVB) and Lysosomal Degradation

Some endocytosed proteins are sorted towards ILV of MVBs through additional steps that involve the cargo sorting machinery ESCRT (Endosomal Sorting Complexes Required for Transport). ESCRT were originally discovered in yeast as part of screen for vacuolar protein sorting (Vps) mutants [53]. There are four distinct ESCRT complexes (-0, -I, -II, III) that, with the exception of ESCRT-III, transport cargo along the endocytic pathway via recognition of cargo ubiquitin through UBDs [54, 55]. ESCRT-0 recognizes and recruits ubiquitinated cargo via Vps27p/Hrs (hepaticocyte growth factor-regulated tyrosine kinase substrate) and Hse1p/STAM
Vps27p/Hrs binds cargo via a UIM [58], while recruiting it to the endosomal membrane through its FYVE domain, which binds the endosome-specific phosphatidylinositol-3-phosphate [59]. The ESCRT-0 component Hrs not only directly interacts with Eps15 (involved in membrane protein endocytosis[60, 61]), but with Tsg101 of ESCRT-II[62, 63]. ESCRT-III complex members do not possess UBDs to recognize ubiquitin – instead ESCRT-III is responsible for recruiting deubiquitinating enzymes (DUBs), which remove ubiquitin before cargo is sequestered into ILVs [64] and the machinery necessary for disassembling ESCRT from the endosomal membrane, including membrane abscission [65].

It should be noted that while a large proportion of proteins that are shuttled towards ILVs and MVBs are destined for degradation, some proteins utilize this pathway to reach their final destination where they are functional. For instance, the vacuolar carboxypeptidase S (CPS) is targeted to the lysosome to be activated [66]. Monoubiquitination is sufficient for CPS vacuolar targeting, while blocking ubiquitination results in its retention in the lysosomal limiting membrane [67].

ii. Protein Sorting at the Trans-Golgi Network (TGN)

Ubiquitin has also been shown to play a role in sorting of proteins directly from the trans-Golgi network (TGN) to the vacuole/lysosome. This was first studied in yeast and later in mammalian cells. For instance, while Gap1 is generally present at the plasma membrane under low nitrogen conditions, it is ubiquitinated by Rsp5 at the TGN in the presence of abundant nitrogen. Ubiquitination targets Gap1 directly from the TGN to the vacuole using Bul1 and Bul2 adaptor proteins [68, 69]. Bul1 and Bul2 promote Gap1 ubiquitination through association of their PY motifs with the WW domains of Rsp5 [70, 71]. Similarly, in the presence of uracil Fur4 is sorted directly from the TGN to the vacuole due to Rsp5 mediated ubiquitination [72].

Ubiquitin sorting from the TGN to lysosome/vacuole is mediated by GGAs (Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor (ARF)-binding proteins). The GGA family (GGA1-3) is involved in the clathrin-dependent sorting of proteins from the TGN to endosomes [73]. GGAs have a modular structure that includes a VHS (Vps27, Hrs, Stam) and GAT (GGA and TOM (target of myb)) domain linked to a GAE domain (γ-adaptin ear) [74]. Each domain plays a unique role in the sorting of proteins from the TGN to the endosomal system [75] and is addressed in greater depth in section 3.4. Important to the role of ubiquitin in
sorting is the GAT domain, which binds Arf:GTP and ubiquitin [76]. For instance, GGA2 binds ubiquitinated Gap1 via its GAT UBD and is essential for its TGN to vacuole sorting [77].

Recently, targeting of proteins directly from the TGN to lysosome was extended to include an E3 ligase dependent, but target-protein ubiquitination-independent mechanism. For instance, our lab demonstrated that lysosomal sorting of LAPTM5 was independent of its ubiquitination, but dependent on LAPTM5 binding to ubiquitinated GGA3 via LAPTM5’s UIM in the presence of Nedd4. Without this interaction, LAPTM5 was retained in the TGN [78]. Similarly, the yeast integral membrane Sna3p was targeted to the yeast vacuole in an ubiquitination-independent fashion, but required the presence of Rsp5 [79, 80].

2.4 Nedd4 family of HECT E3 Ubiquitin Ligases

2.4.1 Nedd4 family architecture

One family of E3 Ubiquitin ligases, are the evolutionarily conserved family of Nedd4 (Neuronal precursor cell-expressed developmentally down-regulated 4) HECT E3 ligases. As outlined in Figure 1.2A, while there is only one Nedd4 member in yeast S. cerevisiae (Rsp5), there are nine members in humans [20]. All Nedd4 family members share a common domain architecture that includes an N-terminal C2 domain, two to four WW domains and a C-terminal catalytic HECT domain (Figure 1.2A,B).

The C2 domain binds phospholipids and directs the ligase to the plasma membrane, endosomes or MVBs [81]. However, it can also mediate direct protein-protein interactions such as the binding of Nedd4-2 to apically localized annexin XIIIb [82] or the growth factor receptor-bound (Grb) family of proteins [83]. Finally, it has also been implicated auto-inhibition of E3 ligase activity in Smurf2 and Nedd4[84, 85].

WW domains are small domains (35-40 amino acid residues) that contain two conserved tryptophan residues (W)[86]. Typically, WW domains of Nedd4 family E3 ligases mediate binding to their substrates by binding L/PPXY (PY) motifs (X = any amino acid) [37, 87, 88]. However, in some cases Nedd4 WW domains can bind to proteins lacking PY motifs, such as FGFR1[89]. In addition, there are PY-motif containing adaptor proteins in both yeast and mammals that serve to link Nedd4 ligases to substrates that lack the canonical L/PPXY motifs. In yeast these proteins are called arrestin-related proteins (ARTs) and enable Rsp5 mediated
ubiquitination of PY-less substrates[90]. Similarly Bsd2 acts as a mediator for Rsp5 mediated trafficking of vacuolar enzymes[91]. This process is also conserved in mammals, where Bsd2 homologues Nedd4 family interacting proteins 1 (NDFIP1) and NDFIP2 link WWP2 to the divalent metal ion transporter DMT1 [92] and Smurf1-mediated degradation of the mitrochondrial antiviral signaling (MAVS) protein during immune signaling [93].

The HECT domain is responsible for the E3s ubiquitin ligase activity. This 350 amino acid domain was first characterized in E6AP (human papilloma virus E6 associated protein) [94]. It is a bi-lobal domain with a conserved c-terminal cysteine residue that is able to form thioester complexes with ubiquitin, while the N-terminal region interacts with the E2 enzyme [95]. PY motifs present in the HECT domain of Nedd4-2 regulate its self-ubiquitination and stability[96].
Figure 1.2: **The Nedd4/Nedd-like Family of E3 Ubiquitin Ligases.** (A) diagrammatic representation of the Nedd4 family of E3 ligases that is conserved across yeast, nematode, fly and mammalian species. All members share a similar domain architecture: C2 domain (can be spliced out, dashed lines), WW domains, and a HECT domain. Diagrams are not to scale [20]. (B) Schematic representation of the relationship between Nedd4 and Nedd4-2 among a variety of species [97]. Reprinted by permission from Macmillan Publishers Ltd: [Cell Death and Differentiation], Nature Publishing Group ©2009. (a) phylogenetic tree (b) conserved modular protein domain structure (green: C2 domain, yellow: WW domains, red: HECT domain). c: chicken, d: fruitfly, h: human, m: mouse, x: frog, z: zebrafish.
Figure 1.2: Nedd4/Nedd4-like Family of Ubiquitin Ligases [20, 97].
2.4.2 Functions of Nedd4 Proteins

The Nedd4 family plays key roles in a variety of cellular processes. The specific functions of Nedd4-1 (referred throughout my thesis as Nedd4) and Nedd4-2 are outlined next.

**Nedd4 (Nedd4-1):**

The yeast *S. cerevisiae* homologue of Nedd4 is Rsp5. Rsp5 is a key player of endocytosis [81] and protein trafficking [79, 98]. Additionally it has been implicated in transcription regulation [99], mitochondrial inheritance [100], response to anesthetics[101], regulation of cellular pH[102], biosynthesis of unsaturated fatty acids[103] and actin cytoskeleton maintenance and dynamics[104].

The *Drosophila melanogaster* dNedd4 has been shown to play a role in neuromuscular junction formation. Specifically, dNedd4 promotes neuromuscular synaptogenesis through the ubiquitin mediated endocytosis of the PY motif containing protein Commissureless (Comm)[105] In fact, dNedd4 isoforms differentially regulate this process [106]. Similarly, Nedd4 is involved in nervous system development in mammals. Nedd4 promotes neurite growth and arborization in mice through the ubiquitination and degradation of the effector kinase Rap2A[107]. Similarly, Nedd4 is essential for the proper interaction between the nerve and muscle, and different Nedd4 knock-out mouse embryos exhibit reduced formation and function of neuromuscular junctions[108].

Mammalian Nedd4 (or Nedd4-1) was originally identified in a gene down-regulated during the development of the mouse central nervous system [109]. Nedd4 is now known to be a positive regulator of cell proliferation and growth. Mouse embryonic fibroblasts (MEFs) from Nedd4 Knock out (KO) mice show reduced mitogenic activity [110]. Furthermore Nedd4 KO results in embryonic lethality at mid gestation, with severe heart defects and vasculature abnormalities[111].

In line with Nedd4’s role in proliferation and cell growth, Nedd4 has been shown to a play critical role in regulating the stability and degradation of cell surface receptors, including the Insulin-like growth factor I receptor (IGF-1R), the Vascular Endothelial Growth Factor Receptor-2 (VEGF-R2), the Endothelial Growth Factor Receptor (EGFR) and the Fibroblast Growth Factor Receptor 1 (FGFR1).
The role of Nedd4 in regulating the tyrosine kinase IGF-1R is controversial. Some have shown that Nedd4 ubiquitinates and downregulates IGF-1R in a ligand-dependent fashion [83, 112-114]. Others have suggested Nedd4 promotes IGF-1R recycling, which could explain why Nedd4 KO MEFs and embryos grow poorly [110]. Similarly, Nedd4 mediates the ubiquitination and degradation of VEGF-R2 during embryonic development [115]. Finally, Nedd4 has been implicated in the regulation of EGFR endocytosis and lysosomal degradation through its ability to ubiquitinate proteins involved in clathrin-mediated endocytosis and degradation of EGFR including: Eps15, Tsg101, Hrs, Secretory Carrier Membrane Proteins (SCAMPs)[116-118] and the ubiquitin binding protein ACK (activated Cdc42-associated tyrosine kinase)[119].

Controversially, Nedd4-1 was proposed to be the proto-oncogenic ubiquitin ligase for the tumor suppressor PTEN [120, 121], whereby ubiquitination regulates PTEN nuclear import and tumor suppression [122]. However, independent studies were unable to detect changes in PTEN levels or stability in mouse embryonic fibroblasts (MEF) from Nedd4 KO mice[123]. Furthermore, WWP2 was shown to regulate PTEN stability[124]. Taken together with the observation that Nedd4 destabilizes the mTOR substrate Grb10 that regulates cell survival pathways, it is unlikely that PTEN is truly a physiological substrate of Nedd4 [125].

Other roles of Nedd4 in cell signaling include its ability to regulate the stability of the guanine-nucleotide exchange factor CNrasGEF [126, 127] and recruitment of Nedd4 to the activated β(2)-adrenergic receptor (β(2)AR) by β-arrestin2 and subsequent Nedd4 mediated β(2)AR ubiquitination and lysosomal degradation [128].

There have been additional roles for Nedd4 proposed in the immune system that include T cell function and viral budding. Nedd4 promotes the conversion of naive T cells into activated T cells through polyubiquitination of Cbl-b upon CD28 co-stimulation[129]. While PY motif containing viral particles (eg. Epstein Barr-, Retro- and Ebola-virus) recruit Nedd4 and other MVB sorting machinery to the plasma membrane for viral egress[130-132].

Most relevant to my PhD, our lab demonstrated a novel mechanism of direct Golgi to lysosome sorting for LAPTM5 that required the binding to Nedd4 via LAPTM5’s PY motifs [78]. While LAPTM5 is ubiquitinated by Nedd4, its lysosomal targeting is independent of its ubiquitination. Instead, LAPTM5 uses its ubiquitin-interacting motif (UIM) to bind to ubiquitinated GGA3, an
adaptor protein, which is required for LAPTM5 sorting to the lysosome. The role of LAPTM5 ubiquitination by Nedd4 remains unknown.

Nedd4-2:

Nedd4-2 has been implicated in the regulation of ion channel stability, through PY-motif dependent mechanisms. For instance, Nedd4-2 ubiquitinates the amiloride-sensitive epithelial sodium channel (ENaC), leading to its internalization and degradation [38, 133, 134]. ENaC is composed of three subunits (α, β and γ), each containing a PY motif to which Nedd4-2 WW domains bind [135]. Liddle syndrome is a hereditary form of hypertension in which ENaC activity and abundance at the plasma membrane is increased due to deletion or mutation of PY motifs in the β or γ subunits of ENaC [136-138]. This precludes Nedd4-2 binding and regulation [139]. Similarly, Nedd4-2 regulates the cell surface stability of additional sodium gated channels (Na+1.1, 1.2, 1.3, 1.5, 1.6, 1.7, 1.8) [140, 141], the Cl-/H+ antiporter (CLC5) [142] and the voltage-gated potassium channel hERG1 [143] through PY-motif dependent mechanisms. Nedd4-2 has also been implicated in TGFβ signaling. Through association with Smad7, Nedd4-2 triggers the ubiquitin-dependent degradation of the TGFβ receptor, while binding to the R-Smad Smad2, induces its degradation [144]. Finally, Nedd4-2 has also been implicated in the downregulation of the neurotrophin induced Trk neurotrophin receptor A (TrkA) in a PY motif dependent process [145].
3 Lysosomes: Function and Biogenesis.

The lysosome was initially described as membrane bound organelle with degradative capacity by Christian De Duve in 1955 [146]. Since then, it has become apparent that the lysosome is a degradative organelle with important secretory and signaling functions (Figure 1.3) [147]. This section summarizes our current understanding of lysosome function and biogenesis.

3.1 Lysosomal degradation & secretion

Exogenous and cytosolic (or intracellular) materials reach the lysosome for degradation via the endocytic pathway [148] or autophagic processes like macroautophagy [149] and chaperone-mediated autophagy (microautophagy) [150, 151]. Digested materials include protein aggregates, oxidized lipids, damaged organelles and intracellular pathogens [147]. Digestion of these materials liberates building blocks for the generation of new cellular components, as well as energy for the nutritional needs of the cell.

The lysosome’s degradative ability is tightly linked to its acidic pH and the abundance of catabolic enzymes. There are over 50 luminal acid hydrolases capable of degrading macromolecules into their more fundamental building blocks [152]. In addition there are intramembrane cleaving proteases (I-CLIPs) that cleave transmembrane substrates through a Regulated Intramembrane Proteolysis (RIP) [153]. Examples of I-CLIPs include the γ-secretase complex with catalytic subunits presenilin 1 and 2 [154], and the Signal-peptide-peptidase-like 2a (SPPLa)[155]. All of these enzymes thrive in the acidic lysosome environment (~pH 5)[156].

The acidic environment is largely generated and maintained by the activity of a proton-pumping V-type ATPase that uses ATP hydrolysis to pump protons into the lysosomal lumen[157]. Other membrane proteins that mediate Ca$^{2+}$ and Na$^+$ release from the lysosome are critical in this process[158]. They include the anion transporter channel 7 (CLC7)[159], the cation transporter mucolipin 1 (MCOLN1)[160] and the two-pore calcium channel 1 (TPC1 and TPC2)[161, 162].

All cells can undergo lysosomal exocytosis[163, 164]. This process involves the Ca$^{2+}$ mediated fusion of lysosomes with the plasma membrane and the release of their contents into the extracellular space[165-168]. This release is critical to a variety of cellular processes[147], including degranulation of cytotoxic T lymphocytes[169], bone resorption by osteoclasts[170],
mechanisms against bacterial and parasitic infection[171-173], platelet function during coagulation[174] and hydrolase release by spermatozoa during fertilization[175]. Exocytosis also serves as a plasma membrane repair mechanism, by which lysosomal membranes seal/repair lesions in the plasma membrane [176, 177]. The process is mediated by vesicle SNARE (v-SNARE) VAMP7, the Ca²⁺ sensor synaptotagmin VII (SYTVII) and RABs on the lysosomal surface that interact with the target SNARE (t-SNARE) SNAP23 and syntaxin4 on the plasma membrane[178, 179].

3.2 Lysosomal signaling

It was recently shown that some lysosomal proteins are key players of nutrient sensing and signaling. These proteins form a complex referred to as the LYNUS (Lysosome nutrient sensing) machinery (Figure 1.3B) [147]. The kinase complex mammalian target of rapamycin complex 1 (mTORC1), a master controller of cell growth[180], is activated at the lysosomal membrane in the presence of amino acids[181]. Specifically luminal amino acids control mTORC1 docking to the lysosome and its subsequent activation [182]. The role of amino acid sensing in mTORC1 activation is explored in greater depth in the final section of this introduction (See section 5).
Figure 1.3: **Main functions of the lysosome and their relationship with key cellular processes[147].**

(A) Lysosomes are involved in degradative (blue), secretory (orange) and signaling (green) processes. Lysosomes degrade and recycle intra- and extracellular material via autophagy and endocytosis, respectively. During autophagy, autophagosomes fuse with late endosomes. This breaks down materials into new cellular components and can satisfy some of the nutritional needs of the cell. In Ca$^{2+}$ dependent exocytosis, lysosomes fuse with the plasma membrane, secreting their contents into the extracellular space and repairing/resealing plasma membranes. Finally, lysosomes scan sense nutrient availability and activate lysosome-to-nucleus signaling pathways that mediate the starvation response and energy metabolism.

(B) The LYNUS (Lysosome nutrient sensing machinery) senses lysosomal nutrient levels through the vacuolar ATPase (v-ATPase) complex and is phosphorylated by mammalian target of rapamycin (mTORC1) on the lysosomal surface. Components of LYNUS include: mTORC1, with regulatory proteins Raptor (regulatory-associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein) and Deptor (DEP domain-containing mTOR-interacting protein), which physically interact with RAG GTPases (RagA or RagB and RagC or RagD), to activate mTORC1 at the lysosomal membrane. The Ragulator complex mediates the activation and docking of Rag GTPases to the lysosomal membrane. Rheb (Ras homologue enriched in brain) is the small GTPase responsible for mTORC1 activation. The v-ATPase mediates amino-acid sensitive interactions between Rag GTPases and Ragulator as the initial step in lysosomal signaling. lysoNa$_{ATP}$ (endolysosomal ATP-sensitive Na$^+$ permeable channel) comprises two pore calcium channel 1 and 2 (TCP1, TCP2) interacts with mTORC1. It is constitutively open during starvation, where it controls the lysosomal membrane potential, pH stability and whole body amino acid homeostasis.

Figure 1.3: Main functions of the lysosome and their relationship with key cellular processes [147].
3.3 Lysosome biogenesis and protein sorting.

The biogenesis of lysosomes is a dynamic process that integrates materials from both the biosynthetic and endocytic pathways into the endo-lysosomal system. Endolysosomal intermediates are categorized based on their content, molecular make-up, morphology and pH [183]. They include primarily Early Endosomes (EE), Late Endosomes (LE) or MVBs and Lysosomes (Figure 1.4).

3.4 Sorting of Lysosomal Proteins

Proteins are sorted to the lysosome by endocytosis or during biosynthesis. This section outlines the key aspects of each pathway.

3.4.1 Endocytosis

Endocytosis can be clathrin-dependent or independent [184]. In either case, plasma membrane proteins are internalized into early endosomes, which are a heterogeneous group of organelles. At this point the default pathway is to recycle plasma membrane proteins back to the plasma membrane or occasionally towards the TGN. This is observed for example with ironbound transferrin receptors. Here the mildly acidic pH of the early endosome triggers dissociation of the transferrin from its receptor and inactivates it. Transferrin is degraded by the lysosome, but empty transferrin receptors are recycled back to the plasma membrane [185, 186]. If however the protein is destined for the degradation in the lysosome, machinery is recruited to sort the cargo into ILVs of late endosomes (or MVBs), which are eventually delivered to the lysosome [29, 187]. This was outlined in detail for ligand-bound receptors such as the EGFR in section 2.3.2. The transition of early endosomes towards late endosomes is characterized by a series of docking, fusion and fission events. Distinctively, early endosomes contain the small GTPase Rab5, while late endosomes contain Rab7. The loss of Rab5 and gain of Rab7 is essential for the recruitment of specific late-endosome effector proteins [188]. Eventually the late endosomes/MVB will fuse with the lysosome, leading to cargo degradation.

3.4.2 Sorting

Unlike the proteins that are delivered to the lysosome for degradation, lysosomal hydrolases and lysosomal transmembrane proteins (LMPs) are synthesized specifically to function at the
lysosome. Upon ER synthesis, lysosomal proteins reach the Golgi and enter the TGN where they are sorted to the lysosome via direct or indirect pathways. Direct targeting involves transport from the TGN to early or late endosomes. In the indirect pathway newly synthesized proteins are sorted from the TGN to the plasma membrane, internalized into early endosomes by endocytosis and subsequently delivered to the lysosome [189]. The sorting fate of lysosomal proteins is dictated by their lysosomal sorting signal. These signals can be post-translational modifications or short internal sequence motifs that are recognized by specific membrane sorting complexes.

i. Sorting of lysosomal hydrolases

Lysosomal luminal hydrolases are targeted to the lysosome primarily via the direct TGN to endosome route. This targeting is mediated by mannose 6-phosphate receptors (MPR) [190, 191]. MPRs recognize and bind to lysosomal hydrolases that have been modified with Mannose-6-Phosphate (M6P) residues in the Golgi. MPRs bound to their cargo are sorted into endosomes through their interaction with the vesicular sorting machinery including GGA and AP complexes [192, 193]. The acidic pH of endosomes disrupts hydrolase-MPR binding, releasing the hydrolase into the endosomal lumen and eventual delivery to the lysosome [194, 195]. The MPR can then be recycled back to the TGN [196]. Some hydrolases are sorted independently of MPRs, by associating with lysosomal transmembrane proteins like Limp2 [197] or Sortilin [198, 199].

ii. Sorting of LMPs

Lysosomal membrane proteins (LMP) can be sorted directly or indirectly to the lysosome from the TGN. However, unlike luminal hydrolases that utilize M6P as a lysosomal sorting signal, LMPs utilize short linear sorting motifs as their lysosomal sorting signal. These are typically tyrosine based (YXXΦ) or dileucine DXXLL or [DE]XXXL[L/I] motifs [200]. Each of these motifs has been implicated in a variety of intracellular sorting processes, including endocytosis, secretion, TGN to endosome and lysosomal targeting [201]. Table 2 provides examples of a range of proteins containing lysosomal targeting motifs.
Table 2 Lysosomal targeting motifs. [78, 200, 202]

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Recognition by</th>
<th>Lysosomal Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine Based</td>
<td>YXXΦ</td>
<td>μ subunits of AP complexes</td>
<td>LAMP1, LAMP2a, CD63, Cystinosin, Acid phosphatase</td>
</tr>
<tr>
<td>Dileucine-type</td>
<td>[DE]XXXL[LI]</td>
<td>μ and/or β subunits of AP complexes</td>
<td>Limp II, NPC1</td>
</tr>
<tr>
<td></td>
<td>DXXL</td>
<td>VHS domain of GGA Proteins</td>
<td>Sortilin, Mucolipin-1</td>
</tr>
<tr>
<td>PY</td>
<td>L/PPXY</td>
<td>WW domains of Nedd4</td>
<td>LAPTM5</td>
</tr>
</tbody>
</table>

Note: X = any amino acid, Φ = large hydrophobic residue

Tyrosine and dileucine motifs that function as lysosomal targeting motifs are typically localized towards the C-terminal end of proteins and within 6-13 amino acids of a transmembrane domain[203, 204]. The specific composition of the motif and surrounding amino acids affects the ability of the motif to act as a lysosomal sorting signal. For instance, in the case of YXXΦ, having a glycine residue or a residue with a small side chain that precedes the conserved tyrosine residue enhances lysosomal sorting, as was seen with Lamp1 [205]. Similarly, having an acidic residue at the [DE] position of the acidic dileucine motif is particularly conducive to lysosomal sorting of MHC class II molecules and Limp2 [206, 207].

Lysosomal acid phosphatase is a prototypic LMP that utilizes the indirect pathway of lysosomal sorting [208]. It is transported from the TGN to the plasma membrane and endocytosed by its C-terminal YXXΦ motif and to be sorted towards the lysosome [209, 210]. In contrast, Lamp1, Lamp2 and CD63 have all been shown to utilize their C-terminal YXXΦ motif for both direct and indirect lysosomal sorting[205, 211, 212]. It is noteworthy that individual components of lysosomal multi-protein complexes, such as the v-ATPase, are targeted to the lysosome independently of one another [213-215].
Adaptor Proteins (AP) and GGA proteins recognize lysosomal cargo proteins and recruit them into budding vesicles. Individual subunits and/or domains contain specific binding regions for membrane lipids, vesicle coats (e.g. clathrin) and other accessory proteins required for vesicle budding and fission (e.g. ARF proteins)\cite{216}. As listed in Table 2 YXXΦ and [DE]XXX[L/I] motifs are recognized by the μ and μ or β subunits of Adaptor Proteins (APs 1-5). APs are largely responsible for sorting cargo proteins into clathrin-coated vesicles (CCVs). To date, five adaptor complexes (AP1-5) have been characterized. Each AP complex localizes to a distinct subcellular location and mediates post-Golgi trafficking (Figure 1.4). APs are heterotetrameric complexes, consisting of two large subunits (γ, α, δ, ε or ζ and β1-5), a medium sized subunit (μ1-5) and a small subunit (σ1-5) \cite{217}. DXXLL motifs, on the other hand, are recognized by the VHS domains of monomeric GGA (GGA1-3) proteins. GGA1-3 mediate clathrin-dependent sorting of proteins from the TGN to endosomes by binding DXXLL motif containing proteins through their VHS domains \cite{73}. 
Figure 1.4: **Diagram of endosomal trafficking pathways and machinery adapted from** [217]. AP complexes 1-5 are indicated in different colors. AP-1 and AP-2 promote the assembly of clathrin *in vitro*[218, 219] and bridge between clathrin and transmembrane cargo proteins[220]. AP-1 is involved in trafficking between the TGN and tubular endosomes, with evidence supporting bi-directional transport [221]. AP-2 facilitates endocytosis[222]. AP-3 traffics cargo from tubular endosomes to late endosomes and lysosomes[223, 224]. Both AP-4 and AP-5 mediated CCV independent processes [225]. AP-4 [226, 227] traffics proteins from the TGN to endosomes[228]. While AP-5 colocalizes with late endosomal and lysosomal markers, but its specific role in targeting is largely uncharacterized [217]. GGA proteins 1-3 are represented as single entity in pink.
Figure 1.4: Diagram of trafficking pathways and machinery [217].
Table 3 lists examples of LMPs that utilize AP and GGA proteins to sort towards the lysosome. A single motif will interact with a variety of adaptor molecules based on a combination of protein subcellular localization, expression level and cell type. For instance, Lamp1 and Lamp2 contain a similar tyrosine based motif and knockdown of AP-1, 2, 3 and 4 all triggered varying degrees of altered plasma and lysosomal membrane localization [212].

Table 3 AP and GGA Proteins in Endosomal Sorting [189, 228-231]

<table>
<thead>
<tr>
<th>Adaptor Molecule</th>
<th>Direction of Sorting</th>
<th>Examples of Cargo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AP</strong> Hetero-tetramer: Subunits: β, μ, σ and α/γ/δ or ε</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bi-directional transport from Golgi to early Endosome</td>
<td>MPR, gp48/MHC-I, Lamp1, Lamp2</td>
</tr>
<tr>
<td>2</td>
<td>Plasma Membrane Endocytosis</td>
<td>LAP, endolyn, CD1b, MPR</td>
</tr>
<tr>
<td>3</td>
<td>Early endosome to late endosome</td>
<td>Endolyn, CD1b, gp48/MHC-I, Lamp1, Lamp2</td>
</tr>
<tr>
<td>4</td>
<td>Golgi to late endosome</td>
<td>Lamp1, Lamp2, LimpII, CD63</td>
</tr>
<tr>
<td><strong>GGA (1-3)</strong> Monomer 4 Domains: VHS, GAT, Hinge, GAE</td>
<td>Golgi to Lysosome</td>
<td>MPR, Sortilin, Low density lipoprotein receptor-related protein 3</td>
</tr>
</tbody>
</table>

There is increasing evidence that some LMPs require several targeting motifs for lysosomal targeting. For instance LAPTM4a requires two tandemly arranged tyrosine-based signals [232], while Mucolipin-1 (MCLN1) requires two dileucine motifs[233] for proper lysosomal trafficking. Additionally, there is growing evidence that variations of typical targeting motifs, as well as novel unrelated sequences can mediate LMP sorting. For instance, TMEM192 requires 2 dileucine motifs and a c-terminal cysteine residue [155]. CLN3 requires a dileucine motif and an unconventional cluster of acidic amino acids [234, 235]. This is similar to the Ocular Albinism type 1 (OA1) protein that requires an unconventional dileucine-based motif and a novel cytosolic motif for melanosomal targeting [236].
Summary of the various roles of lysosomal membrane proteins. Some lysosomal membrane proteins are involved in the trafficking of newly synthesized hydrolases to the lysosome (LIMP2) and the acidification of the lumen (V-type H+-ATPase complex and Chloride Channel 7 (CLC7)). Other proteins mediate macroautophagy and chaperone-mediated autophagy, by which autophagosomes engulf cytoplasmic components (including organelles and large complexes), fuse with lysosomes and create auto-lysosomes in which degradation occurs. Chaperone-mediated autophagy relies on the selective recruitment and transport of cytosolic proteins across the lysosomal membrane via LAMPs for degradation. Lysosomal exocytosis and plasma membrane repair are calcium- and synaptotagmin 7 (SYT7)-dependent processes that play a role in pathogen entry, autoimmunity and neurite outgrowth. Lysosomal cell death is triggered through the release of luminal cathepsins into the cytosol. Cholesterol homeostasis is regulated by NPC1 (Niemann-Pick C1 protein). Antigen presentation via MHC class II depends on lysosomal proteolytic ability. Finally, exosome release appears to play a role in adaptive immune responses.
Figure 1.5: Major functions of Lysosomal Membrane Proteins (LMPs) [183].

4 Functions of Lysosomal Membrane Proteins

Recent advances in proteomic analysis have identified over 200 lysosomal integral and transmembrane proteins [237, 238]. To date lysosomal membrane proteins (LMPs) have been implicated in a variety of processes including vesicular movement and fusion events, transport, acidification and maintaining lysosomal stability and integrity (Figure 1.5) [239]. However, despite the enormous success in identification of novel LMPs, their physiological functions and sorting determinants remain largely uncharacterized. Elucidating these LMP features will expand our understanding of the lysosomes role in health and disease.

4.1 Functions of Lamps and Limps

The most well characterized lysosomal membrane proteins are also the most abundantly expressed at the lysosome: Lysosome-associated membrane protein 1 (Lamp1) and 2 (Lamp2), Lysosomal integral membrane protein-2 (Limp2) and CD63 (Limp1) [240]. Lamp1 and Lamp2 alone are thought to constitute 50% of all the proteins residing in the lysosomal membrane [241].

Their heavily glycosylated luminal domains form a protective glycocalyx [242] that separates the proteolytic contents of the lysosomal lumen from the rest of the cell [243]. Since their initial characterization the role of these proteins has expanded beyond that of maintaining lysosomal membrane integrity.

Lamp2 has been implicated in autophagosome maturation [244-246]. Lamp2a has been shown to mediate the selective uptake and degradation of cytosolic proteins by chaperone-mediated autophagy [247, 248]. Double Lamp1/Lamp2 KO mice are embryonic lethal and embryonic fibroblasts revealed defects in autophagosome maturation [249-251].

Limp2 implicated in lysosome/endosome biogenesis, whereby its overexpression results in the accumulation of swollen endosomes and lysosomes containing cholesterol [252, 253]. Interestingly, Limp2 was recently shown to be the responsible for the M6PR-independent transport of β-glucocerebrosidase (β-GC) to the lysosome [197]. Limp2 associates with β-GC early in the ER (i.e. early in the secretory pathway), and the complex is trafficked from the TGN to the lysosomes, where acidity leads to the dissociation of the complex. Limp2 binds to β-GC via a coiled-coil domain within its luminal domain [254]. Interestingly Limp2 was also shown to
play a role in the degradation of Listeria, by regulating trafficking and fusion events between late endosomes and lysosomes[255].

CD63 (or Limp1) is a tetraspanin protein [256]. Tetraspanins are typically expressed at the cell surface, where they play roles in cell morphology, motility, invasion, fusion and signaling[257]. However, unlike most tetraspanins, CD63 is enriched at the late endosome/lysosomal membrane[258]. CD63 KO mice have normal lysosomal function, however CD63 appears to play a role regulatory activity in platelet adhesion and in kidney physiology. Furthermore, Kidney cells showed abnormal intracellular lamellar inclusions [259]. CD63 has also been implicated in regulating the trafficking of proteins, however the detailed mechanisms through which it achieves is less clear.

4.2 Lysosomal Membrane Proteins (LMP) in Disease

A variety of current disorders that have been linked to defects in LMPs are listed in Table 4 [260]. These disorders largely arise from mutations in the LMP itself, which affect protein function or preclude LMP binding to lysosomal sorting machinery. The majority of disorders associated with LMP dysfunction or altered targeting give rise to lysosomal storage disorders (LSDs). LSDs result in disruption of lysosomal function or biogenesis and lead to the accumulation of undegraded substrates in endosomes and lysosomes. Eventually this build-up compromises cellular function, particularly in cells in which protein turn-over is normally elevated [261].

Some characteristic changes in the lysosomal compartment observed during LSDs have been observed in other pathologies as well. For instance, swollen lysosomes are also linked to changes in lysosomal composition (Lamp1/Lamp2) and positioning during malignant transformation [249, 262, 263]. Enlarged lysosomes are prone to lysosome-mediated cell death [264], which is characterized by an increase in membrane permeability that releases luminal cathepsins into the cytoplasm, which in turn trigger cell death [265]. Changes in the function and targeting of LMPs can therefore trigger negative downstream effects and contribute to disease pathology.

Elucidating the function and sorting determinants of yet uncharacterized LMPs will allow us to assign them roles in critical lysosome mediated pathways. This in turn will expand our understanding of the lysosome’s role in health and disease.
Table 4 Diseases of altered LMP function/sorting [260].

<table>
<thead>
<tr>
<th>LMP</th>
<th>LMP Role</th>
<th>Disease associated with LMP Mutation</th>
<th>Typical Disease Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLN3</td>
<td>unknown function (metabolite transport suggested)</td>
<td>Juvenile neuronal ceroid lipofuscinosis</td>
<td>Neurodegeneration, neuronal lipofuscin accumulations</td>
</tr>
<tr>
<td>CLN7</td>
<td>Metabolite transport</td>
<td>Late infantile neuronal ceroid lipofuscinosis</td>
<td>Neurodegeneration, neuronal lipofuscin accumulations</td>
</tr>
<tr>
<td>CLC7; OSTM1</td>
<td>Proton and anion (Cl–) transporter</td>
<td>Malignant infantile osteopetrosis</td>
<td>Osteopetrosis, bone fractures and growth abnormalities, anemia, bleeding problems</td>
</tr>
<tr>
<td>Cystinosin</td>
<td>H+ and L-cysteine symport</td>
<td>Cystinosis</td>
<td>Kidney failure, acidosis, photophobia, blindness, diabetes</td>
</tr>
<tr>
<td>HGSNAT</td>
<td>Lysosomal acetyl import and transfer to heparan sulfate</td>
<td>Mucopolysaccharidosis type IIIC</td>
<td>Neurological symptoms, progressive dementia, aggressive behavior, hyperactivity, seizures, deafness, vision loss</td>
</tr>
<tr>
<td>LAMP-2</td>
<td>Autophagy, lysosomal fusion and transport</td>
<td>Danon disease</td>
<td>Muscle atrophy, cardiomyopathy and mental retardation</td>
</tr>
<tr>
<td>LIMP-2/SCARB2</td>
<td>Chaperone and transport of β-glucocerebrosidase</td>
<td>Action myoclonus-renal failure syndrome</td>
<td>Epilepsy, glomerulosclerosis and kidney failure</td>
</tr>
<tr>
<td>LMBRD1</td>
<td>Lysosomal export of cobalamin</td>
<td>Cobalamin F-type disease</td>
<td>Developmental delay, stomatitis, glossitis, seizures and methylmalonic aciduria</td>
</tr>
<tr>
<td>NPC1</td>
<td>Lipid, cholesterol and Ca2+ transport</td>
<td>Niemann-Pick type C</td>
<td>Hepatosplenomegaly, thrombocytopenia, ataxia, dysarthria, dysphagia</td>
</tr>
<tr>
<td>MCOLN1</td>
<td>Cation channel</td>
<td>Mucolipidosis type IV</td>
<td>Psychomotor retardation, corneal opacity, retinal degeneration, ophthalmological abnormalities</td>
</tr>
<tr>
<td>Sialin</td>
<td>Lysosomal export of sialic acids and acidic hexoses; asparagine and glutamine import</td>
<td>Salla disease</td>
<td>Nystagmus, hypotonia, cognitive impairment</td>
</tr>
</tbody>
</table>
Figure 1.6: **LAPTM Family Architecture.** A schematic representation of LAPTM family member architecture. The putative number of transmembrane domains is indicated by purple rectangles: five for LAPTM5, four for LAPTM4a and LAPTM4b. Dileucine, tyrosine-based and PY motifs are indicated in each LAPTM member. Only LAPTM5 contains an ubiquitin interacting motif (UIM). LAPTM4b exists as a 35kDa and 24kDa isoform (arising from leaky scanning), which differ only by an N-terminal stretch of 91 amino acids. # aa = number of amino acids encoded by the wildtype protein.
Figure 1.6. LAPTM Family Architecture.

LAPTM5
# of aa 262

LAPTM4A
# of aa 233

LAPTM4B
35kDa 317

35kDa

24kDa 226

Putative TM Domain
YXXΦ motif
L/PPXY motif
Dileucine or VL motif
4.3 LAPTM Proteins

The LMPs of greatest interest to my thesis belong to the Lysosome-Associated Protein TransMembrane (LAPTM) family. This family comprises three members that were named according to their putative number of transmembrane domains: LAPTM4a, LAPTM4b and LAPTM5 (Figure 1.6). LAPTMs are conserved in vertebrates, insects and nematodes [232]. LAPTM4a and LAPTM4b are ubiquitously expressed, while LAPTM5 is expressed in immune cells [266-269]. LAPTM4a and LAPTM4b are most closely related with ~43% amino acid sequence conservation; while LAPTM4a and LAPTM4b are more distantly related to LAPTM5 (~25% and 23%). Sequence conservation is particularly strong in the putative α-helical transmembrane segments and c-termini, which could support conserved function and/or sorting properties [232, 269].

4.3.1 LAPTM Sorting

All LAPTM family members contain a variety of putative lysosomal targeting motifs in their C-termini (Figure 1.6). When I began my PhD only LAPTM5 and LAPTM4a lysosomal sorting had been previously investigated. Our lab demonstrated that LAPTM5 was sorted to the lysosome via the TGN. LAPTM5 lysosomal sorting is dependent on the ability of its PY motifs to bind Nedd4 [78]. However, although LAPTM5 is ubiquitinated, ubiquitination is not required for lysosomal targeting. Instead, lysosomal targeting depends on LAPTM5’s ability to bind via its UIM to ubiquitinated GGA3. LAPTM4a was shown to require two tandemly arranged tyrosine-based motifs to sort to the lysosome [232], however the role of PY motifs in its sorting had not been examined. None of LAPTM4b lysosomal sorting determinants were known, nor had its lysosomal localization been confirmed.

4.3.2 LAPTM Function

LAPTM5 was discovered in a screen identifying genes that are preferentially expressed in hematopoietic tissues [266]. LAPTM5 is a negative regulator of cell surface expression of the B and T cell receptor[270, 271]. It is a positive regulator of pro-inflammatory signaling pathways in macrophages [272]. LAPTM5 expression is down regulated through DNA methylation in multiple myeloma [273] and neuroblastoma [274].
LAPTM4a was identified in a yeast complementation screen that searched for mammalian proteins capable of overcoming a thymidine transport deficiency in yeast [267]. In mammals, LAPTM4a is ubiquitously expressed, and localizes to the late endosome and lysosomal compartment [267, 275]. Initial studies demonstrated that LAPTM4a overexpression in drug-sensitive yeast altered their sensitivity to a variety of compounds including nucleosides and nucleobase analogs, antibiotics, anthracyclines, ionophores and steroid hormones, much like a multidrug transporter would [276]. Additional studies have also implicated LAPTM4a in transport processes. For instance, LAPTM4a enhances glutathione and endocytic marker uptake in Yeast [277] and in mammalian systems LAPTM4a was shown to interact with the human organic cation transporter 2 (hOCT2) [278]. In line with its potential role as a transporter, LAPTM4a was down-regulated in myeloid leukemia patients who responded to chemotherapy [279]. LAPTM4a mRNA is differentially expressed under a variety of conditions. Up-regulation is observed in the rat incisor enamel organ [280], in response to nerve growth factor exposure in rat PC-12 cells [281] and during follicular development [282-284]. Finally, LAPTM4a was identified as a potential candidate gene for human essential tremor [285].

LAPTM4b was originally identified as a novel gene up-regulated in human hepatocellular carcinoma (HCC) [286]. Subcellular fractionation and immunohistochemical analysis determined LAPTM4b subcellular localization to be intracellular and membrane bound [268, 287]. LAPTM4b mRNA is highly expressed in normal adult heart and skeletal muscle tissue, as well as the testis, ovary, prostate, placenta, kidney, lung, liver, pancreas, spleen, colon and small intestine [268, 269]. Leaky scanning of the gene transcript results in two primary LAPTM4b isoforms: 24kDa (short) and 35kDa (long) [268]. The long and short isoform differ only by a stretch of 91 amino acids at the N-terminus (Figure 1.6 and Figure 2.1).

Following the original cloning, it was demonstrated that both LAPTM4b isoforms were overexpressed ~3.5 fold in HCC liver tissues relative to normal liver tissue [287, 288]. Since then, LAPTM4b mRNA was shown to be up-regulated in HCC derived cell lines [286] and tissues [268, 286], pituitary adenomas [289], solid lung tumors [269], colon carcinoma [269] and breast cancer tissues [290]. The RNA probes used throughout these studies do not distinguish between LAPTM4b isoforms.
Although both 24kDa and 35kDa LAPTM4b are expressed in a variety of HCC derived cell lines and tissues[287, 288], the vast majority of studies have focused on the 35kDa isoform. Table 5 summarizes the cancer cell lines and tissues in which 35kDa LAPTM4b protein was shown to be over-expressed.

Table 5: Detection of 35kDa LAPTM4b overexpression by Immunohistochemistry

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Cell Line</th>
<th>Tissue Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallbladder Carcinoma</td>
<td>GBC-SD[296]</td>
<td>Human [297]</td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>PC3M, PC[288]</td>
<td>Human [298]</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>BE1, LH7[288]</td>
<td>Human [291]</td>
</tr>
<tr>
<td>Breast Carcinoma</td>
<td>BT549, MDA-MB-231[299]</td>
<td>Human [291, 299, 300]</td>
</tr>
<tr>
<td>Ovarian Carcinoma</td>
<td></td>
<td>Human [301-303]</td>
</tr>
<tr>
<td>Cervical Carcinoma</td>
<td></td>
<td>Human [304]</td>
</tr>
<tr>
<td>Endometrial Carcinoma</td>
<td></td>
<td>Human [305]</td>
</tr>
<tr>
<td>Metastatic Ovarian Tumor</td>
<td></td>
<td>Human [306]</td>
</tr>
<tr>
<td>Pancreatic Cancer Tissue</td>
<td></td>
<td>Human [307]</td>
</tr>
<tr>
<td>Colorectal Carcinoma</td>
<td></td>
<td>Human [291, 308]</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td></td>
<td>Human [291]</td>
</tr>
<tr>
<td>Melanoma</td>
<td>WM451, WM983A[288]</td>
<td></td>
</tr>
</tbody>
</table>

In line with the overexpression of the 35kDa protein in a variety of cancers, 35kDa LAPTM4b is an independent prognostic marker for poor overall and disease-free survival of breast cancer [300], gall bladder carcinoma [297], ovarian cancer [301], extrahepatic cholangiocarcinoma [295] and hepatocellular carcinoma [293, 309]. In most cases, LAPTM4b expression level inversely correlates with tumor differentiation [268, 291]. Furthermore, inoculation of nude mice with cells overexpressing 35 kDa LAPTM4b showed increased tumorigenesis [57, 309-311].

The poor prognosis has been linked to the ability of 35kDa LAPTM4b to enhance microvilli formation, serum-independent growth, migration, invasive potential, growth rate, cell cycle progression and expression of cell cycle regulatory factors such as cyclins (D1 and E), transcription factors (c-myc, c-jun, c-fos), as well as decrease expression of cell cycle inhibitors p27 (cyclinD1/CDK4 inhibitor) and p16 (cyclinE/CDK2 inhibitor) [57, 309, 312, 313].
Additionally, overexpression of 35kDa LAPTM4b has also been linked to increased autophagy, which can help promote cell survival [311, 314]. 35kDa LAPTM4b has a unique 91 amino acid N-terminus that contains a PPRP motif [268]. This motif is essential in promoting cell proliferation and metastatic potential of HCC cells [312] and mediates binding to the p85α regulatory subunit of PI3K [315].

What role the 24kDa LAPTM4b, which lacks the key PPRP motif, plays in any of the above processes is unknown.

Like LAPTM4a, the 35kDa LAPTM4b has been implicated in small molecule transport. Breast cancer patients overexpressing LAPTM4b develop de novo resistance to anthracyclines[299] and breast cancer cells overexpressing LAPTM4b showed reduced anthracycline sensitivity. Others proposed that the 35kDa LAPTM4b associates with the multidrug transporter p-glycoprotein (MDR1) thereby facilitating drug efflux and increased cell resistance to a chemotherapeutic drugs[315]. The role 24kDa LAPTM4b plays in these processes is unknown.

5 Regulation of mTORC1 by amino acids

In order to grow and/or proliferate, cells need to sense the availability of nutrients and energy. Contrastingly, cells need to be able to suppress growth during unfavorable conditions, such as during starvation and low energy [316]. Mammalian TOR complex 1 (mTORC) and mTORC2 are at the heart of this balance. Both mTORC1 and mTORC2 regulate metabolism through growth factor receptor signaling. While mTORC2 largely promotes cytoskeletal rearrangements and pro-survival signaling, mTORC1 promotes protein synthesis, cell cycle progression and cell growth, while inhibiting autophagy [180]. Furthermore, unlike mTORC2, mTORC1 has been shown to be responsive to a much larger number of upstream signals: growth factors, stress, energy status, oxygen and amino acids[180]. As such, mTORC1 has become known as the master regulator of cell growth. The latter portion of my PhD demonstrates a role for LAPTM4b in amino acid activation of mTORC1. Here I summarize the role of amino acid sensing in mTORC1 activation at the lysosome.
5.1 mTORC1, a Master Regulator of Cell Growth

Rapamycin is an antifungal, anticancer and immunosuppressive compound[317]. Mutant TOR1 and TOR2 in yeast showed rapamycin resistance[318, 319], and mTOR was later isolated as the mammalian target of rapamycin [320-322]. mTOR is an atypical serine/threonine kinase of the phosphoinositide 3-kinase (PI3K)-related kinase family. The catalytic mTOR subunit forms complexes with two distinct subsets of proteins to form mTORC1 and mTORC2 (Table 6). mTORC1 is composed of six components, while mTORC2 is composed of seven components. These two distinct TOR complexes were shown to be differentially inhibited by rapamycin and to regulate different pathways in both yeast and mammals [323-325]. Figure 1.7 summarizes the various pathways in which mTORC1 and mTORC2 have been implicated, including their upstream regulators and their downstream effectors. The focus of my work has been on amino acid activation of mTORC1. Activation by mTORC1 (by other signals) and mTORC2 has been reviewed elsewhere (see[180, 316]).

Table 6 mTORC1 and mTORC2 complexes: components and function. Adapted from [180].

<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
<th>mTORC1</th>
<th>mTORC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR</td>
<td>Serine/Threonine Kinase</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Deptor</td>
<td>mTOR inhibitor [326]</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>mLST8</td>
<td>Positive regulator of mTORC1 activity[327]</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Tti1/Tel2</td>
<td>Scaffold that regulates assembly and stability of mTORC1/2[328]</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Raptor</td>
<td>Scaffolding protein. Assembly, localization and substrate binding of mTORC1 [329, 330]</td>
<td>✔</td>
<td>x</td>
</tr>
<tr>
<td>Pras40</td>
<td>mTORC1 inhibitor[331, 332]</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>Rictor</td>
<td>Scaffolding protein. Assembly, localization and substrate binding of mTORC2[323, 325]</td>
<td>x</td>
<td>✔</td>
</tr>
<tr>
<td>mSin1</td>
<td>Scaffold protein. Assembly, localization and interaction with SGK1[333, 334]</td>
<td>x</td>
<td>✔</td>
</tr>
<tr>
<td>Protor 1/2</td>
<td>Increases mTORC2-mediated activation of SGK1[332, 335, 336]</td>
<td>x</td>
<td>✔</td>
</tr>
</tbody>
</table>

✔ = present in complex; x=not present; Deptor = DEP domain containing mTOR-interacting protein; mLST8 = mammalian lethal with sec-13 protein 8; Raptor = regulatory-associated protein of mammalian target of rapamycin; Pras40 = proline-rich Akt substrate 40kDa; Rictor = Rapamycin-insensitive companion of mTOR; mSin1 = mammalian stress-activated map kinase-interacting protein 1; Protor 1/2 = protein observed with rictor 1 and 2.
Figure 1.7: **The mTOR signaling pathway from** [180]. This diagram illustrates the complexity of mTOR signaling. Tumor suppressors are indicated in green, oncogenes in red. RTK = Receptor Tyrosine Kinase.

mTORC2 (orange) is largely activated via the growth factor signaling pathway. mTORC2 regulates: actin cytoskeletal dynamics and organization and cell survival/metabolism through FoxO gene transcription.

mTORC1 (blue) is activate by growth factors, amino acids, stress, energy status and oxygen. It promotes protein synthesis (including cap-dependent translation and elongation, ribosome/mRNA biogenesis, rRNA, tRNA transcription, 5’TOP mRNA translation) through the phosphorylation of its downstream effectors S6K1 and 4E-BP1. It promotes lipid synthesis through promoting SREBP1/2 mediated gene transcription, as well as enhances energy metabolism through the upregulation of glycolytic and oxidative genes that trigger glucose metabolism and mitochondrial oxidative metabolism. On the other hand, mTORC1 inhibits autophagy by blocking autophagosome formation and inhibits lysosome biogenesis through regulation of TFEB subcellular localization.

Figure 1.7: The mTOR Signaling Pathway from [180].
5.2 mTORC1 function

When activated, mTORC1 phosphorylates a wide variety of substrates. Collectively mTORC1 activation promotes protein synthesis, lipogenesis, and energy metabolism, but inhibits autophagy and lysosome biogenesis. The best characterized substrates of mTORC1 include the Ribosomal S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding protein (4EBP) [337]. These two proteins are key players of initiating protein synthesis, through enhancing transcription and translation. Specifically, mTORC1 activation leads to the transcription of genes of translation, cell proliferation, invasion and metabolism [338, 339]. During nutritional sufficiency mTORC1 inhibits autophagy and lysosome biogenesis by direct phosphorylation of the unc-51-like kinase 1 (ULK1) and autophagy-related protein 13 (ATG13) which are subunits of the autophagy initiating ULK1 complex, inhibiting autophagosome formation [340-343]. Also, mTORC1 phosphorylates the microphthalmia-transcription factor E (MiT/TFE) member EB (TFEB) which regulates the transcription of the coordinated lysosomal expression and regulation (CLEAR) network. Overexpression of TFEB increases lysosome biogenesis and degradation of complex molecules [344, 345]. mTORC1 phosphorylation of TFEB prevents its nuclear translocation and inhibits CLEAR network transcription [346, 347]. Finally, mTOR regulates its own activity by degrading Deptor through ubiquitination by the E3 ligase SCF(βTrCP) [348-350].

5.3 Upstream regulation of mTORC1 activation

A glance at Figure 1.7 demonstrates that most factors that regulate mTORC1 activation (mitogen and growth factor signaling, energy levels, oxygen availability and genotoxic stress) converge upon the TSC-complex (tuberous sclerosis 1, TSC2 and TBC1D7). The TSC is an up-stream inhibitor of mTORC1 [351]. TSC1 stabilizes TSC2[352, 353] and TBC1D7 promotes the TSC1 and TSC2 interaction as well as the GTPase-activating protein (GAP) activity of TSC2 towards the small GTPase Rheb (Ras homologue enriched in brain) [354]. As such, TSC2 mediates the critical inhibitory role of the TSC [355-360]. Rheb is the immediate upstream activator of mTORC1. Rheb-GTP directly binds mTORC1 and stimulates its kinase activity[357-359, 361]. Rheb is targeted to the lysosomal/endosomal membranes by a c-terminal CAAX motif (Cysteine–Aliphatic-Aliphatic-terminal amino acid) [181, 362-364]. Rheb’s lysosomal localization is a key feature of amino acid regulated activation of mTORC1.
Interestingly, mTORC1 activation remains sensitive to the availability of amino acids in mouse embryonic fibroblasts isolated from TSC2−/− mice [365, 366]. Amino acid mediated mTORC1 activation is regulated by the lysosomal v-ATPase, the Ragulator complex and Rag GTPases at the lysosome, downstream of TSC, but upstream of mTORC1 activation. These players were identified by biochemical and genetic screens[362, 367] and importantly, immune-isolated lysosomes showed that lysosomes themselves contain all the machinery required for sensing amino acids and activating Rag GTPases [182].

It was recently demonstrated that mTORC1 deactivation is an acute, active process in response to upstream stimulation, as opposed to a dampening due to signal dissipation[368]. Turning off mTORC1 in the absence of amino acids, unlike its activation by amino acids, involves the TSC complex. In the absence of amino acids, Rags cannot recruit mTORC1 because they exist in an inactive conformation (RagA/B\textsuperscript{GDP}-RagC/D\textsuperscript{GTP}). Instead, in this inactive conformation, the Rag complex recruits TSC to the lysosome, which in turn inhibits Rheb and mTORC1 signaling [369]. Similarly, in the absence of growth factors, TSC localizes to lysosomes inhibiting Rheb and mTORC1. However, during Growth Factor stimulation, Akt phosphorylates TSC2 causing its dissociation from the lysosome and precluding TSC2 from inhibiting Rheb [370].

5.4 Amino acids activate mTORC1

Amino acids are required to stimulate protein synthesis in cells [371] and can stimulate mTORC1 activation [372, 373]. It is not clear whether all amino acids, one particular amino acid or amino acid byproducts are being sensed for activation[351]. What is known is that certain amino acids are better mTORC1 activators than others. For instance, leucine and arginine are critical for mTORC1 activation, but not sufficient. If cells are deprived of the remaining 18 amino acids, neither amino acid is sufficient for mTORC1 activation [372]. Similarly, glutamine and other large neutral amino acids like leucine are required for full activation of mTORC1 and the absence of glutamine can reduce mTORC1 signaling[374, 375]. Finally, high levels of leucine have been shown to repress proteosomal degradation, as well as enhance mTORC1 signaling to promote growth[376, 377]. Considering leucine’s strong ability to activate mTORC1, it is often used as the proteotypic activator of mTORC1 for experiments.

The amino acids that activate mTORC1 can be both intracellular (autophagic/degradative pathway) and extracellular in origin. How each of these pools activates mTORC1 is not well
defined. However, in either case it is clear that amino acids cannot readily diffuse across the proteolipid barrier of the plasma membrane or intracellular compartments. Amino acids must be transported by transporters of the solute carrier (SLC) gene superfamily (SLC1, SLC6, SLC7, SLC36, SLC38 and SLC43)[378]. Amino acid transporters are classified based on the types of amino acids they transport. mTORC1 signaling is activated by system L and A transporters acting together as tertiary transport systems. System L transporters like LAT1-4F2hc (SLC7A5/SLC3A2) import mostly branched-chain amino acids (eg. leucine) in exchange for other intracellular amino acids (eg. glutamine) (see Figure 4.1). Intracellular amino acids (eg. glutamine) are provided by System A transporters like SNAT2(SLC38A2) that are coupled to the electrochemical gradient established in an ATP-dependent fashion across the plasma membrane by Na+/K+ pumps[379]. In fact, tertiary transport was recently shown to be the rate-limiting step of mTORC1 activation. It was shown that L-glutamine import by SLC1A5 (ASC family ASCT2) is coupled to glutamine efflux by SLC7A5/SLC3A2 regulating mTORC1 activation and autophagy, as well as coordinating cell growth and proliferation [380].

Interestingly, there is also a role for intracellular amino acids in mTORC1 activation. Cells starved of amino acids that are treated with cycloheximide (which blocks protein synthesis), activate mTORC1 signaling through the accumulation of intracellular pools of amino acids [381-383]. This supported the idea that sensing amino acid availability could occur from within the cell, independent of extracellular cues [362]. Regardless of whether amino acids were extracellular or intracellular in origin, their lysosomal accumulation triggers mTORC1 activation and this process is outlined below[182, 362].

5.5 An inside-out mechanism of lysosomal mTORC1 activation in response to amino acids

Figure 1.8 illustrates the current model in which amino acids in the lysosomal lumen are sensed and activate mTORC1 by an inside-out mechanism[182]. Amino acids in the lysosomal lumen are sensed by an unknown mechanism and signal to the lysosomal v-ATPase. The v-ATPase is linked to Ragulator, which is the scaffolding protein that links Rag GTPases to the lysosomal surface and activates them through GEF activity[181]. The v-ATPase/Ragulator interaction is amino-acid sensitive. The presence of luminal amino acids triggers a structural rearrangement of the v-ATPase-Ragulator-Rag GTPase complex. Ragulators’s GEF activity towards Rag GTPases
is enhanced. Rag GTPases function as obligate heterodimers: RagA or RagB will dimerize with RagC or RagD[367, 384-386]. They recruit mTORC1 to the lysosomal surface based on their “active” nucleotide loading state: RagA/B-GTP [362]. Thus, Ragulator promotes the formation of an active Rag GTPase conformation on the lysosomal surface (Rag(A/B)-GTP-Rag(C/D)-GDP)[387]. This recruits mTORC1 to the lysosome, leading to mTORC1 activation by the small GTPase Rheb. In fact, mTORC1 is constitutively localized at the lysosome in cells or animals that constitutively express GTP-locked-RagA/B [362, 367, 388]. The role of ATP hydrolysis of the v-ATPase and amino-acid sensitive interactions with Ragulator were shown to be critical in this inside-out signaling of amino acids to mTORC1. The v-ATPase’s ability to establish a proton gradient, however, was dispensable (reviewed in [351, 389]).
Figure 1.8: **mTORC1 amino acid sensing pathway from [351]**.

(A) Under low amino acid conditions Ragulator is found in an inhibitory state with the v-ATPase, and Gator1 exerts its GAP activity towards RagA, keeping this GTPase in the inactive GDP-bound state that is not sufficient to recruit mTORC1. Insulin signaling inhibits TSC translocation to the lysosomal surface where it functions as a GAP for Rheb, inactivating this G protein.

(B) Upon amino acid stimulation, Gator1 may be inhibited by Gator2, and Ragulator and v-ATPase undergo a conformational change, unleashing the GEF activity of Ragulator towards RagA, while the folliculin complex promotes RagC GTP hydrolysis. The active Rag heterodimer (RagA-GTP/RagC-GDP) recruits mTORC1 to the lysosomal surface where it interacts with and is activated by Rheb. The large “?” indicates the fact that the mechanism by which EAA such as Leu enter the lysosome to activate mTORC1 is unknown.

A) Low Amino Acid Levels

B) High Amino Acid Levels

Figure 1.8: mTORC1 amino acid sensing pathway from [351].
While this model is widely accepted, it does not reconcile the observation that radioactively labeled amino acids accumulate in the lysosome within 10 minutes of their addition to cells, but mTORC1 can be activated after only 3 minutes [182, 362, 389]. Furthermore, this model does not explain how Leu enters the lysosome in the first place to activate mTORC1. How Leu enters the lysosome to activate mTORC1 is at the focus of my thesis Chapter 4.

It should be noted that a variety of cytosolic amino acid sensors have been identified and implicated in amino acid sensing upstream of mTORC1. These include leucyl-tRNA synthetase (LRS), glutamate dehydrogenase (GDH), and unbranched chain amino acid receptors 1 and 2 (UBR1-2), inositol polyphosphate multikinase (IMPK), mitogen-activating protein kinase kinase kinase-3 (MAP4K3), vacuolar sorting protein (VPS34) and Ras-like protein A (RalA) [378].

5.6 Amino acids trigger subcellular re-localization of mTORC1

Amino acids trigger colocalization of mTORC1 with its activator Rheb [181, 362, 389]. In the absence of amino acids, mTORC1 resides diffuse in the cytoplasm. Upon addition of amino acids, mTORC1 translocates to the lysosomal surface mediated by Raptor, where it is thought to interact with the GTPase Rheb [181, 362]. Interestingly, disruption of the lysosomal membrane with detergents or ionophores inhibits amino acid dependent recruitment of mTORC1 to the lysosome[182]. Controversially the lysosomal amino acid transporter PAT1 (proton-assisted amino acid transporter) has been reported to export amino acids from the lysosomal lumen [390] whereby its overexpression has been proposed to drain the lysosomal lumen of amino acids and thereby inactivate mTORC1 signaling [182]. Others have suggested that PAT1 overexpression activates mTORC1, while its knock-down inhibits it. They proposed PAT1’s ability to regulate mTORC1 activation was not dependent on active amino acid transport, but instead on its ability to relay an amino acid mediated signal [391].

5.7 Regulators of amino acid mTORC1 signaling

Given the central role of Rag GTPases in recruitment of mTORC1 to the lysosome, it is not surprising they are the targets of a variety of modulators including tumor suppressor folliculin (FLNC), the GATOR1 & 2 complexes and SH3B4 (SH3 (Src homology 3 domain)-binding protein 4). These modulators all target the GTP/GDP states of Rag GTPases to regulate amino
acid dependent mTORC1 activation. For instance, FLNC and FN1PII promote mTORC1 activation during conditions of amino acid sufficiency through their GAP activity for RagC and RagD [392]. On the other hand the GATOR subcomplexes GATOR1 (subunits: DEPDC5, Nprl2, Nprl3) and 2 (subunits: Mios, WDR24, WDR59, Seh1L, and Sec13) are negative regulators of Rag GTPases. GATOR1 suppresses mTORC1 activation through its GAP activity for RagA/B, while GATOR2 negatively regulates GATOR1[393]. Similarly, SH3BP4 acts as a negative modulator of amino acid mediated mTORC1 activation through increasing RagB GTP hydrolysis and preventing RagB GDP dissociation. [394]. Finally, the cytoplasmic tRNA charging enzyme LRS (Leucyl tRNA-synthetase) has been shown to detect cytoplasmic Leucine levels, directly bind and act as a GAP to Rag GTPases [395, 396].

5.8 Amino acid activation of mTORC1 in Disease

Interestingly, a variety of disorders have been linked to hyperactive or dysfunctional mTORC1 activation. Lack of mTORC1 signaling can cause muscle atrophy/wasting and is linked to ageing[397]. On the other hand, upregulated mTORC1 signaling has been observed in cancer and diabetes, typically within the context of growth factor signaling and through inactivating mutations of negative mTOR regulators such as TSC1-TSC2 and PTEN and deregulation of PI3K and Akt[316]. Interestingly, players in amino acid regulated mTORC1 activation are being identified as mutated in cancer. GATOR1 inactivating mutations lead to hyperactive mTORC1 signaling and render cells insensitive to amino acid starvation[393]. Similarly, it is already known that some amino acid transporters are upregulated in cancer [398, 399]. Thus, identification of additional players that regulate mTORC1 activation will provide an opportunity to develop novel therapies for disorders arising from deregulated mTORC1 signaling.
6 Project Goals

Prior to the start of my PhD, the role of Nedd4 in LAPTM4 sorting had not been investigated and the physiological function of LAPTM4b (24kDa) was largely uncharacterized. As such the goals of my PhD were threefold:

i. Characterize the potential role of PY motifs and Nedd4 in LAPTM4 lysosomal sorting, given the role of PY motifs and Nedd4 in LAPTM5 targeting,

ii. Determine whether PY motifs and Nedd4 proteins are conserved lysosomal sorting determinants of other PY motif containing lysosomal membrane proteins beyond the LAPTM family.

iii. Characterize the physiological role of 24kDa-LAPTM4b through identification and validation of novel 24kDa-LAPTM4b binding partners through affinity purification and mass spectroscopic analysis.
Chapter 2
A role for the Ubiquitin Ligase Nedd4 in the intracellular targeting of LAPTM4 proteins


I designed and performed all experiments presented in this chapter.
1 Abstract

The lysosome associated protein transmembrane (LAPTM) family is comprised of three members: LAPTM5, LAPTM4a and LAPTM4b, with the latter previously shown to be overexpressed in numerous cancers. While our lab had previously demonstrated the requirement of the E3 ubiquitin ligase Nedd4 for LAPTM5 sorting to lysosomes, the regulation of sorting of LAPTM4 proteins remained less clear. Here it is shown that LAPTM4a and LAPTM4b are localized to the lysosome, but unique to LAPTM4b, a fraction of it is present at the plasma membrane and its overexpression induces the formation of actin-based membrane protrusions. It is demonstrated that LAPTM4s, like LAPTM5, are able to co-immunoprecipitate with the E3 ubiquitin ligase Nedd4, an interaction that is dependent on LAPTM4 PY motifs and plays a role in membrane sorting. Accordingly, in Nedd4 knockout mouse embryonic fibroblasts (MEFs), LAPTM4a and LAPTM4b show reduced lysosomal localization. Moreover, lack of PY motifs leads to enhanced missorting of LAPTM4b to the plasma membrane instead of the lysosome. Together these results suggest that while some requisites of LAPTM5 lysosomal sorting are conserved among LAPTM4 proteins, LAPTM4a and LAPTM4b have also developed distinct sorting requirements.

2 Introduction

The Lysosome Associated Protein Transmembrane (LAPTM) family of proteins include LAPTM4a, LAPTM4b (with two major isoforms: 35kDa and 24kDa [268]) and LAPTM5. LAPTM4a and b are closely related with ~43% amino acid sequence conservation, and more distantly related to LAPTM5 (~23-25% conservation) (Figure 2.1B). Sequence homology is strong in the putative α-helical transmembrane segments and C-termini and suggests conserved function and/or sorting of these proteins [232, 269]. LAPTM4a and LAPTM4b are ubiquitously expressed, while LAPTM5 is expressed in immune cells [266-269, 275, 276]. LAPTM5 has been implicated in regulation of B cell and T cell receptor surface expression [270, 271], is downregulated in multiple myeloma [273] and is a positive regulator of pro-inflammatory signaling pathways in macrophages [272]. Interestingly, both LAPTM4 proteins play a role in multidrug resistance. When overexpressed in yeast, LAPTM4a confers multidrug resistance as a small molecule transporter capable of altering yeast sensitivity to small molecules, such as nucleosides and nucleobase analogs, antibiotics, anthracyclines, ionophores and steroid
hormones [276]. LAPTM4b may mediate multidrug resistance by interacting with the multidrug resistance protein MDR1 [315]. Moreover, LAPTM4b is overexpressed in various cancers [268, 269, 286, 288, 289, 291, 293, 295, 297, 301, 304, 309, 315] and has been implicated in the tumorigenic process [57, 309, 312, 313, 315, 400]. Overexpression of LAPTM4s may enhance the proliferative and/or detoxification potential of cells, likely supporting their cancerous transformation or maintenance. Thus, identifying LAPTM4s sub-cellular localization and the factors that regulate their sorting could serve as the basis for strategies to counteract deleterious consequences of their overexpression.

All LAPTM proteins are assumed to localize to the late endosomes and lysosomes, which has been confirmed for LAPTM5 [78, 266] and LAPTM4a [267, 275]. LAPTM5s contain several putative lysosomal targeting motifs in their C-termini, including tyrosine based (YXXΦ), PY (L/PPXY) and dileucine ([DE]XXXL[LI]) motifs (Figure 2.1A). With the exception of PY motifs, these motifs are recognized by major adaptor molecules involved in trafficking from the Golgi to the lysosome (directly or indirectly via the plasma membrane) including the adaptor proteins AP1 to AP4 and GGA1-3 [200]. APs and GGAs bind cargo, protein coats and other accessory proteins for transport to and from specific compartments [229, 401]. Our lab recently demonstrated that lysosomal targeting of LAPTM5 is dependent on its C-terminal PY motifs, ubiquitin interacting motif (UIM) and an interaction with the E3 ubiquitin ligase Nedd4 [78]. Nedd4 proteins are comprised of a C2 domain, 3-4 WW domains that bind substrates by interacting with PY motifs, and a ubiquitin ligase HECT domain [7]. This mode of sorting is also used by the yeast vacuolar protein Sna3p [79]. Interestingly, both LAPTM4a and b contain PY motifs, but their role in targeting has not been investigated to date.

Here the lysosomal and plasma membrane sorting of LAPTM4a and LAPTM4b is investigated, and it is shown that their PY motifs, as well as Nedd4, participate in their subcellular sorting. LAPTM4a and b, like LAPTM5, are present in the late endosomal and lysosomal compartments, but unlike other LAPTM members, LAPTM4b is also expressed at the plasma membrane, a site that is favoured upon mutation of its PY motifs.
3 Materials and Methods

Cell Lines, Antibodies and Reagents

Hek293T cells (Obtained from ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected using the calcium phosphate transfection method[402]. Mouse embryonic fibroblasts (MEF) (WT and Nedd4-/- MEF) were generated as described earlier[123]. MEF cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml glutamine. They were transfected according to the manufacturer’s protocol using ESCRTV transfection reagent (Sigma-Aldrich). For confocal imaging: Mouse monoclonal [H4A3] to Lamp1 (Abcam), rat anti-mouse Lamp1 (BD Pharmingen), Normal Goat Serum (Jackson ImmunoResearch Laboratories Inc.), Alexa-Fluor 647 conjugated ConcanavillinA (ConA, Invitrogen), Alexa-Fluor 488 conjugated Phalloidin (Invitrogen), Alexa-Fluor 488 Goat-anti mouse 2° antibody (Invitrogen), Alexa-Fluor 488 Goat-anti rat 2° antibody (Invitrogen). Slides were mounted using Darkocytomation (Dako Corporation). For Immunoprecipitation and Immunoblotting: Peroxidase-conjugated AffiniPure Goat anti-mouse IgG(H+L) (Jackson ImmunoResearch laboratories Inc.), Living Colors mCherry (mCh-) monoclonal antibody (Clontech), GAPDH (A-3, Santa Cruz), anti-human transferrin receptor antibody #13-6800 (Zymed), anti-tubulin (Sigma), anti-β-actin (Sigma), anti-hemagglutinin (HA, Covance), anti-V5 (AbDserotec), anti-penta-His (Invitrogen), anti-Lamp1 (Abcam). Protein-G agarose (BioShop Canada), streptavidin agarose (Thermo Scientific). Nickel-NTA agarose beads (Qiagen). LLnL (N-acetyl-Leu-Leu-norleucinal, Sigma). For biotinylation: EZ-link Sulfo-NHS-SS-biotin (Thermo Scientific). Developed with Western Lightning™ Plus-enhanced chemiluminescence (ECL,Perkin Elmer). miniMACS columns for magnetic affinity chromatography (Miltenyi Biotec Inc.).

Constructs

We obtained cDNA entry clones of human LAPTM4a (GenBank: AAH03158.1) and LAPTM4b (the 24kDa isoform, GenBank: AAH31021.1) from Invitrogen, and used these cDNAs in all our experiments. Site directed mutagenesis was used to mutate the 2nd conserved Proline in the PY-motifs (L/PPXY) to Alanine. LAPTM4a single PY-motif mutants are P208A, P213A, P228A, while the total PY motif mutant is LAPTM4a-3PA. The total PY motif mutant of LAPTM4b is
LAPTM4b-2PA (mutated residues are P205A and P221A). WT and PY motif mutants were N-terminally tagged with mCh (mCherry) or HA (Hemagglutinin) using the Gateway cloning system (Invitrogen). The histidine-tagged ubiquitin construct (His-Ub) was provided by D. Bohmann (University of Rochester Medical Center, Rochester, NY;[403])

**Immunofluorescence Confocal Microscopy**

Hek293T and MEF cells were cultured on poly-D-Lysine coated coverslips in 6-well-plates. At 24hrs post transfection wells were washed 3x with 1ml PBS and incubated 5 min with Alexa-Fluor-647-conjugated ConA (1:1000). After three PBS washes, cells were fixed with 4% Paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and incubated with 1:100 Normal Goat Serum in 3% Skim Milk (30 min). Slides were incubated 1 hr with mouse anti-human (Hek293T) or rat anti-mouse (MEFs) Lamp1 (1:1000) in 3% skim milk. After three PBS washes, cells were incubated with goat anti-mouse or anti-rat Alexa 488 Fluor-conjugated antibody. When applicable, Phalloidin-staining (1:1000) was performed concurrently with the 2\(^{nd}\) antibody. Coverslips were mounted with Dako Cytomation and imaged with a LSM510 confocal microscope at 63x magnification with a 1.4NA oil-immersion objective (Carl Zeiss MicrolImaging, Inc.). Colocalization of Lamp1 and mCh-tagged LAPTM4s was assessed by Volocity 5.4.1 (Perkin Elmer) and expressed in terms of the Pearson’s correlation coefficients.

**Characterization of the Actin-based Membrane Protrusions**

Hek293T cells (untransfected, or transfected with mCh- or mCh-LAPTM4b-WT) were stained with phalloidin as described above. The length of actin-based membrane protrusions was determined using the line tool in Volocity 5.4.1 (Perkin Elmer). Each phalloidin-stained actin-based membrane protrusion was measured per cell and the average length of the protrusions was determined. The average density of protrusions per \(\mu m^2\) was determined by computing the total number of actin-based membrane protrusions per cell divided by the total cell’s surface area. The average length and density of the protrusions indicated per condition (untransfected, mCh- or mCh-LAPTM4b-WT) in Table 8 represent the average length and density of actin-based membrane protrusions of 20 cells.
Co-Immunoprecipitation (Co-IP) Assays

Hek293T cells were co-transfected with mCh-tagged LAPTM4s and V5-tagged Nedd4. At 24 hrs post transfection media was changed. At 48 hrs, cells were lysed on ice in 1ml Lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1.0 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 1 mM PMSF) and centrifuged at 12000 rpm (30 min, 4 °C). 1 mg of lysate was incubated with 1.5 µl anti-V5 antibody and 15 µl protein-G sepharose beads (4 °C, 4 hrs). Tubes were spun at 1000 rpm (3 min), washed 3x with Lysis Buffer and 3x with low salt HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% TritonX-100). Proteins were eluted with 30µl 1xSDS-PAGE sample buffer, resolved on 15% SDS-poly-acrylamide Gels (SDS-PAGE) and transferred to nitrocellulose. Upon cell lysis, 50 µg of fresh cell lysate are set aside and loaded on the same 15% SDS-PAGE gel as Lysate. This serves as a control to confirm and monitor the expression of the transfected proteins. For immunoblotting: the membrane was blocked in 3% Dry Milk (30 min), incubated 1 hr with primary antibody (anti-mCh 1:2500; anti-V5 1:5000; anti-GAPDH 1:1000, anti-Tubulin 1:1000), washed 3x with wash buffer (PBS, 0.1% Triton X-100), incubated with Horseradish peroxidase conjugated goat anti-mouse IgG (45 min) and developed using enhanced chemiluminescence (ECL). All centrifugations were performed using an Eppendorf Centrifuge 5417R.

Cell Surface Biotinylation Assay

Hek293T were transfected with mCh-LAPTM4a-WT, mCh-LAPTM4b-WT or mCh-LAPTM4b-2PA. At 24 hrs post-transfection cells were washed 3x with PBS and incubated with 3 ml of 1 mg/ml EZ-link Sulfo-NHS-SS-biotin in PBS (1 hr, 4 °C). Cells were washed 3x in PBS and lysed in 1mL Lysis Buffer. Lysates were collected and centrifuged at 12000 rpm (30 min, 4 °C). Upon cell lysis, 50 µg of fresh cell lysate are set aside and loaded on the same SDS-PAGE gel as “Lysate.” This serves as a control to confirm and monitor the expression of the transfected proteins. Then 1 mg of supernatant was incubated with 30 µl streptavidin agarose beads (4 hrs, 4 °C). Beads were spun at 2000rpm (2 min), washed 3x with 1ml Lysis Buffer and 3x 1ml Low Salt HNTG. Biotinylated proteins were eluted in 30 µL 1xSDS-PAGE sample buffer (5 min, 100 °C). Samples were resolved by 15% SDS-PAGE and transferred to nitrocellulose. Immunoblotting: membrane was blocked in 3% Dry Milk (30 min), incubated with anti-mCherry
Isolation of Lysosomes using Magnetic Affinity Chromatography

Lysosomes were isolated as previously described by magnetic affinity chromatography using superparamagnetic (Iron-Dextran (FeDex)) particles [404, 405]. Briefly, Hek293T in 20 cm dishes were transfected with 20 µg HA-LAPTM4a or b. At 24 hrs the cells were incubated with 20 ml colloidal iron dextran particles in 20 ml lyophilized media. After 9 hrs, cells were washed 3x with PBS and chased with fresh media overnight. Cells were washed 1x with PBS, trypsinized, collected in 5 mL fresh media and spun at 2000 rpm for 5 min. Lysosomes were isolated at 4°C. The pellet was resuspended in 40 ml homogenization buffer (0.25M sucrose, 4mM imidazole, pH 7.4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 1 mM PMSF) and spun at 2000 rpm for 5 min. The pellet was homogenized in 2 ml homogenization buffer and spun for 10 min. at 2000 rpm. Then 1 ml post-nuclear supernatant (PNS) was collected and loaded onto a miniMACs column on a magnet. The flowthrough (F) was collected and the column was washed 2x with homogenization buffer. The column was removed from the magnet and the lysosomal fraction was eluted with 300 µl Elution Buffer (5mM Tris pH 7.4, 150 mM NaCl, 0.1% Triton). Then 10 µg of untransfected Hek293T cell lystate, PNS, Flowthrough (F), and Lysosomal Fraction (L) were loaded on a 15% SDS-PAGE and transferred to nitrocellulose. An immunoblot was performed as described for Co-IP experiments, with anti-Lamp1 (1:1000) or (1:10 000) anti-HA antibodies.

Ubiquitination Assay

Hek293T cells were transfected by calcium phosphate transfection. HA-LAPTM4 constructs were co-expressed with Histine-Ubiquitin (His-Ub) and/or V5-tagged Nedd4 (WT or Catalytically inactive mutant (CS)). At 24 hrs post transfection cell media was exchanged and at 48 hrs the cells were lysed on ice in lysis buffer supplemented with 50 µM LLnL. Cell lysates were centrifuged at 12 000 rpm (30 min, 4 °C). Then 50 µg of lysates were set aside as loading controls. 2 mg were treated with 1% SDS and boiled for 5 min to dissociate potential LATM4-interacting proteins and diluted 11 times with lysis buffer. Then 30 µL nickel-agarose beads were used to precipitate His-Ubiquitinated proteins at 4°C overnight. Tubes were spun at 2000 rpm (3
min), washed 3x with Lysis Buffer and 3x with low salt HNTG. Proteins were eluted with 30µl 1xSDS-PAGE sample buffer, resolved on 12% SDS-PAGE and transferred to nitrocellulose. The membrane was probed for the presence of ubiquitinated, HA-tagged LAPTM4.

4 Results

4.1 LAPTM4s are highly conserved LAPTM family members

In order to determine the potential for shared determinants of lysosomal targeting across the LAPTM family of proteins, human LAPTM cDNAs were aligned using ClustalW. From this alignment, it is apparent that LAPTM4a and LAPTM4b are the most closely related family members (Figure 2.1B) with 42.5% conserved amino acids. Furthermore, all LAPTM proteins contain a significant number of putative lysosomal targeting motifs in their C-termini (Figure 2.1A) including putative tyrosine based (YXXΦ), PY (L/PPXY) and dileucine ([DE]XXXL[LI]) motifs.

4.2 Both LAPTM4 proteins localize to the lysosome, but LAPTM4b is also present at the plasma membrane

To detect sub-cellular localization of LAPTM4a and LAPTM4b, we generated mCherry-tagged (mCh) LAPTM4a and LAPTM4b (the 24kDa isoform) constructs and expressed them in Hek293T cells (which endogenously express these proteins). Figure 2.2A,B shows that both LAPTM4a and LAPTM4b co-localized significantly with the late endosomal and lysosomal marker Lamp1. Unlike LAPTM4a, however, some LAPTM4b was also detected at the plasma membrane. In support, cell surface biotinylation experiments revealed the presence of LAPTM4b (but not LAPTM4a) at the cell surface (Figure 2.2C). These results suggest that a fraction of LAPTM4b is present at the plasma membrane. In order to biochemically confirm the presence of LAPTM4b in the lysosomal compartment, lysosomes were isolated from HA-LAPTM4b overexpressing cells using magnetic affinity chromatography, employing a previously described method [404, 405]. As expected, the lysosomal fraction isolated is enriched for endogenous Lamp1 relative to an equal amount of post-nuclear supernatant (PNS) input. Similar to Lamp1, HA-LAPTM4b was detected in the lysosomal fraction, as was LAPTM4a (Figure 2.2D).

Interestingly, our results show that cells expressing LAPTM4b also exhibit hair-like membrane protrusions that largely co-stained with the plasma membrane marker Concanavalin A (ConA),
(Figure 2.2A, Table 7, Figure 2.3). In addition, the nature of the protrusions was examined by staining Hek293T cells either untransfected or transfected with mCherry or mCh-LAPTM constructs with the actin-binding protein phalloidin (Figure 2.4). Untransfected, mCh-, LAPTM5 and LAPTM4a -expressing cells showed similar and minimal hair-like protrusions (Figure 2.4A). In contrast, mCh-LAPTM4b expressing cells on average had approximately twice as many and twice as long actin-based membrane protrusions relative to untransfected or mCh-transfected cells (Figure 2.4 and Table 8).
Figure 2.1: **Sequence alignment of the LAPTM family of proteins.** (A) ClustalW [406] amino acid sequence alignment of the LAPTM4 family members LAPTM4a (GenBank:AAH03158.1), 24kDa-LAPTM4b (short) (GenBank: AAH31021.1), 35kDa-LAPTM4b (long) (NP_060877.3) and LAPTM5 (GenBank: AAI06897.1). Putative transmembrane regions (predicted by SMART [407]) are highlighted in yellow, PY motifs are bold and underlined, and the UIM motif of LAPTM5 highlighted in blue. Putative DiLeu motifs are highlighted in green. (B) Percentage of conserved amino acids between human LAPTM family members as determined by alignment using ClustalW. (C) PY motifs in the C-terminal tails of LAPTM4a and LAPTM4b are indicated in bold. Putative Tyrosine-based motifs are underlined. The putative dileucine motif is indicated in green (italicized), with the bold Leu shared with the PY motif. Tyrosine residues shared by tyrosine-based and PY motifs are indicated in red.
Figure 2.1: Sequence alignment of the LAPTM family of proteins.
Figure 2.2: Both LAPTM4 proteins localize to lysosomes, but LAPTM4b is uniquely present also at the cell surface. (A) At 24 hrs post transfection the plasma membrane (ConA, green) of Hek293T cells expressing mCh-LAPTM4a (red, top) or mCh-LAPTM4b (red, bottom) was stained, cells were fixed and incubated with anti-Lamp1 antibodies (blue). White arrows indicate mCh-LAPTM4b in hair-like protrusions. Cells were imaged using LSM510 and subcellular localization was assessed using Volocity 5.4.1. (B) Colocalization of LAPTM4s with the lysosomal marker Lamp1 was expressed in terms of the Pearson’s correlation coefficient. Degrees of freedom are noted as (r), level of significance as (p). n=37 for LAPTM4a and LAPTM4b. (C) Untransfected or transfected (mCh-LAPTM4a-WT or mCh-LAPTM4b-WT) Hek293T cells were subjected to cell surface biotinylation, lysis, pulldown with streptavidin agarose beads and separation on SDS-PAGE. Cell surface biotinylation of the Transferrin receptor (TfR) was used as a positive control. (D) Biochemical evidence for the presence of LAPTM4 proteins in lysosomes: Lysosomal fraction from Hek293T cells overexpressing HA-LAPTM4a or b were isolated as described in the Materials and Methods section. Post nuclear supernatant (PNS) was loaded on a magnetic column, the flowthrough (F) was collected and upon removal of the column from the magnet the lysosomal fraction (L) was eluted. Immunoblotting shows significant enrichment of endogenous Lamp1 in the L fraction, as well as the presence of LAPTM4a or b.
Figure 2.2: Both LAPT4 proteins localize to lysosomes, but LAPT4b is uniquely present also at the cell surface.
Table 7 mCh-LAPTM4b co-stains with membrane-protrusions when overexpressed in Hek293T cells.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total Number of Cells Observed</th>
<th>Cells in which LAPTM4b is PRESENT in actin-based membrane protrusions</th>
<th>Cells in which LAPTM4b is ABSENT from actin-based membrane protrusions</th>
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<tr>
<td></td>
<td></td>
<td># cells</td>
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<td>2</td>
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Table 8 Hek293T cells in which mCh-LAPTM4b is overexpressed have longer and higher density of actin-based membrane protrusions.

<table>
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<tr>
<th>Protrusions Property</th>
<th>Experiment</th>
<th>Untransfected (n=20)</th>
<th>mCh(n=20)</th>
<th>mCh-LAPTM4b (n=20)</th>
<th>Statistical Significance c</th>
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</thead>
<tbody>
<tr>
<td>Average length (µm)a</td>
<td>1</td>
<td>1.963 ± 0.104</td>
<td>2.267 ± 0.189</td>
<td>3.505 ± 0.092</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>1.545 ± 0.086</td>
<td>1.775 ± 0.122</td>
<td>3.383 ± 0.165</td>
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<tr>
<td>Average density</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(# protrusions/µm²)b</td>
<td>1</td>
<td>0.027 ± 0.003</td>
<td>0.024 ± 0.003</td>
<td>0.0560 ± 0.004</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.034 ± 0.003</td>
<td>0.020 ± 0.002</td>
<td>0.0648 ± 0.008</td>
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</table>

a,b The average protrusion length/density is indicated ± SEM

c A student t-test was performed to determine the statistical significance of the difference between average length/density of actin protrusions of mCh-LAPTM4b and untransfected cells.
Figure 2.3: **mCh-LAPTM4b co-stains with plasma membrane protrusions.** Three view fields selected at random of Hek293T cells (Table 7) expressing mCh-LAPTM4b (red) at 24 hrs post transfection are shown. The plasma membrane is stained with ConA (green). Cells were imaged using LSM510. The brightness of the red channel has been increased approximately 2 fold using Volocity 5.4.1.
Figure 2.3: mCh-LAPTM4b co-stains with plasma membrane protrusions.
Figure 2.4: **LAPTM4b, but not LAPTM4a or LAPTM5, is present in actin-based membrane protrusions in Hek293T cells.** (A) Hek293T cells were transfected with mCherry alone, mCh-LAPTM4b-WT, mCh-LAPTM4a-WT or mCh-LAPTM5-WT (red). 24 hrs post transfection cells were fixed and stained with anti-Lamp1 antibody (blue) and Phalloidin (green). (B) Additional images illustrate the changes in actin-based membrane protrusions associated with mCh-LAPTM4b-WT expression (relative to mCherry-alone expressing cells) in two independent experiments (1 and 2). All images were taken with LSM510 and analyzed by Volocity 5.4.1 (Perkin Elmer). White arrows indicate examples of actin-based membrane protrusions in which mCh-LAPTM4b is present.
Figure 2.4: LAPTM4b, but not LAPTM4a or LAPTM5, is present in actin-based membrane protrusions in Hek293T cells.
4.3 Both LAPTM4a and LAPTM4b bind Nedd4 via their PY Motifs

LAPTM5 was previously shown to interact with the E3 ubiquitin ligase Nedd4 via its C-terminal PY motifs [78]. Since PY motifs are also present in LAPTM4a and LAPTM4b (Figure 2.1C), we investigated their ability to bind Nedd4 in cells. Co-immunoprecipitation (co-IP) experiments were performed using cell lysates from Hek293T cells co-expressing V5-tagged Nedd4 and mCh-tagged LAPTM4a or LAPTM4b. Figure 2.5 shows that both LAPTM4a and LAPTM4b co-IP with Nedd4. Moreover, co-IP of LAPTM4a and LAPTM4b with Nedd4 was dependent on the presence of their PY motifs; Mutating all of LAPTM4a’s 3 PY motif or LAPTM4b’s 2 PY motif prolines to alanines (mCh-LAPTM4a-3PA or mCh-LAPTM4b-2PA) abolished binding to Nedd4 (Figure 2.5A,B). LAPTM4a’s second (P213) and third (P228) PY motif appear most important for this interaction, as they are both unable to bind Nedd4 when mutated to Ala, while mutation of the 1st PY motif (P208A) reduces, but does not eliminate, the binding of LAPTM4a to Nedd4 (Figure 2.5A). Together, these experiments suggest that LAPTM4a and LAPTM4b bind Nedd4 in cells, and that this interaction is mediated via the LAPTM4a PY motifs.

4.4 Nedd4 Ubiquitinates both LAPTM4a and LAPTM4b

It was previously shown that Nedd4 binds and ubiquitinates LAPTM5 [78]. Consequently we examined whether Nedd4 was able to ubiquitinate LAPTM4a and/or LAPTM4b. We performed ubiquitination assays on both LAPTM4a (Figure 2.6A) and LAPTM4b (Figure 2.6B) and found that both are ubiquitinated by Nedd4. The specificity of the ubiquitination is confirmed by the decrease in ubiquitination in the presence of catalytically inactive Nedd4 (CS) and through the loss of ubiquitination of PY motif mutants (3PA and 2PA) that cannot bind Nedd4 (Figure 2.6).
**Figure 2.5: LAPTM4a and LAPTM4b bind Nedd4 via their PY motifs.** (A) LAPTM4a’s binding to Nedd4 is dependent on its 2nd and 3rd PY motifs. A Co-IP was performed on whole cell lysates from Hek293T cells co-expressing V5-tagged Nedd4 and mCh-LAPTM4a (WT, 3PA, P208A (208), P213A (213) or P228A (228)). Nedd4 was precipitated using anti-V5 antibodies, samples were separated on SDS-PAGE and transferred to nitrocellulose. Anti-mCherry antibodies were used to detect binding of mCh-LAPTM4s to Nedd4. (B) LAPTM4b binding to Nedd4 is dependent on its PY motifs. A Co-IP was performed on whole cell lysates from Hek293T cells co-expressing V5-tagged Nedd4 and mCh-LAPTM4a-WT, mCh-LAPTM4b-WT or mCh-LAPTM4b-2PA, as in (A). The housekeeping proteins Tubulin and GAPDH were used interchangeably as loading controls for the cell lysates.
Figure 2.5: LAPTM4a and LAPTM4b bind Nedd4 via their PY motifs.
Figure 2.6: Nedd4 ubiquitates LAPT4a and LAPT4b in cells. (A) LAPT4a is ubiquitinated by Nedd4. Hek293T cells co-expressing V5-tagged Nedd4 (WT or catalytically inactive CS mutant) and HA-LAPT4a (WT or 3PA) and His-Ub were lysed, lysate boiled in SDS to dissociate putative interacting proteins, and diluted (see Methods). Proteins tagged by His-Ub were precipitated using Ni-NTA agarose beads, samples were separated on SDS-PAGE and transferred to nitrocellulose. Anti-HA antibodies were used to detect His-Ubiquination of LAPT4a. (B) LAPT4b is ubiquitinated by Nedd4. As in (A), except using WT or 2PA HA-LAPT4b. Actin is used as a housekeeping protein for the lysate loading control.
Figure 2.6: Nedd4 ubiquitinates LAPT4a and LAPT4b in cells.
4.5 LAPTM4a and LAPTM4b require their PY motifs for proper targeting

Because LAPTM4s interact with Nedd4 via their PY motifs and we previously showed that LAPTM5’s PY motifs are required for its proper localization to the lysosome in Hek293T and dendritic cells (DC) [78], we investigated whether the LAPTM4 PY motifs participate in their lysosomal targeting. Hek293T cells were transfected with WT or PY motif mutant LAPTM4a and LAPTM4b. At 24 hrs post transfection the subcellular localization of the LAPTM4 proteins was assessed by confocal microscopy (Figure 2.7A). Quantification revealed that while WT LAPTM4a and LAPTM4b both co-localized with the late endosome and lysosomal marker Lamp1, the PY motif deficient mCh-LAPTM4a-3PA shows significantly reduced co-localization (~24% decrease in colocalization, Figure 2.7B). Interestingly mCh-LAPTM4b-2PA colocalization with Lamp1 was largely unchanged (~2% decrease in colocalization) (Figure 2.7B). This suggests that LAPTM4a and LAPTM4b differ in their dependence on the PY motifs for lysosomal sorting, with LAPTM4a being dependent and LAPTM4b minimally dependent on these motifs.

However, we noticed that while the mCh-LAPTM4b-2PA mutant was still able to localize to the lysosome, its surface expression appeared to have increased. In order to further investigate this observation, an experiment was conducted in which untransfected, mCh-LAPTM4a-WT, mCh-LAPTM4b-WT or mCh-LAPTM4b-2PA expressing Hek293T cells were subjected to cell-surface biotinylation. As seen in Figure 2.7C, there appears to be more mCh-LAPTM4b-2PA at the cell surface than mCh-LAPTM4b-WT. These data support the notion that the two PY motifs of LAPTM4b are important to minimize its missorting to the plasma membrane.
Figure 2.7: LAPTM4a and LAPTM4b require their PY motifs for proper targeting. (A) 24 hrs post transfection the plasma membrane of Hek293T cells expressing mCh-LAPTM4a-WT, mCh-LAPTM4a-3PA, mCh-LAPTM4b-WT or mCh-LAPTM4b-2PA was stained (ConA, blue), cells were fixed, incubated with anti-Lamp1 antibodies (green) and imaged by confocal microscopy. Subcellular localization of LAPTM4 was assessed using Volocity 5.4.1. (B) LAPTM4 colocalization with the lysosomal marker Lamp1 is expressed in terms of the Pearson’s correlation coefficient. Degrees of freedom are noted as (r), level of significance as (p). Graphs illustrate changes in the mean Pearson’s coefficients of mCh-LAPTM4 colocalization with Lamp1 (LAPTM4a n=32, LAPTM4b n=82). Error bars indicate SEM. * denotes p<0.0001. (C) Cell surface expression of mCh-LAPTM4b-2PA is up-regulated relative to that of mCh-LAPTM4b-WT. Cell surface biotinylation was performed on Hek293T cells expressing mCh-LAPTM4a-WT, mCh-LAPTM4b-WT or mCh-LAPTM4b-2PA. Cell surface biotinylation of Transferrin receptor (TfR) served as a positive control, while cell surface biotinylation of mCh-LAPTM4a-WT and GAPDH served as negative controls.
Figure 2.7: LAPTM4a and LAPTM4b require their PY motifs for proper targeting.
4.6 Nedd4 facilitates lysosomal sorting of LAPTM4a and LAPTM4b

Given that all LAPTM4s were able to efficiently co-IP with Nedd4 and Nedd4 is required for the proper localization of LAPTM5 to the lysosome [78], we investigated the role of Nedd4 in LAPTM4 lysosomal targeting using Nedd4 knockout (Nedd4<sup>−/−</sup>) MEFs our lab previously generated [123]. WT or Nedd4<sup>−/−</sup> MEF cells were transfected with mCh-LAPTM4a or mCh-LAPTM4b. After 24 hrs, the cells were fixed and stained with the lysosomal marker (Lamp1) and co-localization of the LAPTM4 protein was assessed using confocal microscopy (Figure 2.8). mCh-LAPTM4a expressed in WT MEFs showed strong colocalization with the lysosomal marker Lamp1 (Figure 2.8A). In contrast, LAPTM4a exhibited severely reduced (by 50%) lysosomal localization when expressed in Nedd4<sup>−/−</sup> MEFs (Figure 2.8B,C). mCh-LAPTM4b expressing WT MEF cells showed some colocalization with the lysosomal marker Lamp1 (Figure 2.8A). Quantification of the colocalization between mCh-LAPTM4b and Lamp1 in Nedd4<sup>−/−</sup> and WT MEFs, revealed a decrease in colocalization of only ~14%. This decrease is much smaller than that observed for LAPTM4a (Figure 2.8B,C), but is nonetheless significant. These results suggest that Nedd4 plays a significant role in the lysosomal sorting of LAPTM4a and a lesser, but still significant, role in that of LAPTM4b.
Figure 2.8: Nedd4 facilitates lysosomal sorting of LAPTM4a and LAPTM4b. (A) WT or Nedd4−/− MEF cells were transfected with (wildtype) mCh-LAPTM4a or mCh-LAPTM4b (red). 24 hrs post transfection, the plasma membrane was stained (ConA, blue), cells were fixed and incubated with anti-Lamp1 antibodies (green). Cells were imaged by confocal microscopy and subcellular localization was assessed using Volocity 5.4.1. (B) LAPTM4 colocalization with the lysosomal marker Lamp1 is expressed in terms of the Pearson’s correlation coefficient. Degrees of freedom are noted as (r), level of significance as (p). (C) The graphs represent the changes in mean Pearson’s coefficients of WT or Nedd4−/− MEF cells expressing mCh-LAPTM4a (n=26) or mCh-LAPTM4b (n=30). Error bars indicate SEM and * indicates p<0.0001.
Figure 2.8: Nedd4 facilitates lysosomal sorting of LAPTM4a and LAPTM4b.
5 Discussion

Our studies here provide evidence that

(i) LAPTM4b, like LAPTM4a and LAPTM5, localizes to lysosomes.

(ii) both LAPTM4a and LAPTM4b can co-IP with the ubiquitin ligase Nedd4 and can be ubiquitinated by it, albeit at different strengths.

(iii) sorting of LAPTM4a (and to a lesser extent LAPTM4b) is dependent on interaction with Nedd4, similar to our previous observation with LAPTM5.

However, despite these commonalities, we also show that LAPTM targeting is not entirely conserved: LAPTM4b, unlike other LAPTMs, also localizes to the plasma membrane (and appears to enhance the formation of actin-based membrane protrusions) and its dependence on Nedd4 for lysosomal sorting is weaker than that of LAPTM4a. Moreover, LAPTM4b sorting to the plasma membrane is enhanced upon of loss of its PY motifs, suggesting that Nedd4 normally prevents it from sorting to the cell surface.

Our current studies, along with our (and others’) earlier work, indicate that all LAPTM proteins reside primarily within the endo-lysosomal system. While localization was previously shown for LAPTM5 [78, 266] and LAPTM4a [267, 275], LAPTM4b localization had been assumed to be lysosomal from its association with membrane fractions and immunohistochemical staining of cancerous tissues [268, 287, 297, 301, 309]. The lysosomal localization of LAPTM4 proteins supports their putative roles in small molecule transport ([276, 315], as sequestration of bioactive compounds to this organelle precludes their activity [275, 276, 315]. The detection of LAPTM4b at the plasma membrane is in accord with the observation that it is able to bind to MDR1 (a plasma membrane expressed multidrug transporter) [315] and agrees with the expression pattern of other multidrug transporters which are found simultaneously at the plasma membrane and internal organelles (Golgi/Endosomes/Lysosomes) [408, 409]. Since LAPTM4b is the only LAPTM member present at the cell surface, it likely has some unique functions. One such unique function may include the formation of membrane protrusions. The actin-based membrane protrusions induced by LAPTM4b are reminiscent of filopodia associated with the epithelial-mesenchymal transition (EMT) and metastasis of cancerous cells [410-412]. It is possible that
filopodia growth contributes to the increased invasive potential and metastasis associated with LAPTM4b overexpressing cancers and cells [309, 313, 400]. While the membrane protrusions we observed were likely enhanced by LAPTM4b overexpression, such overexpression is common in numerous cancers and likely contributes to the metastatic potential of LAPTM4b -expressing cells, as described above.

We have shown that LAPTM4 proteins, like LAPTM5, coimmunoprecipitates with Nedd4. We believe that this interaction is most likely direct, as this co-IP was abolished by mutating the LAPTM4 PY motifs. While we cannot currently prove that mutation of the PY motifs did not cause other alteration in the LAPTM4 proteins that resulted in loss of binding to Nedd4, the PY motif mutants appear to be expressed at the right size and appear stable, suggesting that they are not misfolded.

Our localization studies suggest Nedd4 plays differing roles for each LAPTM member. In the case of LAPTM5, we previously established that the loss of Nedd4 or functional PY motifs, resulted in LAPTM5 Golgi retention [78]. This stands in contrast with the phenotype observed for LAPTM4s: in the absence of PY motifs or Nedd4 LAPTM4a exits the Golgi, but co-localization with Lamp1 positive vesicles decreases (~50%). Similarly, the loss of PY motifs in LAPTM4b, or lack of Nedd4, does not affect Golgi exit and only partially affects lysosomal localization. Instead, the absence of PY motifs enhances the proportion of LAPTM4b at the cell surface. The role of Nedd4 in regulating lysosomal versus plasma membrane sorting described here is reminiscent of nutrient-dependent sorting of Gap1 permease in yeast, which is controlled via Gap1 ubiquitination by Rsp5, the S. cerevisiae orthologue of Nedd4 [68, 69]. In addition, while LAPTM5 possesses a UIM motif that is also necessary for its lysosomal sorting (by interacting with ubiquitinated GGA3) [78], LAPTM4 proteins lack UIM motifs. In accord, our preliminary data show that unlike LAPTM5 [78], LAPTM4a cannot bind ubiquitin. Thus, despite sequence similarities between the LAPTM family members, the regulation of their sub-cellular localization is not identical (Figure 2.9).

In fact, the greater the LAPTM4 sequence differs from that of LAPTM5, the smaller the role of Nedd4 appears to be in its sorting. This relationship may be secondary to the number and context of LAPTM4 PY motifs. Like LAPTM5, LAPTM4a contains three PY motifs, whereas LAPTM4b contains only two. While a single PY motif is sufficient to bind a WW domain, it is
possible that having numerous PY motifs could enhance binding avidity. Furthermore, the context of the PY motifs may affect Nedd4’s ability to mediate targeting. Based on Figure 1B, we speculated that other LAPTM4 C-terminal putative lysosomal targeting motifs played a role in addition or in combination with PY motifs. Appendix A demonstrates that LAPTM4b utilizes both a C-terminal dileucine and tyrosine motif for its subcellular targeting.

It is possible that access to lysosomal targeting motifs is spatiotemporally controlled. For instance, if a tyrosine-based motif or a dileucine motif has a higher affinity for their binding partner than a neighboring or overlapping PY motif, the PY motifs may become obscured. This is particularly relevant when considering the LAPTM4b PY motifs, which share residues not only with a potential dileucine targeting motif, but also a putative tyrosine based motif. Indeed, two tandemly arranged tyrosine-based (YXXΦ) motifs were previously proposed to be involved in LAPTM4a lysosomal sorting[232]. However, the substitution mutation made to the tyrosine of the 2nd YXXΦ motif simultaneously disrupted a PY motif (Figure 1B) raising the possibility that a disrupted PY motif may have contributed (wholly or in part) to the observed missorting. Nonetheless, involvement of additional putative lysosomal targeting motifs may explain why ~50% and 86% of LAPTM4a and LAPTM4b, respectively, were correctly sorted to lysosomes, despite the absence of Nedd4 in our MEFs.

Having numerous lysosomal targeting motifs may mediate efficient lysosomal delivery even if one or more lysosomal targeting pathways are impaired [413, 414]. Such a mechanism for LAPTM4s remains speculative, but would be relevant considering the potential consequences of LAPTM4 mislocalization with respect to small molecule transport [276, 315] and/or cell signaling [57, 309, 312, 313, 315].

Additionally, while ubiquitination of LAPTM4b occurs in the presence of ectopically expressed Nedd4, and is reduced in the presence of a catalytically inactive Nedd4(CS)(Figure 2.6), LAPTM4b is also ubiquitinated in Hek293Ts that are not transfected with Nedd4. This suggests that either endogenous Nedd4 (expressed in these cells) or/and other E3 ligases, is/are responsible for this ubiquitination. Potential other E3s may include additional Nedd4 family members, which also possess WW domains capable of binding PY motifs [7]. If other Nedd4 family members bind to and/or ubiquitinate LAPTM4b, this could also contribute to the different localization patterns observed when WT LAPTM4b is expressed in Nedd4−/− MEFs (Figure 2.8A)
and LAPTM4b-2PA in Hek293Ts (Figure 2.7A). While the absence of PY motifs results in an accumulation of LAPTM4b at the cell surface without altering lysosomal localization, the absence of Nedd4 leads to a minor decrease in LAPTM4b lysosomal co-localization without affecting its membrane expression. In such a scenario, we speculate that a LAPTM4b PY motif mutant might be unable to bind any Nedd4 family member via their WW domains. Other alternative motifs could then target LAPTM4b to the lysosome and play a role in the accumulation of LAPTM4b at the plasma membrane. In the absence of Nedd4 in the Nedd4^{-/-} MEFs, other Nedd4 family members could potentially assist in the clearance of LAPTM4b from the plasma membrane.

Figure 2.9 summarizes the findings of Chapter 2. Our results demonstrate that LAPTM4s like LAPTM5 require functional PY motifs and the presence of Nedd4 for their proper subcellular localization. However, the degree to which LAPTM sorting is dependent on Nedd4 and PY motifs varies between members, with LAPTM5 showing the greatest and LAPTM4b the least dependence on PY motifs and Nedd4 for lysosomal sorting. Uniquely LAPTM4b is also present at the plasma membrane and its expression is upregulated there in the absence of functional PY motifs.
Figure 2.9: **Schematic representation of LAPTM sorting determinants.** Schematic representation of the contribution of Nedd4 to lysosomal sorting of LAPTM proteins (tested in Hek293T cells), depicting varying dependency on Nedd4, with LAPTM5 being highly dependent (and also collaborating with GGA3), LAPTM4a exhibiting intermediate dependency (~50%), and LAPTM4b minimally dependent on Nedd4. ? represents other (unknown) factors.
Figure 2.9: Schematic representation of LAPTM sorting determinants.
Chapter 3
PY motifs and Nedd4 proteins as conserved lysosomal sorting determinants.

The work presented in this chapter constitutes unpublished data.

I designed and performed all experiments presented in this chapter.
1 Abstract

LAPTM proteins are a family of lysosomal transmembrane proteins capable of binding to the E3 ubiquitin ligase Nedd4 via their PY motifs. It was previously demonstrated that although all LAPTMs required their PY motifs and Nedd4 to target to the lysosome, the degree of the dependence varied significantly. Upon mutation of their PY motifs, LAPTM5 was retained in the Golgi, LAPTM4a localization to the lysosome decreased, while the expression of LAPTM4b at the plasma membrane was enhanced. Whether or not the dependence of the LAPTM proteins on PY motifs and Nedd4 for lysosomal targeting was a unique feature of the family, or a conserved mechanism for a variety of lysosomal membrane proteins remained to be determined. Here I identify additional lysosomal membrane proteins (LMPs) containing putative PY motifs and examine their ability to bind to Nedd4. Transmembrane Protein 55B (TMEM55B) is identified as a novel-binding partner of Nedd4 in HeLa cells. This interaction is mediated via TMEM55B’s PY motif, as a PY motif mutant (P66A) no longer co-immunoprecipitates with Nedd4 in HeLa cells. TMEM55B localizes to the lysosome and Golgi in HeLa cells, however P66A lysosomal colocalization is reduced. Additionally I show that another candidate protein, the multidrug transporter ABCB6, is able to bind to the Nedd4 family members WWP2 and Smurf1. Together these data suggest that PY motifs and Nedd4 could serve as sorting determinants of additional LMPs.

2 Introduction

Recent advances in proteomic analysis have identified over 200 lysosomal integral and transmembrane proteins [238]. Membrane proteins are key players in basic lysosomal functions and have been implicated in small molecule import/export, vesicle movement/fusion/fission events, enzymatic activities and acidification[260]. The specific lysosomal targeting mechanisms of individual lysosomal transmembrane proteins remain largely uncharacterized. However, those proteins whose lysosomal sorting determinants have been determined tend to fall within predictable categories (Table 9). Lysosomal membrane sorting is based on a conserved short linear sorting motif in a cytosolic domain [189]. The most well characterized motifs include the dileucine-based motifs (DXXLL or [DE]XXX[L/I]) and the tyrosine-based motifs (YXXΦ), which tether the protein to vesicular trafficking machinery such as the GGA and adaptor proteins (AP) (reviewed in section 3.4 of the Introduction). The role of ubiquitination during endocytic
processes and biosynthesis of plasma membrane proteins has been explored in both yeast and mammals (see section 2.3.2). Interestingly, much less is known about the role of PY motifs and ubiquitin in the lysosomal sorting of resident lysosomal membrane proteins, i.e. proteins that are targeted to the lysosomal limiting membrane as functional entities, rather than for degradative purposes. PY motifs were implicated in the lysosomal targeting of LAPTM proteins through binding of Nedd4 WW domains, and binding to associated trafficking machinery such as GGA3[78, 415]. In this Chapter, I propose to include Nedd4 proteins and PY motifs as conserved determinants of lysosomal membrane protein targeting beyond the LAPTM family.

To this end, I analyzed the 215 proteins obtained from a mass spectrometric analysis devised uniquely to isolate lysosomal integral and transmembrane proteins from rat liver tritosomes [416]. I surveyed the pool of isolated membrane proteins for the presence of putative PY motifs. The goal was to determine whether additional lysosomal membrane proteins utilized PY motifs and Nedd4 or Nedd4 family members for their lysosomal targeting. I specifically began my analysis with the intent to monitor binding of Nedd4 to candidate PY motifs. I also considered the possibility that other Nedd4 family members could bind lysosomal membrane proteins via their WW domains.

In this chapter I identify several putative PY motif containing lysosomal membrane proteins. I confirm TMEM55B as novel binding partner of Nedd4 and demonstrate that binding is mediated via TMEM55B’s PY motif. Furthermore I demonstrate that the PY motif of TMEM55B plays a role in its subcellular localization. Interestingly I show that while Nedd4 binds TMEM55B, it does not ubiquitinate it. Finally, I identify binding of Smurf1 and WWP2 to ABCB6 in HeLa cells, suggesting the potential role for additional Nedd4 family members in LMP sorting.
Table 9 Lysosomal sorting motifs adapted from [200, 202, 415].

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<tr>
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<td>WW domains of Nedd4 family of ubiquitin ligases</td>
<td>LAPTM5</td>
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<td>LAPTM4A</td>
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<tr>
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<td>LAPTM4B</td>
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*Note:* X = any amino acid, Φ = large hydrophobic residue
3 Materials and Methods

Cell Lines, Antibodies and Reagents

HeLa cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100µg/ml streptomycin. Cells were transfected using Polyjet (Frogga, Bioscience, Canada). For confocal imaging: mouse monoclonal [H4A3] to Lamp1 (Abcam), rabbit anti human giantin (Abcam), rabbit anti human calnexin (Abcam), normal goat serum (Jackson ImmunoResearch Laboratories Inc.), Alexa-Fluor 647 conjugated Concanavillin A (ConA, Invitrogen), Alexa-Fluor 488 Goat-anti mouse 2° antibody (Invitrogen), DAPI (Molecular Probes). Slides were mounted using Darkocytomation (Dako Corporation). For Immunoprecipitation and Immunoblotting: Living Colors mCherry (mCh-) Monoclonal Antibody (Clontech), anti-β-actin (Sigma), anti-Hemagglutinin (HA, Covance), anti-V5 (AbD Serotec), anti-Flag (Sigma). Peroxidase-conjugated AffiniPure goat anti-mouse IgG(L chain only) (Jackson ImmunoResearch laboratories Inc.), Anti-FLAG M2 affinity agarose (Sigma), LLnL (N-acetyl-Leu-Leu-norleucinal, Sigma), MG132 (Boston Biochem). Developed with Western Lightning™ Plus-enhanced chemiluminescence (ECL,Perkin Elmer).

Constructs

We obtained the Human ORFeome cDNA entry clones (pDONR223) for nine candidate proteins: ALDH2 (aldehyde dehydrogenase 2 family) GenBank: BC002967.1, VDAC1 (voltage-dependent anion channel 1) GenBank: BC008482.1, CANT1 (calcium activated nucleotidase 1) GenBank: BC017655.1, RPN1 (ribophorin 1) GenBank: BC010839, BAIAPL1 (BAI1-associated protein 2-like 1, IRTKS) GenBank: BC013888, P2RX4 (purinergic receptor P2X, ligand-gated ion channel, 4) GenBank: BC033826, ABCB6 GenBank: BC000559.2, FAM3C (family with sequence similarity 3, member C) GenBank: BC046932, TMEM55B (transmembrane protein 55B) GenBank: BC002867. Each construct was N-terminally tagged with Flag using the Gateway cloning system (Invitrogen). Site directed mutagenesis was used to mutate the 2nd conserved Proline in TMEM55B’s PY-motif to Alanine (PPPY>PAPY). This PY mutant is referred to as P66A. WT and P66A-TMEM55B were N-terminally tagged with mCherry or Flag.
Identification of PY Motif containing lysosomal proteins

The amino acid sequences of 215 proteins identified in a screen of rat liver tritosomes [416] were manually examined for the presence of PY motifs, by searching for the presence of L/PPXY sequences. Conservation of identified PY motifs in human proteins, was determined by sequence comparison using DNAassist (v3.0, University of the Free State). The membrane topology of candidate proteins was examined using the online tool TMpred by ExPASy (Bioinformatics Portal, Switzerland).

Immunofluorescence and Confocal Microscopy

HeLa cells were cultured on poly-D-Lysine coated coverslips in 6-well-plates. Cells were transiently transfected with cDNA according to the manufacturer protocol (Polyjet, Frogga Bioscience, Canada). At 24hrs post transfection wells were washed 3x with 1ml PBS and incubated 5 min with Alexa-Fluor-647-conjugated ConA (1:1000). After three PBS washes, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and incubated with 1:100 Normal Goat Serum in 3% Skim Milk (30 min). Slides were incubated 1 hr with mouse anti-human (HeLa) Lamp1 (1:1000), rabbit anti-human Giantin (1:2000) or rabbit anti-human Calnexin (1:1000) in 3% Skim milk. After three PBS washes, cells were incubated with goat anti-mouse or anti-rabbit Alexa 488 Fluor-conjugated 2° antibody and briefly stained with DAPI. Cover slips were mounted with Dako Cytomation. Images were acquired using a Quorum WAveFX-X1 spinning disc confocal system at 60x magnification with an Olympus S-Apo 60x/1.35 oil objective (Quorum Technologies Inc., Guelph, Canada). Colocalization of mCh-tagged TMEM55B with various organelle markers was assessed by Volocity 6.0.1 (Perkin Elmer) and expressed in terms of the Pearson’s correlation coefficients.

Co-Immunoprecipitation (Co-IP) Assays

HeLa cells were co-transfected with Flag-tagged candidate cDNAs and V5-tagged Nedd4. 24 hrs post transfection media was changed. At 48 hrs, cells were lysed on ice in 1ml Lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 1.0 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 1 mM PMSF) and centrifuged at 12000 rpm (30 min, 4 °C). 1 mg of lysate was incubated with 15 µl anti-Flag beads (4 °C, 4 hrs). Tubes were spun at 1000 rpm (3 min), washed 3x with Lysis Buffer and 3x with
low salt HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% TritonX-100). Proteins were eluted with 30µl 1xSDS-PAGE sample buffer, resolved on 15% SDS-Polyacrylamide Gels (SDS-PAGE) and transferred to nitrocellulose. For immunoblotting: the membrane was blocked in 3% Dry Milk (30 min), incubated 1 hr with primary antibody, washed 3x with wash buffer (PBS, 0.1% Triton X-100), incubated with Horseradish peroxidase conjugated secondary antibody and developed using enhanced chemiluminescence (ECL).

**Ubiquitination Assay**

mCherry-TMEM55B was co-expressed with HA-Ubiquitin (HA-Ub) and/or V5-tagged Nedd4 (WT or Catalytically inactive mutant (CS)) in HeLa cells. 48 hrs post-transfection cells were lysed on ice in lysis buffer supplemented with 50 µM LLnL and 20 µM MG132. Cell lysates were centrifuged at 12000 rpm (30 min, 4 °C). 50 µg of lysates were set aside as loading controls. 2 mg were treated with 1% SDS and boiled for 5 min to dissociate potential TMEM55B-interacting proteins and diluted 11 times with lysis buffer. 15 µL anti-HA agarose beads were used to precipitate HA-Ubiquitinated proteins at 4°C for 4 hrs. Tubes were spun at 1000 rpm (3 min), washed 3x with Lysis Buffer and 3x with low salt HNTG. Proteins were eluted with 30µl 1xSDS-PAGE sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was probed for the presence of ubiquitinated, mCherry-TMEM55B.

4 Results

4.1 Identification of candidate LMPs with putative PY motifs.

All LAPTM members utilize PY motifs to traffic to the lysosome. We set out to examine the possibility that other unrelated lysosomal transmembrane proteins also contain PY motifs. To this end the amino acid sequences of the 215 lysosomal proteins identified in a mass spectrometric analysis of rat liver tritosomes [416] were examined for the presence of putative L/PPXY motifs.

Examination of the 215 published rat amino acid sequences of lysosomal membrane proteins, revealed 23 candidate proteins containing putative PY motifs (Table 10). Of these 23 proteins, only 20 had conserved PY motifs in their rat and human orthologs. This constitutes
approximately 10% of the sample examined and to our knowledge is the first examination of PY motif frequency across a defined organelle sample.

Table 10: Candidate Rat LMPs with PY Motifs.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession Number</th>
<th>Putative PY motif</th>
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</thead>
<tbody>
<tr>
<td>UDP glycosyltransferase 2 family, polypeptide B</td>
<td>13928718</td>
<td>PPSY, LPWY</td>
</tr>
<tr>
<td>UDP-glucuronosyltransferase 2 family, member 5</td>
<td>34876712</td>
<td>PPSY, LPWY</td>
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<tr>
<td>Niemann Pick C1</td>
<td>6679104</td>
<td>PPVV</td>
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<tr>
<td>Aldehyde dehydrogenase</td>
<td>13929028</td>
<td>LPQY</td>
</tr>
<tr>
<td>Apyrase (soluble calcium-activated nucleotidase 1)</td>
<td>21426787</td>
<td>PPGY</td>
</tr>
<tr>
<td>Arachidonic acid epoxygenase (CYP2C8)</td>
<td>13929204</td>
<td>PPPY</td>
</tr>
<tr>
<td>CYP2C29</td>
<td>9506529</td>
<td>PFPY</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>6978747</td>
<td>LPIY</td>
</tr>
<tr>
<td>Macrophage expressed gene 1 *</td>
<td>12018298</td>
<td>LPPY*</td>
</tr>
<tr>
<td>Ribophorin I</td>
<td>6981486</td>
<td>LPYY</td>
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<tr>
<td>Phosphatidylinositol 4-kinase type II</td>
<td>16758554</td>
<td>PPEY</td>
</tr>
<tr>
<td>Purinergic receptor P2X4</td>
<td>13928806</td>
<td>LPRY</td>
</tr>
<tr>
<td>Similar to RIKEN cDNA 1300006M19 (BAI1-associated protein 2-like 1)</td>
<td>34870394</td>
<td>PPDY</td>
</tr>
<tr>
<td>Molecular transport ATP-binding cassette, sub-family B (MDR/TAP), member 6</td>
<td>18034785</td>
<td>LPGY</td>
</tr>
<tr>
<td>Similar to transmembrane 9 superfamily protein member 4</td>
<td>34859018</td>
<td>PPQY</td>
</tr>
<tr>
<td>Voltage-dependent anion channel 1</td>
<td>6755963</td>
<td>PPTY</td>
</tr>
<tr>
<td>5’nucleotidase, ecto *</td>
<td>11024643</td>
<td>LPSY*</td>
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<tr>
<td>Lysosomal acid phosphatase</td>
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<td>Hypothetical protein XP_236205 (SidT2)</td>
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<td>Similar to hypothetical protein 5031407H10 (MON1B)</td>
<td>34851786</td>
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* PY motifs are not conserved between rat and human amino acid sequences. Yellow = able to bind Nedd4.
The predicted membrane topology of the 20 candidate proteins was examined using TMpred.
Table 11 short lists 9 candidate proteins whose predicted membrane topology suggests a potential cytosolic location of the putative PY motif. Cytosolic localization of the PY motif is assumed to be a prerequisite for binding to Nedd4 WW domains.
### Table 11: Candidate Human LMPs with putative cytosolic PY Motifs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name</th>
<th>GenBank ID</th>
<th>Putative PY Motif</th>
<th>Molecular Weight (kDa)</th>
</tr>
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<tbody>
<tr>
<td>Purinergic receptor P2X, ligand-gated ion channel, 4</td>
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<td>LPRY</td>
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<td>Calcium activated nucleotidase 1 Ribophorin I</td>
<td>CANT1</td>
<td>BC017655.1</td>
<td>PPGY</td>
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<tr>
<td>Aldehyde dehydrogenase 2 family</td>
<td>ALDH2</td>
<td>BC002967.1</td>
<td>LPSY</td>
<td>68.3</td>
</tr>
<tr>
<td>BAI1-associated protein 2-like 1</td>
<td>BAIAP2L1</td>
<td>BC013888</td>
<td>PPDY</td>
<td>57.5</td>
</tr>
<tr>
<td>Molecular transport ATP-binding cassette, sub-family B (MDR/TAP), member 6</td>
<td>ABCB6</td>
<td>BC000559.2</td>
<td>LPSY</td>
<td>92.7</td>
</tr>
<tr>
<td>Voltage-dependent anion channel 1</td>
<td>VDAC1</td>
<td>BC008482.1</td>
<td>PPTY</td>
<td>30.8</td>
</tr>
<tr>
<td>Transmembrane protein 55B</td>
<td>TMEM55B</td>
<td>BC002867</td>
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<td>30.0</td>
</tr>
<tr>
<td>Family with sequence similarity 3, member C</td>
<td>FAM3C</td>
<td>BC046932</td>
<td>PPRY</td>
<td>24.7</td>
</tr>
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</table>

Together this analysis confirmed that putative PY motifs are present in additional lysosomal membrane proteins. Whether or not additional lysosomal membrane proteins utilize PY motifs and/or Nedd4 in their subcellular trafficking was addressed next.

### 4.2 Nedd4 binds to TMEM55B

We next examined whether or not Nedd4 was able to bind to the lysosomal membrane proteins with putative cytosolic PY motifs (Table 11). We co-expressed the candidate Flag-tagged lysosomal proteins (ALDH2, VDAC1, CANT1, RPN1, BAIAPL1, P2RX4, ABCB6, FAM3c or TMEM55B) together with V5-tagged Nedd4. We included Flag-tagged YY1 as a positive control for Nedd4 binding[417]. Immunoblot analysis revealed binding of Nedd4 to TMEM55B, but to none of the other candidate proteins (Figure 3.1A, B).

This data suggests that another lysosomal membrane protein, namely TMEM55B, is able to co-immunoprecipitate (co-IP) with Nedd4 in HeLa cells.
Figure 3.1: **Nedd4 binds to TMEM55B in HeLa cells.** Co-IP was performed on whole cell lysates from HeLa cells co-expressing V5-tagged Nedd4 and Flag-tagged candidate proteins. Candidate proteins in (A) included ABCB6, ALDH2, BAIAPL1, CANT1, RPN1, P2RX4. In (B) they included ABCB6, FAM3c, RPN1, TMEM55B, VDAC1. Candidate proteins were IP-ed with anti-Flag agarose and binding of V5-tagged Nedd4 to the candidate was assessed by SDS-PAGE and Immunoblot analysis. Flag-tagged YY1 was included as a positive control for Nedd4 binding.
Figure 3.1: Nedd4 binds to TMEM55B in HeLa cells.
4.3 ABCB6 binds to other Nedd4 Family Members

Since we did not see binding for eight out of nine candidate proteins to Nedd4, I tested the possibility that other Nedd4 family members may bind these proteins. I thus repeated the co-IP experiments as above, however I included additional Nedd4 family members, which also possess WW domains capable of binding PY motifs [7]. My results show that while Nedd4 (Nedd4-1), Nedd4-2, Smurf2 and Itch did not bind ABCB6, WWP2 and Smurf1 did (Figure 3.2).

This data suggests that Nedd4 family members WWP2 and Smurf1 are able to co-immunoprecipitate with specific LMPs in HeLa cells.
Figure 3.2: **WWP2 and Smurf1 bind ABCB6 in HeLa Cells.** co-IP was performed on whole cell lysates from HeLa cells co-expressing V5-tagged Nedd4 family members (V5-tagged E3 Ligase) and Flag-tagged ABCB6. ABCB6 was immunoprecipitated with anti-Flag agarose and binding of Nedd4 family members was assessed by SDS-PAGE and immnoblotting analysis as indicated. Note: Here, Nedd4 is referred to as Nedd4-1 to distinguish it from Nedd4-2.
Figure 3.2: WWP2 and Smurf1 bind ABCB6 in HeLa Cells.
4.4 TMEM55B’S PY Motif Mediates Binding to Nedd4

Since Nedd4 was able to co-immunoprecipitate with TMEM55B in HeLa cells, I next examined whether the binding was mediated via TMEM55B’s PY motif. To this end, a PY motif mutant (P66A) of TMEM55B was generated in which I substituted the conserved proline of the putative PY motif with an Alanine (PYPY>PAPY) (Figure 3.3A). This substitution should preclude Nedd4 binding to TMEM55B, if the interaction is mediated via the PY motif.

Indeed, preliminary data indicates that while Nedd4 is able to co-immunoprecipitate with WT TMEM55B, this interaction is lost upon co-expression of the P66A TMEM55B mutant with Nedd4 (Figure 3.4). The loss of observed binding is not likely due to protein misfolding, since P66A-TMEM55B runs at its predicted molecular weight and is not retained in the ER (Figure 3.5).

Thus, these data suggest that Nedd4 binds TMEM55B via its PY motif.
Figure 3.3: **The inositol polyphosphate 5-phosphatase TMEM55B.** TMEM55B is a Type 1 phosphatidylinositol 4,5-bisphosphate 5-phosphatase [418]. (A) Annotated TMEM55B amino acid sequence (Accession: BC002867). The conserved CX₅R motif is predicted to be the active site of its phosphatase activity. (B) TMEM55B catalyzes the degradation of PtdIns-4,5P₂ by removing the 4-phosphate.
A

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<td>G</td>
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<td>AWAFV ILLAVLCLGR AL</td>
<td>YW</td>
<td>ACMKV</td>
<td>S HPV</td>
</tr>
</tbody>
</table>

**Red**: Putative PY Motif  
**Purple**: Putative YXXΦ Motif  
**Yellow**: Predicted TM Domains  
**Blue**: CX₃R motif (Active Site)

B

**Phosphatase Activity:**

\[
1\text{-phosphatidylinositol-4,5-bisphosphate} + H_2O = 1\text{-phosphatidyl-inositol 5-phosphate} + \text{phosphate}
\]

**Figure 3.3**: The inositol polyphosphate 5-phosphatase TMEM55B.
Figure 3.4: **Nedd4 binds TMEM55B via its PY motif.** Co-immunoprecipitation was performed on whole cell lysates from HeLa cells co-expressing V5-tagged Nedd4 and Flag-tagged WT- or PY- mutant (P66A) TMEM55B. TMEM55B was immunoprecipitated with anti-Flag agarose and the binding of V5-tagged Nedd4 was assessed by SDS-PAGE and immunoblot analysis.
Figure 3.4: Nedd4 binds TMEM55B via its PY motif.
Figure 3.5: **P66A is not retained in the ER.** (A) Confocal images of HeLa cells expressing WT or P66A mCherry-TMEM55B. Cells were stained for their plasma membrane (ConA, blue), nucleus (DAPI, cyan) and ER (Calnexin, green). Colocalization was assessed using Volocity Software. (B) Colocalization of mCh-TMEM55B the ER marker Calnexin was quantified, graphed and tabulated in terms of the Pearson’s coefficient. Degrees of freedom are noted as (r), level of significance as (p). Error bars indicate SEM.
Figure 3.5: P66A is not retained in the ER.
4.5 The PY motif of TMEM55B Contributes to Its Lysosomal Localization.

Since Nedd4 binds TMEM55B via its PY motif, I wanted to determine whether the PY motif played a role in TMEM55B trafficking to the lysosome.

GFP-TMEM55B was previously shown to localize to late endosomal and lysosomal membranes in Cos7 and HeLa cells [418]. Similarly, our WT mCherry-TMEM55B, much like LAPTM4b used here as a positive control, colocalized significantly with the lysosomal marker Lamp1 in HeLa cells (Figure 3.6).

When I compare the localization of WT and P66A TMEM55B, both proteins localize to the lysosomal compartment (Figure 3.7A). However, there is a decrease in the degree of Lamp1 colocalization for the P66A mutant (Figure 3.7B). While the overall pattern of P66A staining is vesicular, not all vesicles colocalize with the Lamp1 marker. There is a 28% decrease in colocalization of P66A with Lamp1, relative to WT TMEM55B.

Because TMEM55B staining appeared consistently clustered for both WT and P66A proteins, I also stained for colocalization with the Golgi marker Giantin (Figure 3.8). There was no change in Giantin colocalization.

Together these data suggest TMEM55B’s PY motif contributes in part to its lysosomal localization.
Figure 3.6: **mCherry-TMEM55B localizes to lysosomes.** (A) Confocal images of fixed HeLa cells expressing mCh-TMEM55B or mCh-LAPTM4b. Cells were stained for the plasma membrane (ConA, blue), nucleus (DAPI, cyan) and lysosome (Lamp1, green). Colocalization was assessed using Volocity Software. (B) Colocalization of mCh-TMEM55B and mCh-LAPTM4b with Lamp1 was quantified, graphed and tabulated in terms of the Pearson’s coefficient. Degrees of freedom are noted as (r), level of significance as (p). Error bars indicate SEM.
Figure 3.6: mCherry-TMEM55B Localizes to Lysosomes.
Figure 3.7: **P66A shows decreased lysosomal colocalization relative to WT TMEM55B.** (A) Confocal images of HeLa cells expressing WT or P66A mCherry-TMEM55B. Cells were stained for their plasma membrane (ConA, blue), nucleus (DAPI, cyan) and Lysosomes (Lamp1, green). Colocalization was assessed using Volocity Software. (B) Colocalization of mCh-TMEM55B with the Lamp1 marker was quantified, graphed and tabulated in terms of the Pearson’s coefficient. Degrees of freedom are noted as (r), level of significance as (p). Error bars indicate SEM. *p<0.0001 (student t-test).
Figure 3.7: P66A shows decreased lysosomal colocalization relative to WT TMEM55B.
Figure 3.8: TMEM55B's PY motif does not affect Golgi localization. (A) Confocal images of HeLa cells expressing WT or P66A mCherry-TMEM55B. Cells were stained for their plasma membrane (ConA, blue), nucleus (DAPI, cyan) and Golgi (Giantin, green). Colocalization was assessed using Volocity Software. (B) Colocalization of mCh-TMEM55B the Golgi marker was quantified, graphed and tabulated in terms of the Pearson’s coefficient. Degrees of freedom are noted as (r), level of significance as (p). Error bars indicate SEM.
Figure 3.8: TMEM55B's PY motif does not affect Golgi localization.
4.6 Nedd4 does not ubiquitinate TMEM55B in HeLa cells.

Typically substrates that are bound by Nedd4 are subsequently ubiquitinated. Since Nedd4 binds TMEM55B via its PY motif I performed an ubiquitination assay to determine whether Nedd4 ubiquititates TMEM55B.

Whether TMEM55B was co-expressed with HA-ubiquitin alone or in combination with HA-Ubiquitin and WT-Nedd4, I was unable to detect TMEM55B ubiquitination in HeLa cells (Figure 3.9).

This result suggests that while Nedd4 is able to bind to TMEM55B via its PY motif, it does not lead to its ubiquitination.
Figure 3.9: **Nedd4 does not ubiquitinate TMEM55B.** Whole cell lysates of HeLa cells co-expressing V5-Nedd4 (WT or catalytically inactive mutant (CS)), mCh-TMEM55B and/or HA-Ubiquitin were used for detection of TMEM55B ubiquitination. HA-Ubiquitin tagged proteins were co-immunoprecipitated with anti-HA Agarose and separated by SDS-PAGE. TMEM55B ubiquitination was monitored for with anti-mCherry antibodies.
Figure 3.9: Nedd4 does not ubiquitinate TMEM55B.
5 Discussion

I show that TMEM55B binds Nedd4 via its PY motif and that TMEM55B localization is altered in the absence this motif. Importantly, I also demonstrate the binding of additional Nedd4 family members, WWP2 and Smurf1, to another LMP, namely ABCB6.

I identified putative PY motifs in ~20% of LMPs isolated previously by a proteomic approach[416]. The PY motifs of nine of these candidates were predicted to be cytosolic and therefore their ability to bind Nedd4 was investigated. The function of these candidate proteins was varied, including roles in metabolism (ALDH2[419] and CANT1[420]), biosynthesis (RPN1[421]), membrane signaling (BAIAPL1[422] and P2RX4[423]), secretion (FAM3c, [424]), molecular transport (ABCB6[425] and VDAC1[426]) and membrane composition (TMEM55B [418]). I was only able to show that TMEM55B binds Nedd4 via its PY motif in HeLa cells.

TMEM55B is also known as “Type 1 phosphatidylinositol 4,5-bisphosphate 4-phosphatase.” It catalyzes the degradation of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P(2)) to phosphatidylinositol-5-phosphate (PtdIns-5-P) [418]. PtdIns-5-P is a phosphoinositide expressed in plasma, endosomal and nuclear membranes, where it plays roles in signal transduction, membrane trafficking and regulation of gene expression [427]. TMEM55B was previously shown to localize to the lysosome[418], which we confirmed in our HeLa cells. Interestingly we also detected mCherry-TMEM55B at the Golgi. This correlates with the observation that PtdIns-5-P is enriched at the Golgi, where it regulates vesicle trafficking [428]. To date no imaging has been performed on endogenous TMEM55B, nor has its role at during vesicular trafficking been investigated.

As with LAPTMs, loss of TMEM55B’s single PY motif did not eliminate lysosomal localization of the protein. This suggests that other lysosomal targeting motifs must exist to ensure lysosomal delivery of the protein. Indeed, much like LAPTMs, TMEM55B contains additional putative lysosomal targeting motifs: a Tyrosine-based and VL-Motif (Figure 3.3A). TMEM55B’s PY motif tyrosine (PPPYP) overlaps with the tyrosine of the putative tyrosine based motif (YSPL). Because I mutated the conserved proline in the PY motif (PPPYP>PAPY) my localization analysis should be specific to PY motif. The contribution of additional sorting
motifs in TMEM55B localization has not been investigated. The presence of additional putative sorting motifs opens up the possibility that TMEM55B is bound by additional sorting machinery (including GGA and AP proteins)[200], and that its subcellular distribution is likely spatiotemporally regulated.

Interestingly others observed that in response to DNA damage TMEM55B can translocate from its cytoplasmic location to the nucleus and trigger increases in nuclear PtdIns-5-P levels [429]. Increases in PtdIns-5-P levels are associated with increased acetylation and stabilization of p53, and thereby regulate p53-dependent apoptosis. PtdIns-5-P bind the plant homeodomain (PHD) finger of ING2 (inhibitor of growth protein-2), which leads to ING2-dependent activation of p53[430]. I did not observe nuclear localization of TMEM55B, however I also did not induce DNA damage. The mechanism regulating this translocation is not yet understood. Nedd4 family ubiquitin ligases had previously been seen to translocate from the cytoplasm to the nucleus. For instance WWP1 nuclear localization is regulated by the Notch receptor [431]. More importantly yeast Rsp5 was shown to contain nuclear localization and export signals, allowing it to shuttle between the nuclear and cytoplasmic compartments[432]. Upon sequence analysis, TMEM55B does not appear to contain any known nuclear signal sequences. It might be of interest to investigate a potential role for Nedd4 in this translocation mechanism, particularly under conditions of nuclear stress, such as induction of DNA damage, which have previously been shown to lead to TMEM55B nuclear translocation.

While binding of Nedd4 to PY motif-containing proteins generally results in their ubiquitination, this is not always the case. In a protein array screen for unique Nedd4 substrates our lab determined that ~30% of proteins bound by Nedd4 are not ubiquitinated by it. For instance, MAP2 (Microtubule-associated protein 2) was found to bind Nedd4, but was not ubiquitinated in HeLa cells [433]. This is similar to what I observed for TMEM55B: it was bound by Nedd4, but not ubiquitinated by it in HeLa cells. In the case of LAPTM5, ubiquitination occurred, however it was not necessary for its translocation to the lysosome[78]. Instead, it was the ubiquitination of the GGA3 protein that dictated translocation to the lysosome through binding to LAPTM5’s UIM. It is possible that additional proteins associate with the TMEM55B-Nedd4 complex and that instead these proteins are ubiquitinated by Nedd4.
Finally, while I only identified one novel Nedd4 binding partner, I demonstrated that Nedd4 family members WWP2 and Smurf1 can bind to the putative PY motif in another lysosomal transmembrane protein: ABCB6. ABCB6 belongs to the superfamily of ATP-binding cassette (ABC) transporters and is localized to the plasma membrane and lysosome [434]. Its lysosomal targeting determinants have not been examined to date. Neither WWP2, nor Smurf1 has previously been implicated in LMP trafficking or function. WWP2 regulates PTEN and SMAD levels [124, 435], while Smurf1 has been mostly implicated in TGF-beta signaling[436]. While further investigation is required, co-immunoprecipitation of ABCB6 with WWP2 and Smurf1 in HeLa cells opens the possibility that other Nedd4 family members might play a role in targeting and/or function of lysosomal membrane proteins.

In summary, Chapter 3 we have demonstrated that PY motifs and Nedd4 likely play a conserved role in lysosomal membrane protein targeting. Furthermore, I identified novel Nedd4 family binding partners (TMEM55B and ABCB6) in which the functional consequences of the interaction remain to be explored. Identifying PY motifs as key players in lysosomal membrane protein trafficking is important in expanding our understanding of lysosomal biogenesis.
Chapter 4
The lysosomal protein LAPTM4b recruits the Leu Transporter to lysosomes to activate mTORC1

The work presented in the chapter is collaborative in nature and currently being prepared for publication.


My individual contribution to the work presented in this chapter include the identification and validation of the LAT1-4F2hc interaction, generation of the LAPTM4b-KD HeLa cell lines, CHX stability experiments and all immunofluorescence imaging. I also assisted in the generation of all bacterial and mammalian expressions constructs, purification of bacterially expressed proteins, optimization of mTORC1 EAA stimulation conditions and lysosomal isolation protocols. Contributions to this work by Dr. Persaud are indicated in Figures 4.4, 4.7, 4.8, 4.10, 4.11, 4.13, 4.14 and 4.15.
1 Abstract

mTORC1, a master regulator of cellular growth, stimulates protein synthesis and inhibits autophagy. It is activated downstream of growth factors and energy signaling, and depends on intracellular essential amino acids (EAA) such as Leu. mTORC1 activation takes place at the lysosomal membrane, and involves V-ATPase stimulation by intra-lysosomal EAA (inside-out activation), leading to activation of the Ragulator, RagA/B-GTP and mTORC1 via Rheb-GTP. How Leu enters the lysosomes is unknown. Here we identified the lysosomal protein LAPTM4b as a binding partner for the Leu Transporter, LAT1-4F2hc (SLC7A5-SLAC3A2). LAPTM4b recruits the plasma membrane localized LAT1-4F2hc to lysosomes, leading to uptake of Leu into lysosomes and stimulation of mTORC1 activation via V-ATPase following EAA stimulation. These results provide the first demonstration of localization and function of the Leu transporter at the lysosome, and help explain the inside-out activation of mTORC1 by EAA in this organelle.

2 Introduction

The Ser/Thr kinase complex, mammalian Target Of Rapamycin 1 (mTORC1), is a master regulator of energy metabolism, cell and animal growth, autophagy and numerous diseases, including cancer [180, 437]. It is a key mediator of protein translation (and a suppressor of autophagy) downstream of activated growth factor receptor, energy mediators, and amino acid influx[438]. Its localization in the cell is largely dictated by the availability of nutrients[439].

Influx of essential amino acids (EAA), such as Leu, into cells is mediated by the LAT1-4F2hc(CD98) (aka SLC7A5/SLC3A2, or System L) transporter [440, 441] in exchange for Glutamine efflux [380] and results in the recruitment of the mTORC1 complex to the lysosomal membrane by Rag GTPases [181, 367]. Leu/EAA influx into cells leads to their subsequent entry into lysosomes by unknown mechanism(s), where they activate the H⁺ATPase (v-ATPase) that is located at the lysosomal membrane and where it is bound to the Ragulator complex, itself tethered to the lysosomal membrane. Such v-ATPase activation takes place by an intra-lysosomal (inside-out) mechanism [182]. This, in turn, induces activation of the Ragulator-bound RagA/B (RagA/B-GTP), resulting in mTORC1 activation by Rheb-GTP [182, 357, 362].
Together, this nutrient sensing machinery is referred to as the LYNUS (Lysosomal Nutrient Sensing) system[147].

Activation of mTORC1 stimulates phosphorylation of S6K and 4E-BP, leading to enhancement of protein translation [442]. mTORC1 activation also leads to inhibition of AMPK and autophagy [443]. A critical unsolved question is how does Leu/EAA enter the lysosomes in the first place to promote activation of mTORC1 via v-ATPase?

LAPTM4b has several splice isoforms, including a 35 and a 24 kDa isoforms that are identical except for a 91 residue N terminal extension of the former [268]. The 35 kDa isoform is known to be overexpressed in numerous tumor types and its gene amplified in breast cancer [444, 445]. It was proposed to promote cancer by stimulating the PI3K/Akt pathway[315]. Whether the short (24kDa) isoform of LAPTM4b, studied here, is also involved in promoting cancer is not known. LAPTM4b is mainly localized at the lysosomal membrane but a fraction of the protein is also found at the plasma membrane; LAPTM4b lysosomal localization is mediated, in part, by its interaction with the ubiquitin ligase Nedd4 ([415], Chapter 2)

In search for LAPTM4b (24 kDa isoform) binding partners using mass spectrometry, we identified 4F2hc/CD98 (SLC3A2) and LAT1 (SLC7A5) as LAPTM4b associated proteins (Figure 4.1). Chapter 4 is a collaborative effort that provides evidence that LAPTM4b recruits the Leu transporter LAT1-4F2hc to lysosomes, enhances Leucine uptake into lysosomes and stimulates mTORC1 activation upstream of the v-ATPase.
Figure 4.1: **Diagramatic representation of LAPT4b, 4F2hc and LAT1 in a cell.** 4F2hc binds LAT1 via a disulfide bond (S-S), and together LAT1-4F2hc function as complex of the System L family of transporters that allow influx of EAA across the Plasma Membrane (PM). While LAPT4b is depicted at the lysosome, some of the LAPT4b proteins are also expressed at the Plasma Membrane (PM). aa = amino acid length of cytoplasmic regions of proteins.
Figure 4.1: Diagramatic representation of LAPTM4b, 4F2hc and LAT1 in the cell.
3 Materials and Methods

Cell Lines, Antibodies and Reagents

Unless indicated otherwise, HeLa cells (ATCC) were cultured in full media (DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100µg/ml streptomycin). Cells were transfected using Polyjet (Frogga, Bioscience, Canada). Cells were starved overnight in RPMI 1640 medium w/o amino acids, sodium phosphate (Powder) (US Biological). Cells were stimulated with RPMI supplemented with 1xMEM (Minimal essential medium) (Life Technologies). Concanamycin A (Sigma) was used as indicated. The following antibodies were used: mouse monoclonal [H4A3] to Lamp1 (Abcam), rabbit anti-human Giantin (Abcam), rabbit anti-human Calnexin (Abcam), normal Goat Serum (Jackson ImmunoResearch Laboratories Inc.), Alexa-Fluor 647 conjugated Concanavilllin A (ConA, Invitrogen), Alexa-Fluor 488 goat-anti mouse or anti-rabbit 2° antibody (Invitrogen) and DAPI (Molecular Probes). Slides were mounted using Darkocytomation (Dako Corporation). For Immunoprecipitation and Western Blotting we used Living Colors mCherry (mCh-) monoclonal antibody (Clontech), anti-ß-actin (Sigma), anti-Hemagglutinin (HA, Covance), anti-FLAG M2 affinity agarose (Sigma), anti-HA agarose (Pierce), rabbit anti-phospho-p70 S6 Kinase (pS6K-Thr389 or pp70) (Cell Signaling), mouse S6K (p70) SC-8418 (Santa Cruz), rabbit LAT1 (Cell Signaling), goat anti-CD98 (4F2hc) (C-20) sc-7095 (Santa Cruz) and rabbit anti-ATP6V0c (ab104374) (Abcam). We employed horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG(L chain only) or Rabbit anti-Goat IgG (H+L) 2° antibodies (Jackson ImmunoResearch laboratories Inc.). Blots were developed with Western Lightning Plus-enhanced chemiluminescence (ECL, Perkin Elmer). Radioactive Leucine uptake was monitored with [3H]-Leucine (Perkin-Elmer).

Constructs

We obtained the Human ORFeome cDNA entry clones (pDONR223) for LAT1 (GenBank: BC039692), 4F2hc (GenBank: BC003000) and Lamp1 (GenBank: BC021288). Each construct was N-terminally tagged with Flag and/or mCherry using the Gateway cloning system (Invitrogen). LAPTMM4b truncation mutants were subcloned from the LAPTMM4b pDONR223 vector (GenBank: AAH31021.1): ΔC-LAPTMM4b (amino acids 1-181), ΔN-LAPTMM4b (amino acids 21-226), ΔNΔC-LAPTMM4b (amino acids 21-181). N-terminal GST fusion constructs for
bacterial expression were generated by subcloning of LAT1 and 4F2hc from pDONR223 vectors into the N-terminal-GST pDEST15 vector: N-terminus of 4F2hc (GST-N-4F2hc, amino acids: 1-121), N-terminus of LAT1 (GST-N-LAT1, amino acids 1-47) and C-terminus of LAT1 (GST-C-LAT1, amino acids 408-507).

**Identification of LAPTM4b binding partners by affinity-tag immunoprecipitation (IP) and Mass Spectrometry (MS).**

The protocol designed for affinity-tag IP of HA-LAPTM4b-WT and sample preparation for tandem MS was modified from a previously established protocol [446]. Briefly, HeLa cells were transiently transfected with HA-LAPTM4b-WT for 48 hrs. Cells were lysed on ice in Lysis Buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 1.0 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 1 mM PMSF) and HA-LAPTM4b was immunoprecipitated with anti-HA Agarose beads. Beads were washed three times with lysis buffer and with low salt HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% TritonX-100). Bound proteins were eluted with 0.1% Trifluoroacetic acid (TFA) and peptides were generated by trypsin digestion (Biolab, TPCK, #P8101S). Peptide fragments were desalted using Pierce® C-18 Spin Columns (Thermo Scientific, #89870, Lot#NJ176772). The eluted peptide mix was analyzed by Shotgun Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.3.0.339) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Scaffold (version Scaffold_3.6.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications.

**Immunofluorescence and Confocal Microscopy**

HeLa cells were cultured on poly-D-Lysine coated coverslips in 6-well-plates and transiently transfected with the appropriate cDNA. 24hrs post transfection wells were washed 3x with 1ml PBS and incubated 5 min with Alexa-Fluor-647-conjugated ConA (1:1000). After three PBS washes, cells were fixed with 4% Paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and incubated with 1:100 Normal Goat Serum in 3% Skim Milk (30 min). Slides were incubated 1 hr with mouse anti-human Lamp1 (1:1000), rabbit anti-human Giantin (1:2000) or rabbit anti-human Calnexin (1:1000) in 3% Skim milk. After three PBS washes, cells were
incubated with goat anti-mouse or anti-rabbit Alexa 488 Fluor-conjugated 2\textsuperscript{0} antibody and briefly stained with DAPI. Cover slips were mounted with Dako Cytomation. Images were acquired using a Quorum WAveFX-X1 spinning disc confocal system at 60x magnification with an Olympus S-Apo 60x/1.35 oil objective (Quorum Technologies Inc., Guelph, Canada). Colocalization of organelle markers and tagged proteins was assessed by Volocity 5.4.1 (Perkin Elmer) and expressed in terms of the Pearson’s correlation coefficients.

**Co-Immunoprecipitation (Co-IP) Assays**

HeLa cells were co-transfected with the indicated cDNA constructs. At 48 hrs, cells were lysed on ice in 1ml Lysis buffer and centrifuged at 12 000 rpm (30 min, 4 °C). 1-2 mg of lysate was incubated with 15 µl anti-Flag or anti-HA agarose beads (4 °C, 4 hrs). Tubes were spun 1000 rpm (3 min), washed 3x with Lysis Buffer and 3x with low salt HNTG. Proteins were eluted with 30µl 1xSDS-PAGE sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblots were developed with ECL. Images were analyzed using the digital Li-COR Biosciences System.

**In Vitro binding of LAPTM4b to LAT1-4F2hc**

GST-tagged proteins (GST-N-4F2hc, GST-N-LAT1 and GST-C-LAT1) were purified as previously described [417]. *E.coli* (BL21DE3) were transformed and 100mL Luria Broth (LB) grown at 37°C to an absorbance of A\textsubscript{590} of 0.6. Expression was induced with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Promega) for 3 hrs at 30°C. Cells were lysed by sonication in Sonication Buffer (137 mM NaCl, 2.7 mM KCl, 10mM KH\textsubscript{2}PO\textsubscript{4}, 0.5 mM Na\textsubscript{2}HPO\textsubscript{4}.7H\textsubscript{2}O, pH 7.4, 0.1M PMSF and 100µg/ml leupeptin, 100 µg /ml aprotinin, 100 µg /ml pepstatin, 1 mM PMSF, Lysozyme and DNAse), 0.15% Triton-X 100 was added to lysates, which were then clarified by centrifugation (30 min, 10 000 rpm, 4°C). GST-tagged proteins were affinity purified by incubation with glutathione-sepharose resin (Amersham Biosciences) at 4°C for 1.5 hrs. The N-terminus of LAPTM4b was synthesized as a N-terminally biotinylated peptide (Biotin-MKMVAPWTRFYSNSCCLCCHVRTGT) by JPT Innovative Peptide solutions (Berlin, Germany). Binding of the biotinlyated-peptide to GST-fusion proteins was assessed as described previously [89]. 2 µg of GST-fusion protein (LAT1 or 4F2hc termini) immobilized on glutathione-sepharose resin were incubated with 10 mM biotinylated-peptide in PBS at 4°C for 1.5 hrs. All samples were washed three times with HNTG (50mM HEPES (pH7.5), 150 mM
NaCl, 0.1% Triton X-100, 10% glycerol). Samples were eluted with 25µL 1xSB and blotted onto nitrocellulose for Immunoblot analysis. Binding was detected by immunoblotting with streptavidin-HRP (Upstate 18-152). All blots were developed with ECL using the digital Li-COR Biosciences System.

**Generation of stable LAPTM4b knock-down (KD) cell lines**

Stable LAPTM4b KD cell lines were generated using pGIPZ-shRNA (Open Biosystems). HeLa cells were transfected with one of three independent pGIPZ-shRNA vectors: V2LHS_175452 (targets LAPTM4b open reading frame (ORF)), V3LHS_340114 (targets ORF), V3LHS_405603 (targets 3’UTR) or pGIPZ-Ctrl (Control, scramble). Transfected cells were cultured in full media supplemented with 1mg/mL Puromycin. GFP-positive colonies were selected and expanded as individual clones. LAPTM4b KD efficiency was monitored by quantitative PCR (SYBR Green, Sigma) with LAPTM4b unique primers F1 = 5’-TGTTACCAGCAATGACACTACG-3’ and R1 = 5’-ATGTCTGCAAAGTCAAGCTG-3’ (Integrated DNA Technologies). Cell proliferation was assayed by monitoring metabolic activity of cells with a colorimetric substrate (Alamar Blue assay kit (Life Technologies)).

**Amino Acid Stimulation and mTOR activation**

HeLa cells were seeded in full media (DMEM, FBS, Puromycin, Antifungal-Antibiotic) of 6 well plates. Where indicated cells were transfected with ATP6V0c-715 siRNA vector (abm). Cells were starved overnight with RPMI 1640 Medium without Amino Acids. Cells were re-stimulated with RPMI supplemented with Essential Amino Acid (EAA) solution for a given time. Where indicated, cells were pre-incubated with Concanamycin A (Sigma) for 1 hr prior to stimulation. Cells were lysed in Lysis Buffer on ice and mTORC1 activation was monitored by SDS-PAGE and immunoblotting as above.

**Radio-Leucine uptake by Lysosomes**

Control-KD (pGIPZ-ctrl stable HeLa cells) or LAPTM4b-KD HeLa cells were grown in four 10 cm dishes. 24 hrs post-seeding each dish was transfected with Flag-Lamp1. At 24 hrs post transfection, cells were starved overnight in RPMI. Cells were re-stimulated with RPMI supplemented with 4miCu [³H]-Leu. Samples were chased for 10 min, at which point uptake into
lysosomes was measured as described by [182]. For each cell type (Control or LAPTM4b-KD), HeLa cells from all four plates were pooled to generate a post-nuclear supernatant (PNS) by lysing cells through a 23G needle in fractionation Buffer (50mM KCL, 90mM K-Gluconate, 1mM EGTA, 5mM MgCl2, 50mM Sucrose, 20 mM HEPES, pH7.4, supplemented with 2.5mM ATP, 5mM Glucose and protease inhibitors (100µg/ml leupeptin, 100 µg /ml aprotinin, 100 µg /ml pepstatin, 1 mM PMSF). Samples were centrifuged for 10 min at 2000 rpm. The resulting light organelle fraction (pellet) was resuspended and topped up to 1 mL of fractionation buffer. 30µl Anti-FLAG M2 affinity agarose were added to each sample and incubated on a rotor at 4°C for 2 hrs. Samples were centrifuged for 1 min at 2000 rpm, washed 3 times with cold fractionation buffer. The affinity beads with bound lysosomes were transferred to scintillation vials using cut pipet tips and [3H]-Leu radioactivity was measured with a scintillation counter (MiKrowin 2000). Each experiment was done in triplicates. Successful enrichment for the lysosomal fraction was monitored by SDS-PAGE of lysates at various stages of the isolation.

**Protein stability assed by cycloheximide (CHX) chase**

A CHX chase was performed to determine stability of endogenous LAT1 and 4F2hc stability in Control-KD, LATPM4b-KD or LAPTM4b-KD cells reconstituted with transiently transfected mCherry-LAPTM4b. Cells were chased with 50µM CHX for 2, 4, 8, 16 or 24 hrs. Cells were lysed and the endogenous levels of LAT1 and 4F2hc were monitored by SDS-PAGE and immunoblotting. The relative abundance of each protein was normalized to the loading control actin. Images were quantified in Image Studio version 3.1.4 (LI-COR).
4 Results

4.1 Identification of Novel LAPTM4b Binding Partners by Mass Spectrometry (MS)

An affinity-tag based MS protocol was utilized to identify novel LAPTM4b binding partners. HA-tagged LAPTM4b was overexpressed and immunoprecipitated (IP) using anti-HA agarose beads from HeLa cell lysates. Proteins that co-immunoprecipitated with LAPTM4b were eluted, trypsin digested and analyzed by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

The top 25 unique hits as defined by the “Number of Assigned Spectra” identified from two independent MS runs are listed in Table 12. Not surprisingly the overexpressed LAPTM4b bait protein was a hit. Interestingly, 4F2 cell-surface antigen heavy chain (4F2hc) encoded by the gene SLC3A2, was a consistent top hit. Furthermore, the Large neutral Amino acids Transporter small subunit 1 (LAT1) encoded by SLC7A5, was also detected. LAT1-4F2hc form a functional amino acid transporter for Leu and other neutral essential amino acids[440, 441].

A number of heat shock proteins, keratins, tubulins, actins, ribonucleoproteins and ribosomal proteins were identified as unique to the LAPTM4b LC-MS/MS samples. However, these proteins are likely false-positives (indicated by * in Table 12) due to their shear abundance within cells, and belong to the most frequently detected genes in the CRAPome (Contaminant Repository for Affinity Purification) [447].

Thus, through affinity purification of HA-LAPTM4b and LC-MS/MS analysis, we newly identified LAT1 and 4F2hc as novel LAPTM4b associated proteins in HeLa cells.
Table 12 Top Hits for novel LAPMT4b binding partners from two LC-MS/MS datasets.

<table>
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<tr>
<th>GENE</th>
<th>Protein Name</th>
<th>TOTAL ASSIGNED SPECTRA</th>
<th>Accession Number</th>
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</thead>
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<td></td>
<td></td>
<td>RUN1</td>
<td>RUN2</td>
</tr>
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<td>HSPA8*</td>
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<td>Tubulin beta chain</td>
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<td>Keratin, type II cytoskeletal 5</td>
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<td>14</td>
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<td>Isoform 2 of Lysosomal-associated transmembrane protein 4B</td>
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<td>TECR</td>
<td>Trans-2,3-enoyl-CoA reductase</td>
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<td>11</td>
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<td>ADP/ATP translocase 2</td>
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<td>12</td>
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* Indicates likely contaminating protein typical of affinity purified LC-MS/MS.

Highlighting: Grey = LAPMT4b bait protein. Yellow = LAPMT4b binding partners that are further studied here.
4.2 LAPTM4b associates with 4F2hc and LAT1 in HeLa Cells

4F2hc and LAT1 were selected for further examination from the list of proteins that associated with LAPTM4b during MS analysis. We validated their association with LAPTM4b through co-immunoprecipitation experiments in HeLa cells. When Flag-4F2hc was co-expressed with HA-LAPTM4b, the two proteins co-immunoprecipitated with one another (Figure 4.2A,B). Similarly, LAT1 and LATPM4b co-immunoprecipitated when Flag-LAT1 was co-expressed with HA-LAPTM4b (Figure 4.2C). This validates the MS data results and shows that 4F2hc and LAT1 are LAPTM4b associated proteins.

4F2hc and LAT1 play essential roles in amino transport of essential amino acids. As such we examined the possibility that LAPTM4b might co-immunoprecipitate with other closely related amino acid transporters, namely System A glutamine transporters ASCT2 (Sodium-Dependent Neutral Amino Acid Transporter Type 2, SLC1A5) and transporter SNAT2 (Sodium-Coupled Neutral Amino Acid Transporter 2, SLC38A2) to which LAT1 is functionally coupled [379, 443].

Figure 4.3 demonstrates that when either mCherry-ASCT2 or mCherry-SNAT2 is co-expressed with HA-LAPTM4b in HeLa cells, neither transporter co-immunoprecipitates with LAPTM4b. This suggests LAPTM4b does not bind non-specifically to all plasma membrane transporters.

Together these data suggest LAPTM4b interacts with LAT1 and 4F2 (System L), but not with closely related System A transporters, in HeLa cells. It does not reveal the nature of the interaction (direct or indirect binding), which was examined in a later section.
Figure 4.2: LAPTM4b co-IPs with 4F2hc and LAT1 in HeLa cells. Hela cells were co-transfected with FLAG-4F2hc or FLAG-LAT1 and HA-LAPTM4b. 48 hrs post transfection cells were lysed on ice and 15 μL of Flag-agarose (A, C) or HA-agarose (B) were incubated with 1.5 mg of crude cell lysate for 4 hrs at 4°C. Samples were separated by SDS-PAGE, transferred to nitrocellulose and probed with the indicated antibodies. MW = molecular weight marker in kDa.
Figure 4.2: LAPTM4b co-IPs with 4F2hc and LAT1 in HeLa cells.
Figure 4.3: **LAPTM4b does not co-immunoprecipitate with ASCT2 (SLC1A5) or SNAT2 (SLC38A2) in HeLa cells.** Hela cells were co-transfected with mCherry-ASCT2 or mCherry-SNAT2 and HA-LAPTM4b. 48 hrs post transfection cells were lysed on ice and 15 µL of HA-agarose were incubated with 1.5 mg crude cell lysate for 4 hrs at 4°C. Samples were separated by SDS-PAGE, transferred to nitrocellulose and probed with the indicated antibodies.
Figure 4.3: LAPTM4b does not co-immunoprecipitate with ASCT2 (SLC1A5) or SNAT2 (SLC38A2) in HeLa cells.
4.3 Co-IP of 4F2hc and LAT1 with LAPTM4b is Independent of EAA Availability.

Influx of essential amino acids (EAA), such as Leucine into cells is mediated by the LAT1-4F2hc complex [440, 441]. In a variety of tissues like skeletal muscle [448] and proliferating T cells [449], increased availability of EAA upregulates AA transporter gene expression. Considering this dynamic relationship based on EAA availability, we examined whether EAA availability affected the interaction of LAPTM4b with LAT1 and 4F2hc.

HeLa cells co-expressing HA-LAPTM4B and Flag-4F2hc, were starved and stimulated with EAA for varying amounts of time. We found that co-IP of LAPTM4b with 4F2hc (Figure 4.4A) and LAT1 (Figure 4.4B) was independent of EAA availability.
Figure 4.4: **LAPTM4b binds 4F2hc and LAT1 independent of amino acid availability.** HeLa cells were co-transfected with HA-LAPTM4b and Flag-4F2hc (A) or mCherry-LAT1(B). After starvation, cells were restimulated with EAA for varying periods of time. (A) Flag-4F2hc was co-immunoprecipitated using anti-Flag agarose beads. (B) HA-LAPTM4b was immunoprecipitated using anti-HA agarose beads. Immunoblots show co-immunoprecipitation of LAPTM4b with 4F2hc (A) or LAT1 with LAPTM4b (B). DMEM = cells grown in full media. h = hrs.

* Figure 4.4A was contributed by Dr. A. Persaud.
Figure 4.4: LAPTM4b binds 4F2hc and LAT1 independent of amino acid availability.
4.4 LAPTM4b co-localizes with 4F2hc and LAT1 in HeLa cells

Upon validating the interaction of 4F2hc and LAT1 with LAPTM4b, we examined the consequence of this interaction.

Ordinarily 4F2hc and LAT1 are localized to the plasma membrane [440]. This was observed for both mCherry-4F2hc and mCherry-LAT1 overexpressed in HeLa cells (Figure 4.5). Strikingly, when mCherry-tagged 4F2hc or LAT1 are co-expressed with HA-LAPTM4b, there is a recruitment of System L components to the lysosome (Figure 4.5). This observation is supported by strong Pearson Correlation Coefficients ($r$) for both mCherry-4F2hc ($r=0.843$, with $n=84$) and mCherry-LAT1 ($r=0.842$, with $n=77$) colocalization with the lysosomal marker Lamp1.

Together, these data suggest that overexpression of LAPTM4b with 4F2hc or LAT1 in HeLa cells recruits System L components to the lysosome.
Figure 4.5: 4F2hc and LAT1 colocalize with LATPM4b in HeLa cells. HeLa cells were transfected with mCherry-4F2hc or mCherry-LAT1 alone or co-expressed together with HA-LAPTM4b. Cells were fixed 24 hrs post transfection and stained for the nucleus (DAPI, cyan) lysosomes (anti-Lamp1, green) and the plasma membrane (PM. ConA, blue) or LAPTM4b (anti-HA, green). All images were acquired by spinning disk confocal and processed in Volocity 5.4.1.
Figure 4.5: 4F2hc and LAT1 colocalize with LATPM4b in HeLa cells.
4.5 LAPTM4b stimulates mTORC1 activation.

Since LAPTM4b recruits LAT1 and 4F2hc to the lysosome (Figure 4.5) and LAT1 function is linked to the activation of mTORC1 [380], we next tested whether LAPTM4b expression could stimulate mTORC1 activation following EAA stimulation. mTORC1 is a Serine/Threonine kinase and ribosomal S6 Kinase (S6K, aka p70) is one of its best characterized substrates [437]. Thus, we assayed whether LAPTM4b expression affected EAA activation of mTORC1 by monitoring phosphorylation of S6K (p70) at residue T389 (p-p70).

In order to effectively study the role of LAPTM4b mTORC1 signaling, we generated several stable LAPTM4b knock-down (KD) cell lines using pGIPZ-shRNA (Figure 4.6). HeLa cells were transfected with one of three independent pGIPZ vectors encoding shRNA to knock-down Laptm4b (V2LHS_175452 (targets Open reading frame (ORF)), V3LHS_340114 (targets ORF), V3LHS_405603 (targets 3’UTR)) or pGIPZ-Ctrl (Control). Clones were individually selected, expanded and LAPTM4b KD efficiency was monitored by quantitative PCR. The efficiency of LAPTM4b KD was variable. We selected LAPTM4b-KD clone 603a for our analyses (unless indicated otherwise). LAPTM4b-KD clone 603a had the highest efficiency of LAPTM4b KD, and furthermore, because the shRNA targets LAPTM4b’s 3’UTR, reconstitution with epitope tagged LAPTM4b was possible.

In order to assess changes in mTORC1 activation, we monitored differences in p70 phosphorylation between Control-KD (Ctrl) and LATPM4b KD HeLa cell lines. We noticed that our 3 hr starvation protocol often retained weak pp70 signal at time 0 (Figure 4.7A). We therefore increased the stringency of our starvation protocol by starving cells overnight before stimulating them with EAA (Figure 4.7B). We observed very little to no background pp70 staining under these conditions, and furthermore were able to observe stronger pp70 activation upon EAA stimulation. Cell viability was not affected by this maneuver (Figure 4.7C). Our results show that the pp70 signal in LATPM4b-KD HeLa cells was consistently lower than that of Control-KD cells.
Figure 4.6: **Generation of LAPTM4b KD HeLa cell lines.** HeLa cells were transfected with one of three independent pGIPZ vectors encoding shRNA to knock-down LAPTM4b: V2LHS_175452 (sh452), V3LHS_340114 (sh114), V3LHS_405603 (Sh603) or pGIPZ-Control (shCtrl). Clones a-c were individually selected and expanded for each vector. LAPTM4b KD was assessed by quantitative PCR. LAPTM4b mRNA levels are expressed relative to LAPTM4b mRNA levels of the pGIPZ-Ctrl cell line.
Figure 4.6: Generation of LAPTM4b KD HeLa cell lines.
Figure 4.7: **Optimizing stable HeLa cell line starvation and EAA stimulation protocols.**

HeLa cells were starved for 3 hrs (A) or overnight (O/N) (B), then stimulated for variable lengths of time with EAA. mTORC1 activation was monitored by staining for pp70 relative to p70. (C) Cell viability for Control-KD (Control) and LAPT4b-KD (KD (603a)) HeLa cells that were starved overnight was assessed by Trypan Blue staining.

*Figure 4.7C was contributed by Dr. A. Persaud.*
Figure 4.7: Optimizing stable HeLa cell line starvation and EAA stimulation protocols.
4.6 LAPTM4b is required for full activation of mTORC1 by EAA.

Having generated the Control-KD and LAPTM4b-KD HeLa cell lines, we were next able to explore the requirement of LAPTM4b to mTORC1 activation in following EAA stimulation.

Firstly, we observed that mTORC1 activation in LAPTM4b-KD cells was impaired compared to mTORC1 activation in Control-KD cells (Figure 4.8A). Interestingly, reconstitution of mCherry-LAPTM4b into LAPTM4b-KD cells rescued mTORC1 activation to levels comparable to those of Control-KD HeLa cells (Figure 4.8B,C). This attenuated mTORC1 activation was observed in additional stable HeLa LAPTM4b KD clones (Figure 4.9), suggesting our results were not due to off-target effects of the knockdown, but rather specific to LAPTM4b KD. Next we observed that LAPTM4b was able to activate mTORC1 in a dose-dependent manner (Figure 4.10A,B). Finally, because it was known that mTORC1 activation correlated with increased cell growth and proliferation [438], we assayed for differences in cell proliferation across Control-KD, LAPTM4-KD and LAPTM4b reconstituted KD cells (Figure 4.10C). We observed that over time LAPTM-KD cells proliferated more slowly than Control-KD. More importantly, LAPTM4-KD cell proliferation was restored to levels similar to Control-KD cells upon LAPTM4b reconstitution.

Together, these data suggest LAPTM4b expression activates mTORC1 signaling in response to EAA stimulation and is in line with mTORC1 activity promoting cell proliferation.
Figure 4.8: **LAPTM4b KD attenuates mTORC1 activation, which is restore upon LAPTM4b rescue.** Control-KD, LAPTM4b-KD (A) and LAPTMb-KD cells with reconstituted mCherry-LAPTM4b (B) were starved overnight and stimulated with EAA (0-60 min). Cells were lysed on ice and mTORC1 activation was monitored by SDS-PAGE and immunoblotting for p-p70. DMEM indicates positive control cells (for p-p70) grown in full media. C) Quantification of p-p70:p70 ratios was done using Li-COR Image Studio software. Each data point represents data from three experiments. Error bars indicate the SEM.

*Figure 4.8 is contributed by Dr. A. Persaud.*
Figure 4.8: LAPTM4b KD attenuates mTORC1 activation, which is restored upon LAPTM4b reconstitution.
Figure 4.9: **Independent LAPTM4b-KD HeLa cell lines show attenuated mTORC1 activation in response to EAA.** Control-KD (Ctrl) and LAPTM4b-KD cell lines (KD452c, 114b, 452a or 603b) were starved overnight and stimulated with EAA between 0-30 min. Cells were lysed on ice and mTORC1 activation was monitored by SDS-PAGE and Immunoblot analysis for p-p70. “DMEM” indicates positive control cells for p-p70 grown in regular media. “Ladder” indicates wells in which molecular weight ladder was loaded.
Figure 4.9: Independent LAPTM4b-KD HeLa cell lines show attenuated mTORC1 activation in response to EAA.
Figure 4.10: **LAPTM4b promotes mTORC1 activation in a dose-dependent manner.** A) Control-KD and LAPTM4b-KD cells (with varying amounts of reconstituted mCherry-LAPTM4b) were starved overnight and stimulated with EAA for 15 min. mTORC1 activation was monitored by Immunoblotting for p-p70. B) Quantification of p-p70:p-70 ratio of blots from (A) using Li-COR Image Studio software. C) Cell Proliferation in Control-KD, LAPTM4b KD and LAPTM4B reconstituted KD cells was monitored by assaying metabolic activity using a colorimetric method (Alamar Blue) over the course of 5 days.

*Figure 4.10 is contributed by Dr. A. Persaud.*
Figure 4.10: LAPT4b promotes mTORC1 activation in a dose-dependent manner.
4.7 LAPTM4b requires active v-ATPase for mTORC1 activation

Since the lysosomal v-ATPase (H\(^{+}\)-ATPase) stimulates mTORC1 activation in response to intralysosome amino acid stimulation [182], we set out to test whether enhancement of mTORC1 activation by LAPTM4b involved the v-ATPase.

First, Control-KD, LAPTM4b-KD and LAPTM4b-reconstituted KD HeLa cells were starved and stimulated with EAA in the presence or absence of the v-ATPase inhibitor Concanamycin A (Figure 4.11A). Concanamycin A is a v-ATPase inhibitor that was shown to impair mTORC1 activation [182]. Upon EAA stimulation LAPTM4b-KD cells responded significantly less to EAA stimulation than Control-KD cells and reconstitution of LAPTM4b-KD cells with mCherry-LAPTM4b rescued the phenotype (Figure 4.11B). Not surprisingly, inhibiting the v-ATPase with Concanamycin A reduced mTORC1 activation for both Control-KD and LAPTM4b-KD cells. Interestingly though, reconstitution of LAPTM4b after its KD was not able to restore mTORC1 activation in the presence of Concanamycin A. This was the first indication that LAPTM4b activation of mTORC1 required v-ATPase activity.

Next, we knocked down the v-ATPase-V0c subunit, which is required for function of the ATPase in mTORC1 activation [182], in Control-KD and LAPTM4b-KD HeLa cells. Similar to inhibition with Concanamycin A, KD of the v-ATPase-V0c subunit prevented mTORC1 activation by LAPTM4b following EAA stimulation (Figure 4.11C,D).

Together these data suggest that LAPTM4b-mediated activation of mTORC1 requires active v-ATPase, and places LAPTM4b upstream of this H\(^{+}\) transporter.
Figure 4.11: **LAPTM4b requires active v-ATPase for mTORC1 activation.** (A) Control, LAPTM4b-KD and LAPTMb-KD cells with reconstituted HA-LAPTM4b were starved overnight and stimulated with EAA for 15 min in the presence or absence of Concanamycin A. Cells were lysed on ice and mTORC1 activation was monitored by SDS-PAGE and Immunoblotting analysis. (B) Quantification of p-p70:p70 ratios was done using Li-COR Image Studio software. Each data point represents data from three experiments. Error bars indicate the SEM. (C) Control-KD and LAPTM4b-KD HeLa cells were transiently transfected with Control or v-ATPase KD shRNA (Control shRNA and ATP6V0c shRNA), in the presence or absence of reconstituted HA-LAPTM4b. All cells were starved overnight and stimulated with EAA for 15 min. Cells were lysed on ice and mTORC1 activation and v-ATPase knockdown were monitored by SDS-PAGE and Immunoblot analysis. (D) Quantification of p-p70:p70 ratios was done using Li-COR Image Studio software. Each data point represents data from three experiments. Error bars indicate the SEM.

*Figure 4.11 is contributed by Dr. A. Persaud.*
Figure 4.11: LAPT4M4b requires active v-ATPase for mTORC1 activation.
4.8 LAPTM4b does not affect the stability of 4F2hc and LAT1

Considering the dramatic recruitment of LAT1-4F2hc to the lysosome upon co-expression with LAPTM4b, we performed a cycloheximide (CHX) pulse chase experiment to test whether this affects stability of endogenous LAT1 and 4F2hc.

Control-KD, LATPM4b-KD or LAPTM4b reconstituted LAPTM4b-KD cells were treated with the protein synthesis inhibitor CHX over a 24-hr period. Cells were lysed and the endogenous levels of LAT-1 and 4F2hc were quantified by immunoblotting (Figure 4.12A). In all cell lines, LAT1 and 4F2hc were degraded very slowly (<25% of the protein pool was degraded by 24 hrs) and moreover there was no significant change in LAT1 or 4F2hc stability in the presence or absence of LAPTM4b (Figure 4.12A, B).

These data suggest that LAPTM4b does not translocate System L to the lysosomes for degradation, and thus does not affect stability of this transporter.
Figure 4.12: **LAPTM4b does not affect LAT1 and 4F2hc Stability.** (A) HeLa cells were grown in full media and chased with 50 μM Cycloheximide (CHX) for 0-24 hrs. Cells were lysed on ice and lysates were separated by SDS-PAGE, immunoblotted and analyzed using Li-COR Image Studio software. (B) & (C) The amount of endogenous LAT1 and 4F2hc was graphed relative to the amount of actin over time. Error bars indicate the SEM of 3 independent experiments.
Figure 4.12: LAPT4b does not affect LAT1 and 4F2hc Stability.
4.9 LAPTM4b promotes Leu uptake into lysosomes

mTORC1 activation by amino acids occurs at the lysosomal membrane by intra-lysosomal EAA activating the v-ATPase [181]. The mechanism by which EAA enter the lysosome to stimulate v-ATPase function is unknown. Our data show that LAPTM4b recruits System L to lysosomes and promotes EAA-induced activation of mTORC1 in HeLa cells. Thus we examined the possibility that LAPTM4b promotes mobilization of LAT1-4F2hc to the lysosome to facilitate entry of EAA into lysosomes.

Analysis of [$^3$H]-Leu uptake across the plasma membrane showed no effect of LAPTM4b (data not shown). In contrast, we found that [$^3$H]-Leu uptake into lysosomes was significantly (~50%) reduced in LAPTM4b-KD HeLa cells compared to Controls, and reconstituted upon rescue of the KD cells with LAPTM4b (Figure 4.13).

Together, these data suggest that LAPTM4b promotes Leu uptake into lysosomes and mTORC1 activation, most likely by recruiting LAT1-4F2hc to the lysosomal membrane.
Figure 4.13: **LAPTM4b promotes Leu uptake into lysosomes.** Radiolabelled $[^3]$H-Leu uptake into the lysosome of Control-KD and LAPTM4b-KD HeLa cells was monitored. HeLa cells were transfected with Flag-Lamp1 or Flag-Lamp1 and mCherry-LAPTM4b (+LAPTM4b). At 24 hrs post-transfection cells were starved overnight and stimulated for 10 min in the presence of 4 miCu $[^3]$H-Leu. Lysosomes were isolated by anti-Flag-Lamp1 Immunoprecipitation [182]. Uptake of $[^3]$H-Leu into lysosomes was quantified by scintillation counting. SEM is indicated for three independent uptake experiments.

*Figure 4.13 is contributed by Dr. A. Persaud. I helped optimize the immune-isolation of lysosomes.*
Figure 4.13: LAPTM4b promotes Leu uptake into lysosomes.
4.10 LAPTM4b N and C termini are dispensable for association with LAT1-4F2hc.

The mode of interaction between LAPTM4b and LAT1-4F2hc (whether direct or indirect) is currently unknown. Figure 4.1 illustrates schematically the predicted membrane orientations of LAPTM4b, LAT1 and 4F2hc. Accordingly, 4F2hc’s N-terminus and LAT1’s N- and C-termini are cytosolic. Similarly, LATPM4b N- and C-termini are cytosolic.

We generated GST-fusion proteins of 4F2hc N-terminus of 4F2 and LAT1 N-and C-termini in BL21 *Escherichia coli*. We synthesized a biotinylated peptide of LAPTM4b N-terminus and incubated it with each of the GST-fusion peptides *in vitro*. However, we did not see binding of LAPTM4b N-terminus to any of the recombinant proteins (Figure 4.14), suggesting binding is not mediated directly by the N-terminus of LAPTM4b.

Next we generated N- and/or C-terminally truncated LAPTM4b constructs for mammalian expression: ΔN-LAPTM4b, ΔC-LAPTM4b and ΔNΔC-LAPTM4b. We repeated the coIP experiments between the various LAPTM4b truncation mutants, LAT1 and 4F2hc, but were unable to disrupt LAPTM4b:LAT1-4F2hc binding (Figure 4.15). Not surprisingly all truncation mutants ΔN-LAPTM4b (Figure 4.16, Figure 4.17), ΔC-LAPTM4b (Figure 4.18, Figure 4.19), and ΔNΔC-LAPTM4b (Figure 4.20, Figure 4.21) were retained in the ER. These results suggest that binding between LAPTM4b, 4F2hc and LAT1 is not mediated by their cytoplasmic regions. They also suggest that LAPTM4B:LAT1-4F2hc binding is taking place already at the ER, possibly during or soon after protein translation.
Figure 4.14: **LAPTM4b N-terminus does not appear to bind the cytoplasmic regions of 4F2hc or LAT1 In Vitro.** GST-tagged recombinant proteins (GST-N-4F2hc, GST-N-LAT1 and GST-C-LAT1) were purified from *E. coli* and immobilized on Glutathione-Sepharose beads. Equal amounts of recombinant proteins (Commassie stain) were incubated with 10 mM of biotinylated LAPTM4b N-terminus peptide (Sup). Binding of the biotinylated peptide to GST-recombinant proteins was assessed by spot blot developed with Streptavidin-HRP. GST alone was included as a negative control for non-specific binding of the biotinylated peptide to recombinant proteins. pd = pull down.

* I helped generate and purify the proteins for Figure 4.14, generated by Dr. A. Persaud.
Figure 4.14: LAPTM4b N-terminus does not bind the cytoplasmic regions of 4F2hc or LAT1 In Vitro.
Figure 4.15: LAPTM4b N and C termini do not appear to mediate binding to LAT1-4F2hc. HeLa cells were co-transfected with FLAG-4F2hc or mCherry-LAT1 and HA-LAPTM4b WT or truncation mutants (ΔN-LAPTM4b (A,B), ΔC-LAPTM4b (C,D) and ΔNΔC-LAPTM4b (E,F)). 48 hrs post transfection cells were lysed on ice and 15 µL of Flag-agarose or 15µL Protein G agarose with 1.5 µL anti-HA antibody were incubated with 1.5 mg of crude cell lysate for 4 hrs at 4°C. Samples were separated by SDS-PAGE, transferred to nitrocellulose and probed with the indicated antibodies.

* I helped generate the cDNA constructs and performed experimental repeats for Figure 4.15 generated by Dr. A. Persaud.
Figure 4.15: LAPTM4b N and C termini do not mediate binding to LAT1 or 4F2hc.
Figure 4.16: ΔN LAPTM4b is retained in the ER. 24 hrs post transfection, HeLa cells expressing WT or ΔN mCh-LAPTM4b (red) were fixed and stained for various organelle markers (green) using primary antibodies: anti-Lamp1 (Lyosomes), anti-Giantin (Golgi) and anti-Calnexin (ER). The plasma membrane and nucleus are indicated in blue (ConA) and cyan (DAPI) respectively. All images were acquired by spinning disk confocal microscope and processed in Volocity 5.4.1.
Figure 4.16: ΔN LAPTM4b is retained in the ER.
Figure 4.17: **Quantification: ΔN LAPTM4b is retained in the ER.** A-C) Changes in WT vs ΔN mCh-LAPTM4b colocalization with various organelle markers (Lamp1 (Lysosome), Giantin (Golgi), Calnexin (ER) was quantified and graphed in terms on the Pearson’s Correlation Coefficient. Error bars indicate SEM. * denotes p<0.0001 D) Table of Pearson’s correlation coefficients. Degrees of freedom are noted as (r), number of cells n = r+2. level of significance as (p).
Figure 4.17: Quantification: ΔN LAPTM4b is retained in the ER.
Figure 4.18: ΔC LAPTM4b is retained in the ER. 24 hrs post transfection, HeLa cells expressing WT or ΔC mCh-LAPTM4b (red) were fixed and stained for various organelle markers (green) using primary antibodies: anti-Lamp1 (Lyososome), anti-Giantin (Golgi) and anti-Calnexin (ER). The plasma membrane and nucleus are indicated in blue (ConA) and cyan (DAPI) respectively. All images were acquired by spinning disk confocal microscope and processed in Volocity 5.4.1.
Figure 4.18: ΔC LAPT4b is retained in the ER.
Figure 4.19: **Quantification: ΔC LAPTM4b is retained in the ER.** A-C) Changes in WT vs ΔC mCh-LAPTM4b colocalization with various organelle markers (Lamp1 (Lysosome), Giantin (Golgi), Calnexin (ER) was quantified and graphed in terms on the Pearson’s Correlation Coefficient. Error bars indicate SEM. * denotes p<0.0001 D) Table of Pearson’s correlation coefficients. Degrees of freedom are noted as (r), number of cells n = r+2. level of significance as (p).
Figure 4.19: Quantification: ΔC LAPTM4b is retained in the ER.
Figure 4.20: ΔNΔC LAPTM4b is retained in the ER. 24 hrs post transfection, HeLa cells expressing WT or ΔNΔC mCh-LAPTM4b (red) were fixed and stained for various organelle markers (green) using primary antibodies: anti-Lamp1 (Lyososme), anti-Giantin (Golgi) and anti-Calnexin (ER). The plasma membrane and nucleus are indicated in blue (ConA) and cyan (DAPI) respectively. All images were acquired by spinning disk confocal microscope and processed in Volocity 5.4.1.
Figure 4.20: ΔNAC LAPT4b is retained in the ER.
Figure 4.21: ΔNΔC LAPTM4b is retained in the ER. A-C) Changes in WT vs ΔNΔC mCh-LAPTM4b colocalization with various organelle markers (Lamp1 (Lysosome), Giantin (Golgi), Calnexin (ER) was quantified and graphed in terms on the Pearson’s Correlation Coefficient. Error bars indicate SEM. * denotes p<0.0001 D) Table of Pearson’s correlation coefficients. Degrees of freedom are noted as (r), number of cells n = r+2. level of significance as (p).
Figure 4.21: Quantification: ΔNΔC LAPT4b is retained in the ER.
5 Discussion

Prior to this work, mTORC1 activation by EAA was shown to be mediated via an “inside-out” mechanism, whereby intra-lysosome EAA activate the v-ATPase and hence mTORC1 [181, 182, 380]. A missing link in this scenario was how EAA enter the lysosome to activate mTORC1. Our results here show that LAPTM4b recruits the Leu transporter LAT1-4F2hc to lysosomes, enhances Leu uptake into lysosomes and stimulates mTORC1 activation upstream of the v-ATPase. Consequently, we propose to amend the current model of EAA mediated activation of mTORC1 to include LAPTM4b as an upstream activator (Figure 4.22).

LAT1 is a bi-directional amino acid transporter [450]. Leu normally enters cells via LAT1 (in exchange for Glutamine efflux) due to higher affinity of this transporter to extracellular relative to intracellular Leu [441]. What drives Leu entry into lysosomes, described here and earlier [182] is currently unknown. Possibilities include: (i) the v-ATPase – mediated accumulation of H⁺ in lysosomes leads to activation of lysosomal hydrolases that then break down peptides/proteins to provide amino acids for transport/exchange by the Leu transporter. (ii) Glutamine transporters in lysosomal membrane, if present, can exchange amino acids for LAT1-4F2hc. Candidates might include SNAT3 (SLC38A3) and SNAT5 (SLC38A5) which transport Na⁺ plus amino acids in exchange for H⁺[451]. (iii) Other transporters, if present at the lysosomal membrane such as Na⁺/H⁺ antiporters might also favor the accumulation of AA into lysosomes. For instance, SLC38A7 was recently identified in a screen for lysosomal membrane transporters [452] although originally characterized as a plasma membrane transporter capable of transporting neutral and cationic AA including Leucine [453].

While the 35kDaLAPTM4b isoform has been shown to promote cancer via PI3K/Akt activation, it is possible that the 24kDa isoform also has a role in cancer promotion by enhancing mTORC1 activation. This is supported by our observation that this LAPTM4b isoform stimulates cell proliferation. Interestingly, System L is overexpressed in many tumor types [399] and tumor growth depends on EAA uptake, particularly Leucine [454-456]. These observations are in line with LAPTM4b:LAT1-4F2hc association promoting Leucine uptake into lysosomes.

The disulfide bond mediated by Cys160 of LAT1 and Cys110 of 4F2hc [457] is required for proper LAT1 localization and function [440]. How LAPTM4b associates with LAT1-4F2hc
(direct or indirect) remains to be determined. To date our studies suggests an early association of LAPTM4b with the LAT1-4F2hc in the ER. It is possible that the interaction could be mediated by transmembrane domain interactions.

Together Chapter 4 has demonstrated a unique role for LAPMT4b as a component of the nutrient sensing machinery during EAA stimulated mTORC1 activation.
Figure 4.22: An expanding model of mTORC1 activation at the lysosome. LAPTM4b stimulates mTORC1 signaling upstream of the v-ATPase. LAPTM4b recruits the Leu transporter LAT1-4F2hc to lysosomes, enhances Leu uptake into lysosomes and stimulates mTORC1 activation upstream of the v-ATPase.
Figure 4.22: An expanding model of mTORC1 activation at the lysosome.
Chapter 5
Thesis Summary and Future Directions
1 Thesis Summary

This thesis intended to characterize the lysosomal targeting determinants and function of LAPTM4 proteins. Indeed Chapter 2 and Appendix A, provide novel insights into the roles of PY motifs and Nedd4 in the lysosomal trafficking of LAPTM4a and LAPTM4b. It is demonstrated that both LAPTM4s require PY motifs and Nedd4 for proper lysosomal sorting. LAPTM4s without functional PY motifs or Nedd4 show decreased lysosomal localization and additionally, LAPTM4b expression at the plasma membrane is up-regulated. In addition, the preliminary data presented in Chapter 3 suggests PY motifs and Nedd4 family members could be conserved determinants of lysosomal sorting of additional LMPs, including TMEM55B and ABCB6. Finally, and most importantly, Chapter 4 demonstrates a novel and critical role for LAPTM4b at the lysosome; LAPTM4b is a critical component of the nutrient sensing machinery required for mTORC1 activation by amino acids. We demonstrate that LAPTM4b mediates this function by binding and recruiting the Leu Transporter LAT1-4F2hc to the lysosome and is critical in the amino acid uptake by lysosomes that triggers the inside-out activation of mTORC1 that is mediated by the v-ATPase and Ragulator.

This chapter focuses on some of the unresolved questions that remain or arose upon completion of the work presented in this thesis.

2 PY motifs and Nedd4 as conserved LMP sorting determinants

In Chapter 2 of this thesis I demonstrated that LAPTM4 protein sorting determinants differ from those of LAPTM5. Unlike LAPTM5, which is retained in the Golgi in the absence of Nedd4 or functional PY motifs[78], LAPTM4a and LAPTM4b lysosomal localization is reduced and LAPTM4b expression at the plasma membrane is enhanced. Furthermore, both LAPTM4a[232] and LAPTM4b (Appendix A) appear to utilize additional dileucine and tyrosine based sorting motifs to target to the lysosome. Why LAPTM proteins evolved different determinants of lysosomal sorting is not known. What is known is that LAPTM5s are differentially expressed: LAPTM5 is primarily expressed in immune cells, while LAPTM4s are ubiquitously expressed [415]). As such, it is conceivable that LAPTM4 proteins are utilized in more general processes, than LAPTM5. Indeed, Chapter 4 demonstrates that LAPTM4b is a key player of mTORC1 activation in response to amino acids. The ability to respond to nutrient availability is common to
all cells. LAPTM5 on the other hand, mediates pro-inflammatory signaling and regulates TCR and BCR expression, which is immune-cell specific [272]. Interestingly, while all LAPTM proteins are lysosomal, their sorting routes appear to differ and this could have significant roles in explaining their physiological functions.

Though I identified PY motifs, tyrosine and dileucine motifs as determinant for LAPTM4b lysosomal sorting, their individual contributions remain unclear. Other LMPs are known to contain multiple targeting motifs. CLN3 utilizes a dileucine motif and an unconventional cluster of acidic amino acids to target to the lysosome and binds both AP-1 and AP-3 [458]. It is likely LAPTM4s will interact with a variety of sorting machinery depending on its cellular localization. Because some LAPTM4b is detected at the plasma membrane AP-2 may be a binding partner. Identification of the adaptors that mediate LAPTM4 lysosomal targeting can be done through in vivo binding experiments of LAPTM4s to AP and/or GGA proteins. Knock-down of identified binding partners should result in LAPTM4 missorting phenotypes comparable to those of the respective LAPTM4 targeting motif mutants. Defining the adaptors involved in LAPTM4b lysosomal sorting will also help delineate the pathway by which the LAPTM4b/LAT1-4F2hc complex reaches the lysosome. If, in fact the complex is formed during biosynthesis, it is possible that AP and GGA proteins that mediate direct TGN to lysosome sorting could be involved.

LAPTM4 proteins, unlike LAPTM5, do not contain a stereotypical conserved UIM that would be able to bind to ubiquitinated GGA3 to mediate their trafficking to lysosomes (Figure 1.6). Preliminary data suggest that LAPTM4s, unlike LAPTM5, are unable to bind ubiquitin or interact with GGA3 (data not shown). They do contain lysine residues and are both ubiquitinated by Nedd4. It is possible that UBD containing proteins mediate the lysosomal targeting of LAPTM4s, however this machinery remains to be elucidated. Total lysine mutants (LAPTM4a: 7K>R mutant. LAPTM4b: 4K>R) could be used to determine whether ubiquitination plays a role in LAPTM4 lysosomal sorting.

An interesting consideration to studying the lysosomal targeting determinants of LAPTM4s, is that LAPTM4s are able to dimerize and form multimeric complexes (unpublished data). This means that even if we overexpress LAPTM4b targeting mutants in mammalian cells to monitor their subcellular localization, it is possible that dimerization with endogenous WT proteins could
mask and compensate for the mutation in the ectopically expressed protein. Perhaps one method to overcome this limitation would be to define the dimerization interface and disrupt it. However, considering the obvious implications of affecting membrane folding, utilizing the newly developed CRISPR-mediated gene targeting [459]. In this system, targeting mutants could be generated and substituted for the WT gene in mammalian cells through genome editing using endogenous DNA repair mechanisms. Together with an endogenous antibody to LAPTM4s, immunofluorescence imaging becomes an extremely powerful tool for monitoring lysosomal sorting determinants.

Though the data presented in Chapter 3 point towards PY motifs and Nedd4 acting as conserved determinants of LMP lysosomal sorting, the data are preliminary and require additional consideration. It remains unclear whether expression of WT TMEM55B in Nedd4 KO MEFs affects TMEM55B localization. More importantly co-immunoprecipitation of endogenous TMEM55B and Nedd4, as well as ABCB6 with Smurf1 and WWP2, would significantly support our hypothesis. Similarly, screening for the binding of the remaining seven putatively cytosolic PY motif containing LMPs to other Nedd4 family members could provide additional evidence in favour of PY motifs and Nedd4 being conserved determinants of LMP sorting.

Interestingly, although we show a dependence of TMEM55B on PY motifs in lysosomal sorting, it is of interest to investigate whether Nedd4 binding to TMEM55B could also be involved in nuclear localization. This is particular relevant given the finding by others that DNA-damage induces a translocation of TMEM55B from a cytoplasmic location (vesicular) to the nucleus. The possible role of PY motifs and Nedd4 in nuclear targeting of LMPs has not been studied.

3 Expanding the model of amino acid mediated mTORC1 activation

While many components of the lysosomal nutrient sensing machinery have been identified and characterized, one of the key questions that remained prior to my investigation was how amino acids accumulate within the lysosomal lumen to activate mTORC1 through v-ATPase and Ragulator. In Chapter 4 we demonstrate that LAPTM4b recruits the Leu Transporter LAT1-4F2hc to the lysosome and is required for the accumulation of intra-luminal amino acids. Although we demonstrated that the LAPTM4b/LAT1-4F2hc interaction is independent of amino acid availability, it remains to be determined whether endogenous LAPTM4b and LAT1/4F2hc
bind and localize to the lysosomal membrane. Furthermore, it remains to be determined whether localization to the lysosomal membrane is constitutive, or much like TSC and mTOC1, whether LAPTM4/LAT1-4F2hc localization to the lysosomal membrane is sensitive to nutritional cues such as amino acid sufficiency or starvation. This is particularly interesting in the context of the observation that radioactively labeled amino acids accumulate in the lysosome within 10 minutes of addition, but mTORC1 can already be activated after only 3 minutes [182, 362, 389]. If LAPTM4b/LAT1-4F2hc is constitutively located at the lysosome, it seems plausible that it would be readily available to allow for rapid uptake of amino acids into the lysosomal lumen. On the other hand, if the complex needs to be recruited to the lysosome, the machinery and conditions that signal this translocation need to be elucidated.

Another question that arose upon identifying LAT1-4F2hc translocation to the lysosome was whether the system L transporter is able to function at this acidic organelle. This is currently under investigation and preliminary data suggest that the acidic environment of the lysosome does not inhibit LAT1-4F2hc transport ability. More importantly, as the pH decreases, the ability of LAT1-4F2hc to uptake radiolabelled Leu into oocytes is unchanged (data not shown). If indeed LAT1-4F2hc is able to function at the low lysosomal pH, one might speculate even small amounts of LAT1-4F2hc at the lysosomal membrane could account for sufficient translocation of Leu into the lysosomal lumen for v-ATPase activation and mTORC1 activation. Currently it is unknown whether there is a threshold minimum amino acid concentration required to activate mTORC1 in an amino acid dependent.

Importantly, LAT1-4F2hc is an obligate exchange transporter. It is only able to transport amino acids if sufficient counter-amino acids are available. At the plasma membrane, LAT1-4F2hc is functionally coupled to the glutamine transporters ASCT2 (SLC1A5)[380] or SNAT2 (SLC38A2), and the K⁺/Na⁺ electrochemical gradient across the plasma membrane[379]. Neither SNAT2, nor ASCT2 co-immunoprecipitated with LAPTM4b. This begs the question: which, if any, SLCs is present at the lysosomal membrane to provide the amino acids required for lysosomal uptake by system L.

Lysosomes are known to contain saturable amino acid transport systems (t, l, and h) for 5-30 μM phenylalanine, leucine or tryptophan/L[378, 460-462]. The specific identity of these amino acid transporters is less well characterized. Recently a variety of potential lysosomal amino acid
transporters were identified during the proteomic analysis of rat liver lysosome-enriched membranes [452]. The majority are amino acid exporters, rather than importers, including PQLC4 which is the cysteine exporter defective in cystinosis [463], PQL2, an exporter of cationic amino acids [464], and SLC38A7, a transporter of neutral and cationic amino acids [453]. A more promising candidate for LAT1/4F2hc functional coupling could be SNAT5 (SLC38A5). ASCT2, LAT1 and SNAT5 are the most substantially up-regulated amino acid transporter mRNAs (ESTs) in normal and cancerous human tissues [399]. SLC38A5 belongs to the system N transport family and is able to counter transport both Na⁺ and H⁺ ions, in exchange for glutamine, alanine, asparagine or histidine [465, 466] Additionally, SNAT5 (SLC38A5) transport has been shown to be driven by H⁺, which is not true of system A transporters like SNAT2 (SLC38A2). This would be of particular interest considering the acidic H⁺-rich lysosomal microenvironment [451]. Preliminary data suggests SNAT5(SLC38A5) colocalizes with LAPTM4b in intracellular vesicles. A role for SNAT5 in amino acid sensing at the lysosome is currently under investigation.

To date, it is unclear whether or not the LAPTM4b/LAT1-4F2hc interaction is mediated directly or indirectly. LAPTM4b N- and C-terminal truncation mutants, as well as loop-region mutants, continue to co-immunoprecipitate with LAPTM4b/LAT1-4F2hc in HeLa cells. It is possible that the interaction is mediated by transmembrane domain interactions and/or that it is indirect. This could be assessed using the membrane yeast two-hybrid (MYTH) system [467]. Delineating the nature of the interaction would provide the framework to disconnect LAPTM4b’s specific role in mTORC1 activation. Additionally, LAPTM4b binding mutants could be used in functional assays of mTORC1 activation, as well as to monitor the role of LATPM4b in tumor formation and metastasis in nude mice.

Interestingly, in Chapter 2, I showed that LAPTM4b requires its PY motifs and Nedd4 to sort to the lysosome. In the absence of PY motifs, its expression at the plasma membrane is enhanced. Testing the LAPTM4b-PY motif mutant’s ability to bind LAT1/4F2hc and its ability to rescue defective mTORC1 signaling in LAPTM4b-KD HeLa cells could provide additional information on the spatiotemporal control on the complex. It can be speculated that LAPTM4b expression at the plasma membrane could reduce the availability of LAT1-4F2hc at the lysosome by retaining LAT1-4F2hc at the plasma membrane. In order to eliminate background signal in LAPTM4b-KD cell lines, it could be useful to generate stable Knock-in, 24kDa-LAPTM4b-WT and PY
mutant expressing cell lines using CRISPR-mediated gene targeting [459]. A role for Nedd4 in this interaction could also be investigated. To date, the role of Nedd4 ubiquitination of LAPTM4b is not understood. It is possible it could have signaling or trafficking consequence for LAPTM4b/LAT1-4F2hc.

Finally, it will be interesting to determine whether the LAPTM4b/LAT1-4F2hc interaction has pathological consequences. Already it is known that LAT1 is upregulated in a variety of cancers (gliomas, breast, lung, prostate, and ovarian cancers) and expression correlates with increased aggressiveness and mortality[399, 468-471]. As outlined in the Introduction, certainly 35kDa-LAPTM4b has been shown to be associated with aggressiveness and mortality in a variety of cancers as well. The role of 24kDa LAPTM4b investigated here remains unclear. As such it will be interesting to determine whether 24kDa LAPTM4b is also capable of promoting tumor growth given its stimulation of mTORC1 activity.

Thus, this thesis has expanded our understanding of the role of PY motifs and Nedd4 in LAPTM4 family lysosomal targeting, and has implicated LAPTM4b as a key component of the nutrient sensing machinery in cells.
References


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Appendix A – Additional LAPTM4b targeting motifs
Unlike for LAPTM5, PY motifs and Nedd4 do not account for 100% of LAPTM4 lysosomal targeting [415]. Therefore I examined the potential role of additional lysosomal targeting determinants in LAPTM4 proteins, including the Dileucine ([DE]XXXL[LI]) and Tyrosine-based (YXXΦ) targeting motifs.

1 Introduction

Dileucine and tyrosine-based motifs are recognized by major adaptor molecules involved in trafficking from the Golgi to the lysosome (directly or indirectly via the plasma membrane) including the adaptor proteins AP1-AP5 and GGA1-3 [200, 217]. APs and GGAs bind cargo, protein coats and other accessory proteins for transport to and from specific compartments [229, 401].

Both LAPTM4a and LAPTM4b have putative Dileucine motifs (Figure 1.6). LAPTM4a’s Dileucine motif is predicted to face the luminal side of endolysosomes and overlap partially with a predicted transmembrane domain. Consequently a role for the putative Dileucine motif in LAPTM4a subcellular trafficking seemed unlikely. LAPTM4b’s c-terminal Dileucine motif on the other hand is predicted to be cytosolic and theoretically accessible to cytosolic sorting machinery. Dileucine motif mutants (DiLeu) were generated for both LAPTM4 proteins. LAPTM4a’s motif was mutated from DSSCLL> DSSCAA, while LAPTM4b’s motifs was mutated from DTTVLL>AXXXXAL, to prevent disruption to LAPTM4b’s adjacent PY motif. DiLeu mutant localization was analyzed by confocal imaging.

Others previously proposed that LAPTM4a requires two tandem YXXΦ motifs for proper sorting to the lysosome [232]. In their analysis, the 2\textsuperscript{nd} and 3\textsuperscript{rd} tyrosine based motifs were critical to LAPTM4a’s sorting to the lysosome (Figure 2.1C). While we do not question the contribution of the 2\textsuperscript{nd} tyrosine motif, we do question that of the 3\textsuperscript{rd}. Firstly, the 3\textsuperscript{rd} motif (YEMA) does not have a large and bulky hydrophobic residue at the Y+3 position. Secondly, tyrosine is the conserved tyrosine of LAPTM4a’s 2nd PY motif (LPT\underline{Y}EMA). In Chapter 2 I showed that this 2\textsuperscript{nd} PY motif mediated Nedd4 binding [415]. The potential role of LAPTM4b’s YXXΦ’s motifs in its targeting had not been examined.

Based on the methodology used by Hogue et al. 2002, there are three putative YXXΦ motifs in LAPTM4b: Y180 (YXXI), Y207 (YXXA) and Y223 (YXXA). Both Y207 and Y223 lack the
conserved hydrophobic residue typical of YXXΦ motifs and furthermore share their tyrosine residue with LAPTM4b PY motifs. As such, I only investigated Y180’s potential role in LAPTM4b subcellular targeting. I generated a single tyrosine motif mutant (Y180A) and analyzed its subcellular localization relative to the WT LAPTM4b by confocal imaging.

Together, Appendix A demonstrates that LAPTM4b’s Dileucine motif and 1st YXXΦ motif contribute to its subcellular localization.

2 Methods

Using site-directed mutagenesis, LAPTM4a and LAPTM4b’s putative DiLeu motifs were mutated. LAPTM4a’s motif was mutated from DSSC LL > DSSCA A. LAPTM4b’s DiLeu was mutated from D TTVLL > AXX XAL. WT and DiLeu-LAPTM4a or LAPTM4b were mCherry-tagged, transiently transfected into HeLa cells on coverslips and fixed at 24 hrs. Fixed cells were stained for the Endoplasmic Reticulum (ER marker Calnexin), as well as the plasma membrane (Concanavilin A) and the nucleus (Dapi). WT and DiLeu-LAPTM4b expressing cells were subsequently stained for Lysosomes (Lamp1), Recycling Endosomes (Transferrin Receptor (TfR)) and Golgi (Giantin). Similarly, LAPTM4b’s YXXΦ motif was mutated by substituting the conserved tyrosine residue for alanine (Y180A). WT and mutant LATPM4b were mCherry-tagged, transiently transfected into HeLa cells on coverslips and fixed at 24 hrs. Fixed cells were stained for the lysosomal compartment (Lamp1) or the Golgi (Giantin), as well as the plasma membrane (Concanavilin A) and the nucleus (Dapi). Z-stack images were obtained via confocal imaging with LSM510 and subcellular localization of LAPTM4s was analyzed using Volocity 5.4.1.
3 Results

3.1 A c-terminal Dileucine motif affects LAPTM4b lysosomal localization.

Upon transient transfection of WT and dileucine mutants DiLeu-LAPTM4a and LAPTM4b in HeLa cells, staining for the ER marker Calnexin revealed DiLeu-LAPTM4a (A-1A), but not DiLeu-LAPTM4b (A-1B) was retained at the ER. As such, I only assessed DiLeu-LAPTM4b’s subcellular localization.

Compared to WT-LAPTM4b, the DiLeu-LAPTM4b mutant appeared to colocalize less with the lysosomal marker Lamp1 (A-2A) and the recycling endosomal marker Transferrin Receptor (TfR)(A-3A), has increased plasma membrane (PM) expression (A-2B) and a more clustered nuclear appearance. Quantification of changes in Lamp1 and TfR colocalization of WT-LAPTM4b and DiLeu-LAPTM4b revealed a reduction of DiLeu-LAPTM4b colocalization with the Lamp1 (~13%, n=86, p<0.0001) (A-2B) and TfR marker (~18%, n = 105, p<0.0001) (A-3B) when compared to WT-LAPTM4b. The amount of LAPTM4b co-localizing with the plasma membrane also increased (~19%, n=86, P<0.0001)(A-2B). Because clustering around the nucleus appeared more pronounced with DiLeu-LAPTM4b, we examined whether there were any changes in Golgi localization. However, there was no significant colocalization with the Golgi marker Giantin by WT or DiLeu-LAPTM4b (WT=0.3966 vs DiLeu=0.3182, n=102) (A-4).

These data suggest that LAPTM4b's dileucine motif plays a role in its subcellular localization. Without a functional dileucine motif, LAPTM4b colocalizes less with the lysosomal marker Lamp1 and the colocalization of LAPTM4b at the plasma membrane appears to increase.
A-1: **The DiLeu-LAPTM4a mutant, but not DiLeu-LAPTM4b mutant is retained in the ER.** (A) 24 hrs post transfection the plasma membrane of HeLa cells expressing mCh-LAPTM4a-WT or mCh-DiLeu-LAPTM4a mutant and (B) mCh-LAPTM4b-WT or mCh-DiLeu-LAPTM4b mutant was stained (ConA, blue) and DAPI (nucleus, light blue). Cells were fixed, incubated with anti-Calnexin (ER) antibodies (green) and stained with DAPI (nucleus, light blue). Imaging by confocal microscopy and analysis by Volocity 5.4.1. (C) Degree of LAPTM4b colocalization with Calnexin is tabulated in terms of the Pearson’s coefficient. Degrees of freedom are noted as (r), level of significance as (p).
A-1: The DiLeu-LAPTM4a mutant, but not DiLeu-LAPTM4b mutant is retained in the ER.
A-2: DiLeu-LAPTM4b mutant shows altered Lamp1 and PM staining compared to WT-LAPTM4b. (A) 24 hrs post transfection the plasma membrane of HeLa cells expressing mCh-LAPTM4b-WT or mCh-DiLeu-LAPTM4b mutant was stained (ConA, blue) and DAPI (nucleus, light blue). Cells were fixed, incubated with anti-Lamp1 (Lysosome) antibodies (green) and stained with DAPI (nucleus, light blue). Cells were imaged by confocal microscopy and colocalization assessed by Volocity 5.4.1. (B) and (C) Changes in LAPTM4b colocalization with Lamp1 and Plasma Membrane (ConA) is graphed and tabulated in terms of the Pearson’s coefficient. Degrees of freedom are noted as (r), level of significance as (p). Error bars indicate SEM. * denotes p<0.0001 (B) Changes in colocalization of mCh-LAPTM4b and Lamp1 (WT, DiLeu n=86). (C) Changes in colocalization of LAPTM4b and ConA (WT, DiLeu n=74).
DiLeu-LAPT4b mutant shows altered Lamp1 and PM staining compared to WT-LAPT4b.
A-3: **DiLeu-LAPTM4b mutant shows altered TfR staining compared to WT-LAPTM4b.**

(A) 24 hrs post transfection the plasma membrane of HeLa cells expressing mCh-LAPTM4b-WT or mCh-DiLeu-LAPTM4b mutant was stained (ConA, blue) and DAPI (nucleus, light blue). Cells were fixed, incubated with anti-Transferrin receptor (TfR, Recycling Endosome) antibodies (green) and stained with DAPI (nucleus, light blue). Cells were imaged by confocal microscopy and colocalization assessed by Volocity 5.4.1. (B) LAPTM4 colocalization with the recycling endosome maker TfR is tabulated and graphed in terms of the Pearson’s correlation coefficient. Degrees of freedom are noted as (r), level of significance as (p). Graphs illustrate changes in the mean Pearson’s coefficients of mCh-LAPTM4 colocalization with TfR (WT, DiLeu n=105). Error bars indicate SEM. * denotes p<0.0001.
A-3: DiLeu-LAPT4b mutant shows altered TfR staining compared to WT-LAPT4b.
A-4: DiLeu-LAPTM4b mutant Golgi colocalization is unchanged compared to WT-LAPTM4b. 24 hrs post transfection the plasma membrane of HeLa cells expressing mCh-LAPTM4b-WT or mCh-DiLeu-LAPTM4b mutant (A) was stained (ConA, blue) and DAPI (nucleus, light blue). Cells were fixed, incubated with anti-Giantin (Golgi) antibodies (green) and stained with DAPI (nucleus, light blue). Cells were imaged by confocal microscopy and colocalization assessed by Volocity 5.4.1. (B) LAPTM4b colocalization with the Golgi marker Giantin is tabulated in terms of the Pearson’s correlation coefficient. Degrees of freedom are noted as (r), level of significance as (p).
A-4: DiLeu-LAPTM4b mutant Golgi colocalization is unchanged compared to WT-LAPTM4b.
3.2 Tyrosine Y180 plays a role in LAPTM4b subcellular localization.

Transient transfection of mCherry-tagged WT and Y180A LAPTM4b mutant into HeLa cells revealed that although Y180A retains a vesicular pattern, it’s co-localization with Lamp1 marker is significantly reduced (~50% decrease in colocalization) (A-5).

These data suggest that disruption of the putative tyrosine based motif at position Y180 of LAPTM4b negatively affects the lysosomal localization of the protein in HeLa cells.
A-5: **Y180 plays a role in LAPTM4b subcellular localization.** 24 hrs post transfection the plasma membrane of HeLa cells expressing mCh-LAPTM4b-WT or mCh-LAPTM4b-Y180A was stained (ConA, blue). Cells were fixed, incubated with anti-Lamp1 (Lysosome) antibodies (green) and stained with DAPI (nucleus, light blue). Cells were imaged by confocal microscopy and colocalization assessed by Volocity 5.4.1. (B) LAPTM4 colocalization with the lysosomal marker Lamp1 is expressed in terms of the Pearson’s correlation coefficient. Degrees of freedom are noted as (r), level of significance as (p). Graphs illustrate changes in the mean Pearson’s coefficients of mCh-LAPTM4 colocalization with Lamp1 (WT n=129, Y180A n=132). Error bars indicate SEM. * denotes p<0.0001.
A-5: Y180 plays a role in LAPTM4b subcellular localization.
4 Discussion

In Appendix A I have shown that additional putative targeting motifs to LAPTM4b’s PY motif, play a role in LAPTM4b subcellular localization. Much like LAPTM4b’s PY motif, LAPTM4b’s Dileucine motif plays a role in its sorting to the lysosome and plasma membrane. The decrease in lysosomal/Lamp1 colocalization observed with the DiLeu-LAPTM4b mutant of ~13% is very similar to that observed when WT-LAPTM4b was overexpressed in Nedd4.1 KO MEFs (~14% in Lamp1 colocalization).

The 50% decrease in colocalization observed with the Y180A mutant of LAPTM4b compared to WT in HeLa cells, suggests that Y180A likely plays a unique role in LAPTM4b subcellular sorting. Interestingly, unlike the dileucine and PY motifs, this YXXO motif does not overlap with other known targeting motifs and sits fairly isolated at the transmembrane proximal region of LAPTM4b’s c-terminus.

LAPTM4b is not the only protein that utilizes more than one sorting determinant to arrive at the lysosome. For example Cystinosin requires a tyrosine based motif and a novel signal sequence (YFPQA) to guarantee its proper delivery to the lysosome[472]. Disruption of either motif results in partial mislocalization of the protein.

Having a variety of lysosomal targeting motifs suggests that either there is a redundancy mechanism to guarantee delivery of LAPTM4b to the lysosome and/or in fact that each putative targeting motif is in fact trafficking LAPTM4b along unique pathways to the lysosome. It is possible that depending on where the protein is localized, different sorting determinants will attract different sorting machinery e.g. targeting motifs and sorting motifs and the plasma membrane will differ significantly from the sorting machinery in the later stages of endosomal sorting. This idea becomes particularly interesting with the new light shed on LAPTM4b function. For instance, should LAPTM4b bind 4F2hc-LAT1 in the biosynthetic route other motifs might become accessible and determine relocation to the lysosome for mTORC1 activation purposes, than if LAPTM4b is trafficking to the plasma membrane where it is triggering actin-based membrane protrusion formation.

The sorting machinery involved in LAPTM4b’s Dileucine and Tyrosine based motif trafficking remains to be elucidated.