Regulation of Lysosomal Adaptation to Nutrient Starvation by Lysosomal TRPML1 and PIKfyve

by

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"The oldest, shortest words – 'yes' and 'no' – are those which require the most thought."

--Pythagoras

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DEDICATION

To my parents and my love Dan,

for their unconditional love, support, and encouragement,

for then, for now, and forever

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LIST OF ABBREVIATIONS

AA	*Amino Acids*
AL	*Autolysosome*
ALP	*Autophagy-lysosomal pathway*
ALR	*Autophagic lysosomal reformation*
AP	*Autophagosome*
Api	*Apilimod*
BAPTA-AM	*1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid*
BK	*Big-conductance Ca^{2+}-activated potassium channel*
CHO	*Chinese hamster ovary*
CLEAR	*Coordinated lysosomal expression and regulation*
Defo	*Deforolimus*
EE	*Early Endosome*
ER	*Endoplasmic reticulum*
Eve	*Everolimus*
FRB	*FKBP-rapamycin binding domain*
GFP	*Green fluorescent protein*
GPN	*glycyl-L-phenylalanine 2-naphthylamide*
HEK293	*Human Embryonic Kidney 293*
Iono	*Ionomycin*
LAMP	*Lysosomal-associated membrane protein*
LE	*Late Endosome*
LEL	*Late endosome and lysosome*
LSD	*Lysosomal storage disorder*
LY	*Lysosome*
LYNUS	*Lysosomal nutrient sensing machinery*
mCh	*mCherry*

MiT/TFE	*microphthalmia-transcription factor E*
MITF	*Microphthalmia-associated transcription factor*
ML-IV	*type IV Mucolipidosis*
ML-SA	*Mucolipin Synthetic Agonist*
ML-SI	*Mucolipin Synthetic Inhibitor*
mTOR	*Mechanistic target of rapamycin*
mTORC1	*Mechanistic target of rapamycin complex 1*
NPC	*Niemann-Pick C*
O/E	*Over-expression*
$PI(3,5)P_2$	*Phosphatidylinositol 3,5-bisphopsphate*
$PI(4,5)P_2$	*Phosphatidylinositol 4,5-bisphopsphate*
PI3P	*Phosphatidylinositol 3-phopsphate*
PI4P	*Phosphatidylinositol 4-phopsphate*
PIKfyve	*Phosphatidylinositol 3-phosphate 5-kinase*
PIP	*Phosphatidylinositol phosphate*
PPP3CB	*Calcineurin catalytic subunit isoform beta*
Rapa	*Rapamycin*
Rheb	*Ras homolog enriched in brain*
ROS	*Reactive oxygen species*
RT-PCR	*Reverse transcription polymerase chain reaction*
S6K	*S6 Kinase*
Seco	*Seco-rapamycin*
SMs	*Sphingomyelins*
Tem	*Temsirolimus*
TFE3	*Transcriptional factor E3*
TFEB	*Transcriptional factor EB*
TPC	*Two-pore channel*
TRPML	*Transient receptor potential mucolipin*
WT	*Wild type*
Zota	*Zotarolimus*

ABSTRACT

Lysosomes play an active role in sensing, signaling, and responding to nutrient availability, in addition to their well-established role in degradation. Lysosomes undergo multifaceted changes in lysosome pH, size, number, and activity, referred to as "lysosomal adaptation", via a transcriptional factor EB (TFEB)-mediated lysosome-to-nucleus signaling pathway. The mechanistic target of rapamycin (mTOR) is a nutrient-sensitive protein kinase that regulates TFEB. When nutrients are abundant, mTOR phosphorylates TFEB on the lysosomal membrane and retains it in the cytosol. When nutrients are deprived, TFEB is dephosphorylated and translocates to the nucleus. Here, I identified two novel components required for lysosomal adaptation: the lysosomal Ca^{2+} release channel TRPML1 and phosphatidylinositol 3-phosphate (PI3P) 5-kinase PIKfyve. Upon starvation, TRPML1 is activated and PIKfyve is inhibited, both triggering the dephosphorylation and subsequent nuclear translocation of TFEB independent of mTOR. This results in lysosomal changes to enhance degradation capabilities. Moreover, the expression level of TRPML1 is potently and rapidly increased. Pharmacological inhibition or genetic deletion of TRPML1 completely abolishes the effects of starvation on boosting the degradation capability of lysosomes, suggesting that TRPML1 is essential for lysosomal adaptation during prolonged starvation. Collectively, lysosomes may adapt to cellular changes under nutrient deprivation by generating a transcriptional response via TRPML1 activation and PIKfyve inhibition.

Modulation of lysosomal function by activating the TRPML1-TFEB pathway may dramatically promote cellular clearance, hence representing a promising therapeutic strategy for many lysosome-related disorders. In the search for small molecule compounds that can activate the TRPML1-TFEB pathway, we found that, unexpectedly, rapamycin, a well-known inhibitor of mTOR and clinically approved drug, directly activated TRPML1 in a mTOR-independent manner, inducing lysosomal Ca^{2+}-dependent nuclear translocation of TFEB. Hence, rapamycin may promote autophagy and lysosome biogenesis via both mTOR-dependent and mTOR-independent

pathways. This new finding may help us develop small molecule rapamycin-like compounds that can induce autophagy with reduced mTOR inhibition for specific clinical purposes.

CHAPTER I [1]

Introduction

I-1 Lysosomes --- The Cell's degradation and signaling center

For a long time, lysosomes were considered to be the main cellular degradation and recycling center (Luzio et al., 2007). Two major pathways have been identified to deliver substrates to lysosomes for degradation: endocytosis for extracellular contents and autophagy for intracellular contents **(Fig. 1.1)** (Luzio et al., 2007). During endocytosis, extracellular materials are internalized and routed from early endosomes to late endosomes and then lysosomes for degradation (Saftig and Klumperman, 2009). During autophagy, protein aggregations and damaged organelles are engulfed by autophagosomes, which then fuse with lysosomes for degradation (Yang and Klionsky, 2009). In the past decade, however, it has become evident that lysosomes are not only the "waste bag" of the cell, but also a signaling center (Lim and Zoncu, 2016). Lysosomes regulate the mechanistic target of rapamycin complex 1 (mTORC1) activity, a master regulator of cell growth, and mediate cell's adaptation to environmental changes (Perera and Zoncu, 2016). To fulfill both roles, lysosomes are heterogeneous and dynamic with extraordinary diversity in pH, activity, localization, shape, size, and number (Bright et al., 2016; Korolchuk et al., 2011; Zhou et al., 2013). Dysfunction of lysosomes contributes to various diseases, including lysosomal storage disorders (LSDs), neurodegenerative disorders, cancer, immune diseases, and cardiovascular diseases (Boya, 2012). Thus, as central and dynamic

[1] Source of the material on TRPML1 presented in the Chapter I is adapted from a book chapter on TRPMLs with me as first-author (***Gao, Q.***, *X. Zhang and H. Xu. 2015. TRPML channels. Handbook of Ion Channels. CRC Press. 10.1201/b18027*) and another book chapter on TRPML1 with me as a co-author (*Wang, W., X. Zhang,* ***Q. Gao****, and H. Xu. 2014. TRPML1: an ion channel in the lysosome. Handbook of Exp Pharmacol. 222:631-645*).

organelles, the regulation of lysosomes is of great interest to researchers in both basic science and clinical fields.

1.1 Characteristics of lysosomes

1.1.1 Basic properties of lysosomes

Lysosomes, as membrane-bound organelles, were first discovered and named by Christian de Duve in 1955 (de Duve, 2005). The name derives from the Greek for "digestive body", since they were initially discovered as the main digestive organelles of eukaryotic cells (de Duve et al., 1955). Lysosomes are characterized by the presence of acid hydrolases, an internal acidic pH, and lysosomal specific membrane proteins (Vellodi, 2005). They host more than 60 different types of acid hydrolases, which break down proteins, polysaccharides, and lipids into building-block molecules: amino acids, monosaccharides, and fatty acids correspondingly (Xu and Ren, 2015). For their optimal activities, most hydrolases require an acid environment. Lysosomes provide this by maintaining an acidic pH (between 4.5 and 5.0), ensuring that degradation by acid hydrolases only takes place in lysosomes (Mindell, 2012). Interestingly, very recent studies demonstrate that not all lysosomes have a very acidic pH. pH in each lysosome ranges from 4.5-7 (Bright et al., 2016; Johnson et al., 2016). A population of terminal storage lysosomes is also identified, in which pH is neutral and acid hydrolases are inactivated (Bright et al., 2016). The availability of terminal storage lysosomes with neutral pH facilitates cell adaptation by fusing with other acidified vesicles and thus increasing degradative activities (Bright et al., 2016).

Lysosomes not only contain a unique inner composition, but also have special surrounding membranes. A complete transport system facilitates the exit of degradation products by hydrolases (amino acids, sugars, heavy metals, lipids) from the lysosomes to the cytosol for reutilization (Pisoni and Thoene, 1991). Multiple channels and transporters also specifically reside on the lysosomal membrane and regulate the influx and efflux of ions. In particular, the vacuolar H^+-ATPase localizes in the lysosomal membrane, pumping H^+ into the vesicles for lysosomal acidification (Finbow and Harrison, 1997). Moreover, lysosome membranes are composed of specific highly glycosylated membrane-associated proteins, which protect them from the lysosomal hydrolases in the lumen, including lysosomal-associated membrane proteins (LAMP-1

and LAMP2), lysosome integral membrane protein-2 (LIMP2) and CD63, which have been commonly used as lysosomal markers **(Fig. 1.2)** (Fukuda, 1991; Luzio et al., 2007).

1.1.2 Morphology of lysosomes

Lysosomes are 0.1-2 μm in size and hundreds in number (Novikoff et al., 1956). They can be visualized via fluorescent labeling of lysosomal proteins under fluorescence microscopy and transmission electron microscopy (EM) imaging as membrane-bound electron-dense bodies due to accumulation of substrates (Neiss, 1983; Novikoff et al., 1956; van Meel and Klumperman, 2008). With advances in super-resolution imaging techniques, accurate visualization of live lysosomes under physiological conditions, especially of very small sizes (30-60 nm) and their dynamics, is becoming possible (Shim et al., 2012; Li et al., 2016).

Lysosomes have variable shapes, sizes and numbers depending on cell types and environmental conditions to serve different functions (Bright et al., 2016; Johnson et al., 2016; Korolchuk et al., 2011). Even though lysosomes are spherical in most cell types, they appear as long, tubular structures, which interconnect as a network in macrophages (Swanson et al., 1987). The area taken up by lysosomes in cells also ranges from a small portion of the cytoplasmic volume (0.5% or less) in fibroblasts to a considerably larger portion in macrophages, which is required for high-demand degradation of internalized materials in these cells (Saftig P et al,. 2006). Moreover, kidney cells of the proximal tubules possess large numbers of lysosomes to rapidly degrade proteins from the filtrate, while the lysosomes are scarce in the cells of the nephron segments downstream to the proximal tubules (Lullmznn-Rauch R et al,. 2005). Lysosomes undergo dynamic morphological changes in response to environmental cues. Under prolonged nutrient deprivation, the shape, size, and number of lysosomes are changed due to biogenesis and autophagic lysosome reformation (ALR) when autolysosomes form tubules, which break into vesicles and mature into new lysosomes (Yu et al., 2010).

1.1.3 Ionic compositions and ion channels/transporters of lysosomes

Ion compositions vary considerably in different organelles. Lysosomes maintain specific ion compositions for their specific physiological functions, including high concentrations of H^+, Cl^-, Ca^{2+}, Na^+, but low K^+ (Wang et al., 2012; Xu and Ren, 2015). Lysosomal low pH is generated to

maintain its degradation capabilities by the vacuolar H^+-ATPase, which pumps protons into the lysosome lumen against its electrochemical gradient using free energy generated from ATP hydrolysis (Ohkuma et al., 1982). The CIC-7 H^+/Cl^- exchanger has also been implicated to be a modulator of endosomal pH via transporting two Cl^- into the cytosol for one H^+ into the lysosomal lumen, generating a counter-ion flux and dissipating an opposing voltage (Mindell, 2012).

Ion movements across the lysosomal membrane are important for lysosomal functions and regulated by a set of ion channels and transporters (Morgan et al., 2011). With the development of the lysosomal patch-clamp technique (Dong et al., 2008), direct studies on lysosomal ion channels under near physiological conditions became feasible, and we have gained a lot of new understandings of lysosomal ion channels. Lysosomal Ca^{2+} release, which is important for lysosomal membrane trafficking and cellular signaling, is mainly mediated by transient receptor potential mucolipins (TRPMLs) (Cheng et al., 2010; Dong et al., 2010a; Dong et al., 2010b). Na^+ is released by two-pore channels (TPC1 and TPC2) and may rapidly depolarize endolysosomal membranes and promote fusion **(Fig. 1.2)** (Wang et al., 2012). Although still controversial, TPCs may also mediate lysosomal Ca^{2+} or H^+ conductance (Patel, 2015). Two K^+-selective channels, transmembrane protein 175 (TMEM175) and big-conductance Ca^{2+}-activated potassium channel (BK), which mediate influx of K^+ into the lysosomes, were reported (Cang et al., 2015; Cao et al., 2015). TMEM175 may mediate a background K^+ leak conductance (Cang et al., 2015) and BK may rapidly and effectively depolarize or reverse lysosomal membrane potential in response to the lysosomal Ca^{2+} release, which in turn regulates lysosomal Ca^{2+} signaling (Wang et al., 2017, In press). Moreover, lysosomes also regulate heavy metals such as Fe^{2+} and Zn^{2+}. TRPML1 mediates Fe^{2+} release from lysosomes and maintains cellular iron metabolism (Dong et al., 2008). To maintain cellular Zn^{2+} homeostasis (Kukic et al., 2014), Zn^{2+} is taken up into lysosomes by zinc transporters (ZnT2 and ZnT4) and released from lysosomes by TRPML1.

Despite remarkable progress in recent years, the molecular identities of many other lysosomal channels remain elusive, which may require proteomic studies and large-scale candidate gene screening. It's also largely unknown how is the overall ion homeostasis of lysosomes maintained, for example, how lysosomes take up Ca^{2+} and how K^+ is removed from lysosomes. Assuming that the diameters of the lysosome and cell are 0.2 and 10 μm respectively, a lysosomal has ~50 fold higher surface/volume ratio than a cell, implying that a much more dramatic change happens in

the ionic composition of the lysosomal lumen upon opening or closing of lysosomal channels. The change in ion concentration results in a change in membrane potential and in turn alters the driving force for other voltage-sensitive conductances (Xiong and Zhu, 2016). Thus, the cross-talk between channels is also an interesting aspect to explore and will greatly enhance our understanding of regulation of the lysosomal ion channels.

1.1.4 Lipid compositions of lysosomes

Lysosomes are enclosed by a lipid-bilayer, which is composed of sphingomyelins, phosphatidylcholines, cholesterol and low abundant phosphatidylinositols (Bleistein et al., 1980; Henning et al., 1970). Together with lysosomal proteins, lipids maintain the integrity of lysosomal membrane to prevent permeabilization and leakage of hydrolases and ions, which may result in cell death. Lysosomal lipids, especially compartment-specific phosphatidylinositols, provide lysosomal identity and regulate lysosomal functions. In the endocytic pathway, the lysosome-specific phosphoinositide, phosphatidylinositol 3,5-bisphopsphate [$PI(3,5)P_2$], is produced on lysosomes from phosphatidylinositol 3-phopsphate (PI3P) by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) **(Fig. 1.2)** (Jin et al., 2016). $PI(3,5)P_2$ provides the identity for lysosomes and mediates specific protein recruitment to ensure proper lysosomal trafficking (Di Paolo and De Camilli, 2006). It also specifically binds and activates lysosome-localized TRPML1 and TPCs (Dong et al., 2010b; Wang et al., 2012). Interestingly, $PI(4,5)P_2$ and PI4P also reside on the lysosomal membrane and participate in the formation of reformation tubules and scission of protolysosomes generated from reformation tubules during autophagy-lysosomal reformation (ALS) (Munson et al., 2015; Rong et al., 2011; Sridhar et al., 2013). Meanwhile, PI3P is demonstrated to play a critical role in tubule scission (Munson et al., 2015).

In addition to the "cytoplasmic-facing" phosphoinositides, which are structural lipids of lysosomes, lysosomal luminal lipids also participate in cell signaling. The amount and distribution of cholesterol in the lysosomes are regulated by the lysosomal targeting proteins Niemann-Pick Cs (NPC1 and NPC2), which mediate cholesterol export to diverse cellular compartments. Mutations in NPC proteins result in accumulation of cholesterol in lysosomes and Niemann-Pick Type C disease, a lysosomal storage disorder (Mukherjee and Maxfield, 2004). Moreover, in NPC

cells, cholesterol accumulation results in sphingomyelins (SMs) accumulating in the lysosome due to insufficient acid SMase (aSMase) activity, which inhibits TRPML1 activity (Shen et al., 2012).

1.2 Lysosomes as the cell's degradation center --- Autophagy-lysosomal pathway (ALP)

Macroautophagy (hereafter autophagy) is a major degradation pathway inside the cell, requiring lysosomes (Glick et al., 2010a; Kaur and Debnath, 2015; Klionsky, 2007). The term 'autophagy' was coined by Christian de Duve over 40 years ago, a bit later after the discovery of lysosomes (Deter and De Duve, 1967) and derives from the Greek meaning "self-eating". Autophagy is a fundamental process in eukaryotic cells and is high conserved from yeast to human (Klionsky, 2007). Autophagy begins with isolated membrane phagophores, which expand to form double-membraned autophagosomes and engulf targeted cargos, such as protein aggregates and damaged organelles. The loaded autophagosomes then fuse with lysosomes to form autolysosomes and the engulfed cargos are degraded into new building blocks by lysosomal hydrolases, which are exported and recycled for cellular use (**Fig 1.1**) (Yang and Klionsky, 2009). Thus, the autophagy pathway can be simplified into three steps: autophagosome formation, autophagosome-lysosome fusion, and lysosomal degradation (Kaur and Debnath, 2015). Via pioneer autophagy studies in yeast, the step-wise regulatory signaling pathways involving more than 30 genes such as *ULK-1* (a mammalian homologue of *Atg1*), class III PI-3 kinase *Vps34*, *Beclin-1* and microtubule-associated protein light chain 3 (*LC3B*) were revealed (Klionsky, 2007). Each step is tightly controlled by a set of molecules and a specific mechanism. Completion of autophagy requires the coordinated regulation for each step and expansion of autophagosomes and lysosomes to meet the needs for degradation. Thus, the autophagy pathway is sometimes referred to as the autophagy-lysosomal pathway (ALP) (Martini-Stoica et al., 2016).

Autophagy is a housekeeping action and is active at a basal level to clear abnormal protein aggregates, and damaged organelles such as mitochondria (Glick et al., 2010). Meanwhile, it is induced and up-regulated under nutrient stress such as amino acid starvation by converting non-essential cell components into new building blocks to form essential proteins, facilitating cell survival (Glick et al., 2010). A well-characterized regulator of autophagy is the mechanistic target of rapamycin (mTOR). When nutrients are abundant, mTOR is active and inhibits autophagy. Upon nutrient withdrawal, inactive TOR acts to induce autophagy by directly repressing uncoordinated 51-like kinases 1 (ULK1) (Russell et al., 2014; Noda and Ohsumi, 1998).

Autophagy is not only regulated at a post-translational level, but also at a transcriptional level (Füllgrabe et al., 2014). More than 20 different transcriptional factors have been demonstrated to regulate autophagy genes (Füllgrabe et al., 2014). Among them, two transcriptional factor: the transcriptional factor EB (TFEB) and zinc-finger protein with KRAB and SCAN domains 3 (ZKSCAN3) have been identified as the important regulators of autophagy and globally coordinate multiple steps during autophagy. ZKSCNA3, a member of zinc-finger transcription factor family, negatively regulates more than 60 autophagic and lysosomal genes under non-stressed conditions, while TFEB, a member of the basic helix-loop-helix Leu zipper family, up-regulates hundreds of autophagic and lysosomal genes under starvation or stressed conditions (Sardiello et al., 2009; Settembre and Ballabio, 2011). Thus, they regulate autophagy and lysosome biogenesis in an opposing manner. Upon starvation, there are parallel ZKSCAN3 repression and TFEB upregulation, which have additive effects on autophagy and lysosome biogenesis (Chauhan et al., 2013). Interestingly, TFEB, and possibly ZKSCAN3, is regulated by mTOR, the well characterized autophagy regulator (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Chauhan et al., 2013), demonstrating that mTOR regulates autophagy via two distinct pathways. TFEB and ZKSCAN3 are also implicated to be regulated by protein kinase C (PKC) in a mTOR-independent manner. Active protein kinase C inactivates glycogen synthase kinase 3 β isoform (GSK3β), which results in reduction in TFEB phosphorylation, nuclear translocation and activation, while PKC activates c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), both of which mediate dephosphorylation, translocation out of the nucleus and inactivation of ZKSCAN3 (Li et al., 2016). Detailed information on TFEB will be described in **I-2**.

Autophagy plays essential roles in survival, development, and homeostasis (Yang and Klionsky, 2010). Dysfunction of autophagy is associated with the pathogenesis of many diseases including cancer, neurodegenerative diseases, infectious diseases, heart diseases and aging due to the accumulation of toxic protein aggregates and damaged organelles (Levine and Kroemer, 2008). Stimulation of autophagy has proven to reduce the levels of aggregated proteins and ameliorate cellular toxicity (Martini-Stoica et al., 2016; Schaeffer et al., 2012). Enhancing autophagy-lysosomal pathway (ALP) is a focal point of therapeutic development. TFEB, which coordinates multiple steps in the ALP, is an attractive therapeutic target (Martini-Stoica et al., 2016).

1.3 The lysosome as a cell signaling center

In addition to the classic role of lysosomes in degradation and recycling of cellular waste, compelling evidence indicates that lysosomes are also an important signaling center for cell metabolism and regulate metabolic homeostasis in response to nutrient availability, hypoxia, and stress (Lim and Zoncu, 2016). The lysosome membrane is home to the lysosomal nutrient sensing machinery (LYNUS), consisting of the mTORC1 complex, Vacuolar H^+-ATPase complex, Rag GTPases, Rag GAPs and Ragulator complex. LYNUS controls the activation of mTORC1 and integrates metabolic signals with intracellular changes **(Fig. 1.3)** (Settembre et al., 2013). Mechanistic target of rapamycin (mTOR) is a master regulator of cell growth in response to nutrient levels and exists in mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is recruited to lysosomal membrane and mediates cell response to amino acids level. Rag GTPases function as a heterodimer and sense nutrient availability. Rag GAPs function as GTPase-activating proteins (GAPs) for Rag GTPases (Bar-Peled et al., 2013). The Ragulator complex, including p14, p18/LAMTOR1, MP1, HBXIP, and C7orf59 proteins, recruits Rag GTPases to the lysosomal surface and functions as guanine nucleotide exchange factor (GEF) for Rag GTPases (Bar-Peled et al., 2012; Sancak et al., 2010). The vacuolar H^+-ATPase complex is proposed to sense the amino acids from the lysosomal lumen by an unclear mechanism and relays the signal to the Rag GTPases via Ragulator and possibly Rag GAPs (Bar-Peled et al., 2013; Zoncu et al., 2011). When amino acids are present, the active form Rag GTPases (GTP-bound RagA/B with GDP-bound RagC/D) recruits mTORC1 to the lysosomal surface and facilitates its activation by small Ras-related GTPase Rheb (Ras homolog enriched in brain), which is controlled by growth factor (Kim et al., 2008; Sancak et al., 2008).

mTOR is also regulated by lysosomes via other pathways. Starvation was shown to trigger perinuclear clustering of lysosomes and in turn reduce mTORC1 signaling. (Korolchuk et al., 2011). Furthermore, in a very recent study, lysosomal cholesterol was shown to activate mTORC1 activity through a positive regulator SLC38A9, which is an amino acid sensor on the lysosomal membrane, and a negative regulator NPC1, which regulates the export of cholesterol from lysosomes (Castellano et al., 2017).

Not only are lysosomes required for mTORC1 activation, but mTOR also affects lysosomal functions (Puertollano, 2014). mTOR mediates the phosphorylation of TFEB, the important

regulator of lysosomes, and retains it in the cytosol (Settembre et al., 2012). Moreover, during long-term starvation, mTOR is reactivated by the accumulation of degraded amino acids, and this reactivation triggers autophagy-lysosomal reformation (ALR) for nascent lysosomes formation from autolysosomes (Yu et al., 2010). mTOR also directly regulates the activity of lysosomal proteins. mTOR binds and inhibits ATP-sensitive Na^+ channels TPCs on lysosomes (Cang et al., 2013) and was suggested to phosphorylate the lysosomal Ca^{2+} channel TRPML1, resulting in its inhibition (Onyenwoke et al., 2015).

These findings expand our view of the lysosome from a simple effector of cellular clearance to a sensor and regulator of cell metabolism, providing exciting and challenging aspects in the field. Studies on identification of novel lysosomal signaling complexes may significantly enhance our understanding of lysosomal functions and may provide us with therapeutic implications for nutrient sensing related diseases.

1.4 Lysosomal adaptations to nutrient starvation

To fulfill degradation and signaling roles, lysosomes are highly dynamic and heterogeneous. They undergo multifaceted changes in ion composition, enzyme activity, position, size, and number to provide the optimal lysosome conditions for cell adaptation in response to environmental changes, which are referred to as "lysosomal adaptation". A well-studied scenario is lysosomal adaptation to nutrient deprivation, upon which mTOR is inhibited and TFEB is activated. Autophagy is then induced and greatly enhanced. To complete autophagy, lysosomes are transported to the perinuclear region to fuse with autophagosomes (Korolchuk et al., 2011; Li et al., 2016). Lysosomes are more acidified and activities of hydrolases inside are promoted to enhance lysosomal degradation capabilities (Zhou et al., 2013). Due to increased fusion between autophagosomes and lysosomes, the size and number of autolysosomes are dramatically increased and lysosome number is reduced in a short period of starvation time (1-2 hr). To adapt, lysosome number and size are recovered via lysosomal biogenesis and autophagic lysosome reformation (ALR) in a longer starvation time **(Fig. 1.4)** (Wang et al., 2015; Yu et al., 2010).

1.5 The lysosome is a new and significant intracellular Ca^{2+} store

Ca^{2+} is one of the most important and versatile second massagers in cells and regulates diverse

cellular processes such as cell motility, gene transcription, muscle contraction and exocytosis (Berridge et al., 2000). To fine-tune these processes, multiple organelles contribute to the regulation of global Ca^{2+} homeostasis and dynamics. The Endoplasmic Reticulum (ER) is the classic and largest Ca^{2+} store in the cell (Mekahli et al., 2011). Mitochondria mediate Ca^{2+} uptake and influence cell survival and cellular signaling (Rizzuto et al., 2012). Recently, the lysosome has been shown to be another significant Ca^{2+} store in the cells with around 0.5 mM Ca^{2+} within the lysosome lumen, 5,000 times more concentrated than in the cytosol (around 0.0001 mM) **(Fig. 1.5)** (Christensen et al., 2002; Lloyd-Evans et al., 2008). Upon physiological stimuli, Ca^{2+} is released from the lysosome lumen to the cytosol through regulation of specific proteins and channels (Shen et al., 2012). Lysosomal Ca^{2+} is a versatile regulator of various cellular processes including late endosome-lysosome fusion, lysosomal exocytosis, phagocytosis, membrane repair, signal transduction, lysosomal transportation and lysosome reformation (Cheng et al., 2014; Garrity et al., 2016; Li et al., 2016b; Pryor et al., 2000; Reddy et al., 2001; Samie et al., 2013). Interestingly, recent evidence suggests that the ER extensively cross-talks with lysosomes. In particular, membrane contacts sites between ER and lysosomes are formed (Phillips and Voeltz, 2016). Consistently, recently lysosomal Ca^{2+} is proposed to be refilled from the ER (Garrity et al., 2016).

The well characterized major lysosomal Ca^{2+} channel is TRPML1. One major cellular function of TRPML1 is to regulate organelles trafficking by releasing Ca^{2+} (Cheng et al., 2010; Dong et al., 2010). Consistently, lysosomal trafficking defects are observed in *TRPML1*$^{-/-}$ cells (Cheng et al., 2010). Moreover, even though still under debate, the two-pore channels (TPCs) have been proposed to mediate NAADP induced Ca^{2+} release (Marchant and Patel, 2013; Raffaello et al., 2016).

Upon elevation of juxtaorganellar Ca^{2+}, Ca^{2+} sensor proteins are recruited to translate Ca^{2+} changes into cellular responses. For example, synaptotagmin VII is the Ca^{2+} sensor mediating lysosomal exocytosis (Czibener et al., 2006; Samie and Xu, 2014). An EF-hand-containing apoptosis-linked gene 2 protein (ALG-2) serves as a Ca^{2+} sensor downstream of TRPML activation and regulates lysosomal positioning (Li et al., 2016b). Recently, the phosphatase calcineurin has been shown to be regulated by lysosomal Ca^{2+} and dephosporylates TFEB, triggering TFEB nuclear translocation (Medina et al., 2015). Considering the diverse roles of lysosomal Ca^{2+}, there are likely other Ca^{2+} sensors that are yet to be identified. The localization of particular groups of

lysosomes and effector, and cofactor availability may determine which Ca^{2+} effector is activated for a particular Ca^{2+} release (Li et al., 2016).

1.6 Lysosomes and diseases

Lysosomes are essential organelles in the cell. They exist in all eukaryotic cells except red blood cells. In other organisms, there are equivalent organelles, for example, vacuoles in yeast. Lysosomes have many essential functions such as degradation, plasma membrane repair, cholesterol homeostasis and antigen presentation (Boya, 2012). Disturbance of lysosomes has a profound impact on cell homeostasis, resulting in lysosomal storage disorders (LSDs). Although these are rare diseases, the frequency in total reaches 1/5000 live births. Besides, the number is underestimated due to the lack of reports from patients with mild phenotypes (Boya, 2012). It raises attention for relevant studies to identify mechanisms and develop treatments. LSDs are typically caused by mutations in genes encoding lysosomal enzymes and lysosomal membrane proteins. A major symptom is accumulated undigested and toxic contents, which results in secondary changes including impairment of autophagy, mitochondria dysfunction and inflammation (Vellodi, 2005). Moreover, toxic accumulations in the nervous system cause variable neurodegeneration in about two-thirds of LSD patients (Platt et al., 2012). In fact, neurons are especially sensitive to lysosomal defects because they are post-mitotic and cannot dilute accumulations via cell division (Ferguson, 2015). Accumulation of substrates due to dysfunction of lysosomes is also a hallmark of many other common neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's Diseases. With the advance of whole genomic studies, lysosomal gene mutations have been identified in many patients with common neurodegenerative diseases. For example, patients with heterozygosity of mutations in glucocerebrosidase, a lysosomal enzyme, are prone to Parkinson's disease (Sidransky et al., 2009). Moreover, dysfunction of lysosomes contributes to the progression of cancers and cardiovascular diseases via modifying activities of cathepsins (Appelqvist et al., 2013).

Treatments for lysosome-related diseases have been the focal point for many lysosomal researchers. Multiple treatments have been developed for LSDs, such as enzyme replacement therapy, pharmacological chaperones and substrate reduction therapy, however, current options are still inefficient or unavailable for most LSDs (Amalfitano and Rastall, 2015). For

neurodegenerative diseases, clearance of aggregate-prone proteins such as huntingtin and alpha-synuclein via enhanced autophagy-lysosomal pathway (ALP) has been a promising treatment (Settembre et al., 2013). With the discovery of TFEB, the important regulator of ALP, activation of TFEB has proved to be a promising treatment (Settembre et al., 2013). Thus, TFEB modulators and activators are studied and perused extensively for treating neurodegeneration and LSDs **(Fig. 1.6)** (Martini-Stoica et al., 2016).

I-2 The important regulator of autophagy and lysosomes --- TFEB

Lysosomes undergo dynamic changes in response to physiological stimuli. Coordination of these changes requires the existence of a regulatory network. Transcriptional factor EB (TFEB), along with other members of the MiT/TFE family are characterized as important regulators that mediate global transcriptional response for lysosomal changes (Raben and Puertollano, 2015; Sardiello et al., 2009). TFEB rapidly and efficiently regulates expression of more than 500 autophagic and lysosomal genes, facilitating lysosomal adaptation to environmental changes (Palmieri et al., 2011; Sardiello et al., 2009; Wang et al., 2015). TFEB is recruited to lysosomal membrane and phosphorylated by mTOR (Settembre et al., 2012; Martina and Puertollano, 2013). TFEB is also regulated by lysosomal Ca^{2+} from TRPML1, which will be detailed described in Chapter II (Medina et al., 2015b; Wang et al., 2015). Activation of TFEB has been a promising development direction for lysosome-related disorders (Ballabio, 2016; Sardiello et al., 2009). Understanding of TFEB regulation will facilitate the discovery of TFEB activators.

2.1 Discovery of TFEB as an important regulator of autophagic and lysosomal genes

Using a computational and systems biology approach, lysosomal genes with very similar expression pattern were characterized, and a specific promoter element with a palindromic GTCACGTGAC 10-base site was identified (Sardiello et al., 2009). This site, also known as E-box, is the target site of the microphthalmia-transcription factor E (MiT/TFE) subfamily, which has four members: TFEB, TFE3, TFEC, and MITF. Among them, transcriptional factor EB (TFEB) preferentially binds to the 10-base DAN sequence, also named as Coordinated Lysosomal Expression and Regulation (CLEAR), and promotes lysosomal genes expression (Sardiello et al., 2009). This was the first evidence to support global regulation of lysosomes. In further genomic and expression analyses, TFEB was also shown to coordinate gene expression involved in

autophagy and many other lysosome-associated processes such as endocytosis, phagocytosis and immune response (Palmieri et al., 2011). Consistently, overexpression of TFEB in cultured cells upregulates expression of lysosomal and autophagic genes, suggesting that the different stages of autophagic-lysosomal pathway are mechanistically linked (Settembre et al., 2011). More studies revealed that not only TFEB, but also MITF and TFE3, other members of the MiT/TFE family, also respond to nutrients level and globally regulate autophagic and lysosomal genes (Martina et al., 2014; Ploper et al., 2015; Raben and Puertollano, 2016). Thus, TFEB, TFE3, and MITF have been identified to be a family of transcriptional factors that regulates autophagic and lysosomal genes globally.

2.2 Function and Regulation of TFEB

TFEB regulates genes involved in the autophagic-lysosomal pathway and other lysosome-related pathways (Palmieri M et al., 2011). More recent studies have shown that TFEB has a more diverse role and regulates other pathways, for example ATF4 and AT4 targets in the unfolded protein response (UPR) (Martina J et al., 2016) and RANKL-PKCβ-TFEB signaling cascade in osteoclasts (Ferron et al., 2013). Moreover, TFEB regulates lipid catabolism by up-regulating peroxisome proliferator-activated receptor α (PPARα) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), both of which mediate lipid breakdown under starvation. Overexpression of TFEB in mouse liver attenuates diet-induced obesity, suggesting a therapeutic strategy for lipid metabolism disorders (Settembre et al., 2013).

TFEB is tightly controlled. In response to nutrient starvation (Roczniak-Ferguson A et al,. 2012), oxidative stress (Zhang XL et al., 2016) and ER stress (Martina JA et al., 2016), TFEB translocates to the nucleus and mediates a global transcriptional response (Settembre et al., 2013). The localization of TFEB is determined by the phosphorylation status of TFEB, as phosphorylated TFEB binds with an adaptor protein 14-3-3 and stays in the cytosol, while dephosphorylated TFEB translocates to the nucleus (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre and Ballabio, 2011). mTORC1 mediates the phosphorylation of TFEB at Ser211 and possibly Ser142 (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012b). Like mTOR, TFEB is recruited to the lysosomal membrane for phosphorylation via active Rag GTPase **(Fig. 1.3)** (Martina and Puertollano, 2013). ERK2 was also suggested to phosphorylate TFEB at Ser142 and

retain it in the cytosol (Cea et al., 2012; Pena-Llopis et al., 2011). In RANKL-PKCβ-TFEB signaling cascade for lysosomal biogenesis in osteoclasts, PKCβ is shown to phosphorylate TFEB at S461 and/or S462, S466 and S468 (Ferron et al., 2013).

TFEB is not only regulated post-translationally, but also transcriptionally. Upon starvation-induced TFEB nuclear translocation, *TFEB* transcription is induced. This allows rapid expression of TFEB, which responds to nutrient availability and forms a positive transcriptional auto-regulatory feedback loop for a sustained response (Settembre et al., 2013).

The function and regulation of TFEB are evolutionarily conserved from *C.elegans* to human, implying a requirement of the TFEB regulatory mechanism for organismal adaptation to challenging nutritional conditions (Settembre et al., 2013). In *C. elegans*, the TFEB homologue, HLH-30, acts in a similar manner to TFEB and drives transcriptional response in lipid metabolism and autophagy in fasting worms, which leads to an extended lifespan (Lapierre et al., 2013; Settembre et al., 2013). A conditional knockout mouse line with TFEB deleted in the liver was generated and has been a powerful tool to understand TFEB at the tissue level (Settembre et al., 2012). However, global loss of function of TFEB in the mouse is embryonically lethal due to defects in placental vascularization (Steingrímsson et al., 1998). Thus, the *C. elegans* model of *HLH-30-null* would greatly facilitate our understanding on TFEB at the organism level.

2.3 TFEB activation as a potential treatment for LSDs and neurodegenerative diseases

With the recent discovery of TFEB as the important regulator of ALP, activation and over-expression of TFEB is an appealing treatment for lysosomal storage disorders and neurodegeneration resulting from accumulation of autophagic substrates in lysosomes, which has already been proven to be effective **(Fig. 1.6)** (Appelqvist et al., 2013). Overexpression of TFEB dramatically reduced GAGs accumulation in Multiple sulfatase deficiency (MSD) and mucopolysaccharidosis type IIIA (MPSIIIA), two severe types of lysosomal storage disorders (Sardiello et al., 2009; Medina et al., 2011), and glycogen load and autophagic vacuoles in Pompe's Disease (Spampanato et al., 2013). Moreover, viral-mediated TFEB gene expression mediated cellular clearance in mouse models of MSD and Pompe's Disease (Medina et al., 2011; Spampanato et al., 2013). Furthermore, TFEB overexpression significantly rescued the disease phenotype in Parkinson's, Alzheimer's, and Huntington's (Ballabio, 2016; Decressac et al., 2013;

Dehay et al., 2010; La Spada, 2012; Lee et al., 2014; Pastore et al., 2013; Polito et al., 2014). Thus, further development of TFEB activators proves to be an attractive therapeutic strategy for lysosome-related disorders (Settembre et al., 2013).

I-3 The lysosomal Ca^{2+} permeable channel --- TRPML1

TRPML1, a member of transient receptor potential mucolipin (TRPML) channel subfamily, is a cation channel predominantly localized on the membranes of late endosomes and lysosomes (LELs) (Hersh et al., 2002; LaPlante et al., 2004). In response to the regulatory changes of the LEL-specific $PI(3,5)P_2$ and other cellular cues, TRPML1 releases Ca^{2+} into the cytosol from the LEL lumen (Dong et al., 2010). Such Ca^{2+} released locally and temporally triggers downstream membrane trafficking events (fission and fusion), conducts signal transduction and maintains lysosomal ionic homeostasis (Cheng et al., 2010). At the cellular level, loss of function of TRPML1 results in lysosomal trafficking defects and cellular lysosomal storage. Human mutations in TRPML1 result in type IV Mucolipidosis (ML-IV), a childhood neurodegenerative lysosome storage disease.

3.1 Expression and subcellular localization of TRPML1

Human TRPML1 (or mucolipin-1/MCOLN1) is encoded by the *MCOLN1* gene localized on chromosome 19 (Bargal et al., 2000; Bassi et al., 2000; Slaugenhaupt et al., 1999; Sun et al., 2000). Although there are two other *TRPML1*-related genes, i.e., *TRPML2* and *TRPML3,* in the human and mouse genomes (Cheng et al., 2010), there is only one gene in *Saccharomyces cerevisiae*, *Dictyostelium*, *C.elegans* and *Drosophila*, *yvc1*, *mcln*, *cup*-5 and *trpml,* respectively. These genes share 30-50% sequence identity with human *TRPML1* and encode the TRPML protein or proteins with similar functions, mediating Ca^{2+} release and regulating trafficking steps (Denis and Cyert, 2002; Fares and Greenwald, 2001; Lima et al., 2012; Shen et al., 2012).

Cellular phenotypes of ML-IV and its mouse model indicate that TRPML1 is predominately localized on the membranes of LELs (Slaugenhaupt, 2002; Venugopal et al., 2007), which is further confirmed by immunostaining and gradient fractionation analysis (Kim et al., 2009; Zeevi et al., 2009). The LEL localization of TRPML1 is instructed by two di-Leucine motifs located separately in the N-terminal and the C-terminal cytosolic tails **(Fig. 1.7)** (Abe and Puertollano,

2011; Pryor et al., 2006; Vergarajauregui and Puertollano, 2006). Recently, TRPML1 was demonstrated to also locate in tubulevesicles in parietal cells, mediating gastric acid secretion of stomach by Ca^{2+}-dependent exocytosis (Sahoo N et al., 2017, In press).

TRPML1 is ubiquitously expressed in mouse tissues, with the highest levels of transcripts in the brain, kidney, spleen, liver, gastric, and heart (Falardeau et al., 2002; Samie et al., 2009; Sahoo N et al., 2017, In Press). Consistent with this expression pattern, loss of TRPML1 results in enlarged late endosomes and lysosomes (LELs) and accumulation of lysosomal storage materials in most cell types of ML-IV patients and *Trpml1* knockout mice (Slaugenhaupt, 2002; Venugopal et al., 2007).

3.2 Protein Structure of TRPML1

Human TRPML1 is a 580-amino acid transmembrane protein (Slaugenhaupt, 2002). Due to lack of a crystal structure, our current knowledge about the topology of TRPML1 is mainly gained from bioinformatic analyses, biochemical and structural studies on other TRP channels (Cheng et al., 2010; Dong et al., 2010). Similar to other TRP channels, TRPML1 consists of six putative transmembrane-spanning domains (TMs; S1-S6) with the amino (NH2 or N)- and carboxy (COOH or C)-terminal tails facing the cytosol **(Fig. 1.7)**. Recently, a partial structure of TRPML1 of the linker between the first and second transmembrane segments (S1-S2) was resolved (Li et al., 2017), which revealed the structural basis for dual Ca^{2+}/H^{+} regulation of TRPML1 using $TRPML1^{Va}$, a constitutively active mutant form of TRPML1. With the development of single-particle electron cryo-microscopy (cryo-EM) technique, high-resolution structural analysis of membrane proteins has become more feasible (Liao et al., 2013). High-resolution full-length ML1 structural may be available in the near future, which may further improve our understanding of the regulation and function of TRPML1.

3.3 The biophysical properties and regulation of TRPML1

The LEL localization of TRPML1 complicates the analysis of the permeation and gating properties of the channel. However, the recent development of the whole-endolysosome patch-clamp technique has allowed direct study of TRPML1 on artificially-enlarged lysosomes, which are induced by vacuolin-1, a small molecule that selectively enlarges lysosomes (Dong et al., 2010;

Huynh and Andrews, 2005; Wang et al., 2012). Using the whole-lysosome recordings, it was shown that TRPML1-mediated currents exhibit strong inward rectification (inward indicates cations moving out of the lysosomal lumen). TRPML1 is permeable to Ca^{2+}, Fe^{2+}, Zn^{2+}, Na^{+}, and K^{+} (Dong et al., 2008; Dong et al., 2009; Xu et al., 2007). The putative channel pore of TRPML1 is predicted to be formed by transmembrane segment 5 (S5), transmembrane segment 6 (S6) and the linker or the so-called "pore-loop" region between S5 and S6 (**Fig. 1.7**), which constitutes the selectivity filter of the channel (Cheng et al., 2010). Replacing two negatively-charged amino acid residues in the pore loop with positively-charged ones ($D^{471}D^{472}$-KK) results in a pore-dead non-conducting channel (Dong et al., 2010a; Grimm et al., 2012; Xu et al., 2007).

Interestingly, a proline substitution at V^{432} ($V^{432}P$ or Va, a mutation at the homologous position in TRPML3 causing the Varitint-Walder (Va) phenotype with pigmentation and vestibular defects in mice (Di Palma et al., 2002; Xu et al., 2007) in the lower part of S5 in TRPML1 results in gain-of-function (GOF) constitutively-active TRPML1 channels at both the plasma membrane and endolysosomal membranes (Xu et al., 2007). The constitutive channel activity caused by Pro substitutions is proposed to be related to locking or facilitating channel conformation at the open state (Dong et al., 2010a; Grimm et al., 2012; Xu et al., 2007). Furthermore, unlike the wild-type TRPML1 channel, $TRPML1^{Va}$ showed a dramatically-increased plasma membrane localization, suggesting that the constitutive release of luminal cations (most likely Ca^{2+}) promotes the delivery of TRPML1 to the plasma membrane, likely via lysosome exocytosis (Dong et al., 2009).

Using whole-endolysosome recordings, endogenous activators and inhibitors have been identified for TRPML1. Phosphoinositides have been shown to regulate TRPML1 in a compartment-specific manner. $PI(3,5)P_2$, a phosphoinositide mainly localized in the LEL, potently activates TRPML1 potentially through a direct binding mechanism (Dong et al., 2010; Zhang et al., 2012). On the other hand, $PI(4,5)P_2$, a plasma membrane-specific phosphoinositide, inhibits TRPML1 (Zhang et al., 2012). Such distinct effects of the two phosphoinositide isomers are well suited to meet the cellular functions of TRPMLs. While $PI(3,5)P_2$ may activate TRPMLs to induce endosomal and lysosomal Ca^{2+} release for the purpose of membrane trafficking, $PI(4,5)P_2$ may prevent the channels being active at the plasma membrane (PM). Likewise, Sphingomyelin, a lipid that is abundant at the PM and hydrolyzed in the lysosome, inhibits TRPML1 (Shen et al., 2012). Similarly, overload of lysosomal adenosine may inhibit TRPML1 and impair lysosome

function, resulting in cell death in B-lymphocytes (Zhong et al., 2017). Furthermore, TRPML1 was recently identified as a reactive oxygen species (ROS) sensor in the lysosome in response to oxidative stress. When there is an elevation of ROS levels due to mitochondrial damage, TRPML1 is activated and triggers Ca^{2+} dependent TFEB nuclear translocation, which promotes clearance of mitochondria and removal of excess ROS (Zhang et al., 2016).

3.4 Modulation tools of TRPML1 function: synthetic agonists and antagonists

To further investigate the activation mechanisms of TRPML1, several synthetic small molecule compounds have been identified as TRPML specific agonists and antagonists (Chen et al., 2014; Grimm et al., 2010; Shen et al., 2012; Wang et al., 2015; Zhang et al., 2016). Of them, optimized Mucolipin Synthetic Agonists (ML-SAs) robustly activate TRPML1 at low micromolar or even nanomolar concentrations with a response comparable to or better than $PI(3,5)P_2$, and Mucolipin Synthetic Antagonists (ML-SIs) specifically inhibit TRPML1 currents (Zhang et al., 2016). These agonists and antagonists are helpful not only in investigating the gating mechanisms of TRPML1, but also in probing the biological functions of the channel **(Fig. 1.7)** (Grimm et al., 2012; Zhang et al., 2016). Moreover, these small molecules may be used to restore channel function and rescue lysosome-related diseases with reduced TRPML1 activity, such as diseases associated with loss of function of *fig4* and Niemann-Pick type C (Chen et al., 2014; Shen et al., 2012; Wang et al., 2015; Zou et al., 2015).

3.5 Physiological and cellular functions of TRPML1

TRPML1 mediates lysosomal Ca^{2+} release and regulates many lysosomal membrane trafficking steps, including fusion and fission events through the endocytic pathway, autophagosome-lysosome fusion, phagocytosis, exocytosis (Cheng et al., 2010; Cheng et al., 2014; Li et al., 2016; Samie et al., 2013). For example, TRPML1 regulates lysosome tabulation and reformation via TRPML1-ALG2-dynein signaling pathway (Li et al., 2016). Lysosomal Ca^{2+} release from TRPML1 also affect many important cellular signaling pathways. The TRPML1 homolog in *Drosophila* is required for TORC1 activation by promoting vesicle fusion (Wong et al., 2012). In mammalian cells, even though still controversial, Li et al. demonstrated that modulation of TRPML1 activity directly affected mTORC1 activity (Li et al., 2016). Furthermore, TRPML1 regulates Ca^{2+}-dependent TFEB activation in response to nutrient availability and

cellular stress, such as starvation and increased ROS, mediating necessary lysosomal adaptation (Medina et al., 2015; Zhang et al., 2016). Moreover, Ca^{2+} release from LELs is believed to play an essential role in the transduction of extracellular signals such as glucose-induced insulin secretion in β-cells, smooth muscle contraction, T lymphocyte activation, and neurotransmitter release (Galione et al., 2009).

Lysosomal Ca^{2+} may also couple with activation of other Ca^{2+} dependent channel, such as big conductance Ca^{2+}-activated K^+ channel (BK) channel, which is recently shown to function on lysosomal membrane in additional to previous well-established locations (Cao et al., 2015). Additionally, TRPML1 may regulate global Ca^{2+} dynamics by evoking global Ca^{2+} signaling and Ca^{2+} entry related with ER, potentially via the ER-lysosome membrane contacts sites (Kilpatrick et al., 2016).

Besides Ca^{2+}, TRPML1 is also permeable to Fe^{2+} and Zn^{2+}, and may participate in the regulation of the cellular homeostasis of these heavy metals (Dong et al., 2008). Cells that lack TRPML1 exhibit a cytosolic Fe^{2+}-deficiency and an overload of lysosomal Fe^{2+}, suggesting that TRPML1 contributes to transporting iron out of the lysosomes (Dong et al., 2008). Similarly, the permeability of TRPML1 to Zn^{2+} and elevated Zn^{2+} levels in *TRPML1*$^{-/-}$ cells, are suggestive of an essential role of TRPML1-mediated lysosomal Zn^{2+} transport (Cuajungco and Kiselyov, 2017; Eichelsdoerfer et al., 2010; Kukic et al., 2013).

3.6 TRPML1 KO animal models and related diseases

Mutations in TRPML1 cause mucolipidosis type IV (ML-IV), which is a severe autosomal recessive lysosomal storage disease (LSD) characterized clinically by neuromotor retardation, retinal degeneration, corneal opacity, iron-deficiency anemia and gastric abnormality (Berman et al., 1974). Different from other LSDs with reduced lysosomal hydrolase activities, ML-IV has metabolic defects. At the cellular level, enlarged vacuolar structures and accumulation of membranous lipids were detected. In NP-type C cells (NPC), which is another lysosomal storage disease, decreased activity of sphingomyelinase (SMase) in those cells leads to accumulation of SMs on the lumen side of the membrane, which greatly inhibits TRPML1 activity and results in defects in Ca^{2+} release from TRPML1 and Ca^{2+}-dependent lysosomal trafficking (Shen et al., 2012), suggesting that deregulation of TRPML1 channel in the lysosome may be a primary

pathogenic mechanism that causes secondary lysosome storage in NPC.

Using genetic knockout (KO) approaches, animal models of ML-IV have been established in mice, *C. elegans* and *Drosophila*, providing opportunities to better understand the underlying pathogenic mechanisms at the organism and cellular levels, and to develop potential therapeutic strategies for ML-IV. The first murine model of TRPML1 KO displays neurological, gastric, and ophthalmological abnormalities that are reminiscent of the clinic features of ML-IV patients (Venugopal et al., 2007). They are great model systems for us to understand TRPML1's function in the tissue or organism level.

Based on our current understanding on TRPML1, many potential therapeutical ways have been proposed. Upon further improvement, small molecule activators of TRPML1 might be able to be applied to restore abnormal lysosomal storage in MLIV patients, Niemann-Pick type C disease, Fig-4 deficiency and HIV-associated dementia (Bae et al., 2014; Chen et al., 2014; Shen et al., 2012; Zou et al., 2015). Meanwhile, it was shown that high-protein diets could rescue the defects induced by loss of function of *dTRPML* in *Drosophila* (Wong et al., 2012). Thus, it is of great interest to investigate the effect of amino acid supplementation in reducing MLIV manifestations. With the finding of TFEB as the important regulator of the autophagy-lysosomal pathway, it would be worthwhile to test whether TFEB activation can be helpful for MLIV (Martini-Stoica et al., 2016).

I-4 The goals of this thesis

Lysosome functions and adaptation are subject to a global transcriptional control by nutrient-sensitive TFEB via a lysosome to nucleus signaling pathway. Modulation of lysosomes, especially enhancement of autophagy-lysosomal pathway via TFEB, is a promising therapeutic strategy for lysosome-related diseases, which requires comprehensive understanding of TFEB regulation. Considering the important role of lysosomal Ca^{2+} and $PI(3,5)P_2$ on lysosomal functions and their reported changes in response to nutrient availability, my major aim was to identify the role of Ca^{2+} and $PI(3,5)P_2$ in regulating TFEB in response to nutrient availability. Screening for small molecules to treat lysosome-related diseases, TFEB/TFEB activators, is my secondary aim (**Fig. 1.8**).

I-5 Figures

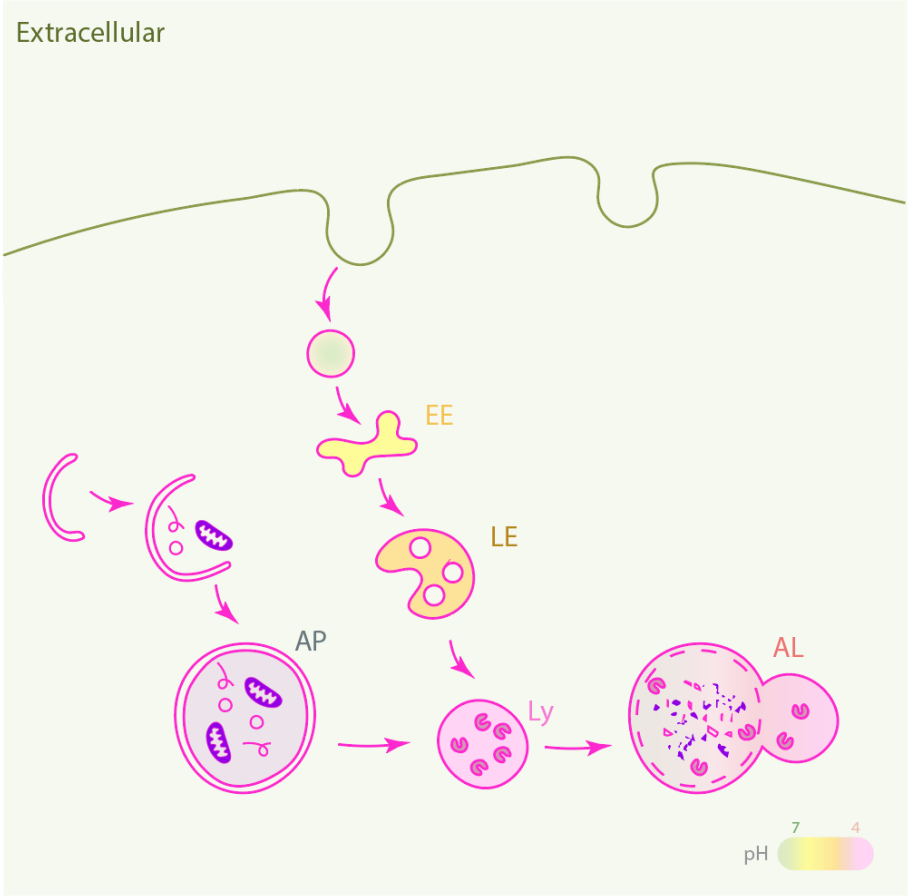

Figure 1.1 Pathways for substrate delivery to lysosomes.

Two major pathways have been identified to deliver substrates to lysosomes for degradation: endocytosis for extracellular content degradation and autophagy for intracellular contents degradation. During endocytosis, cargos are routed from early endosome (EE), late endosomes (LEs) and then lysosomes for degradation. Along the endocytic pathway, the intravesical pH drops from 6.0-6.5 in the early endosomes to 4.5-5.5 in the late endosomes and lysosomes. Upon initiation of autophagy, a phagophore with an isolated membrane is formed. The phagophore then expands to engulf targeted cargo, such as protein aggregates and damaged organelles, and forms a double-membraned autophagosome (AP). The loaded autophagosome then fuses with the lysosome (LY), and contents in the autophagosome are degraded by lysosome-resident hydrolases in the newly formed autolysosome (AL).

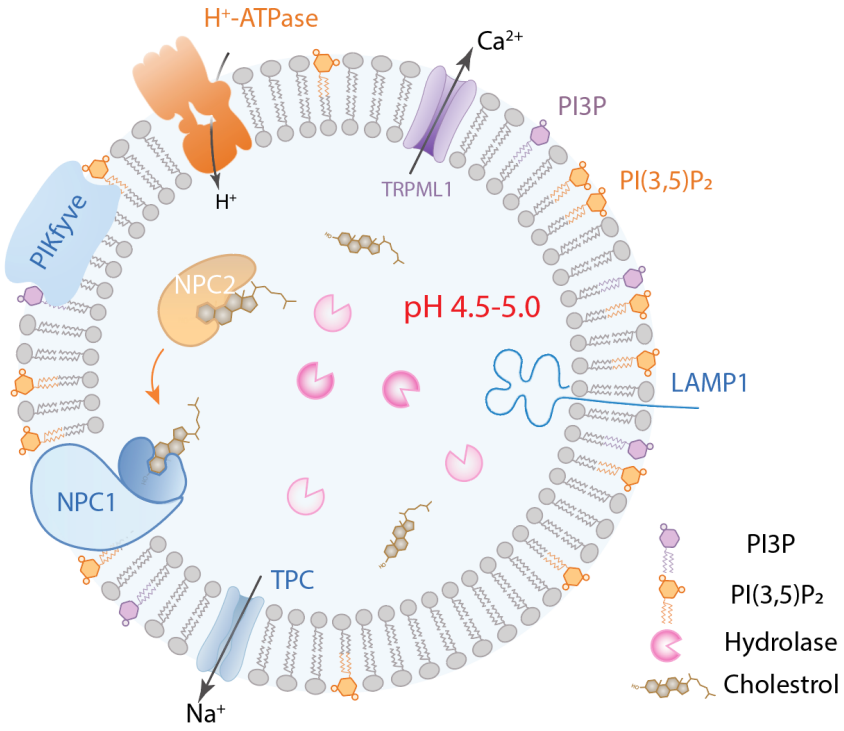

Figure 1.2 Characteristics of lysosomes.

Lysosomes are maintained at acidified pH, by a proton pump vacuolar H^+-ATPase, which is required for optimal lysosomal hydrolase activity. Lysosomes are identified by the localization of lysosome-specific structural proteins, such as LAMP1/2. Phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$] is a lysosomal-specific phosphoinositide, which is generated by PI3P 5 kinase PIKfyve from phosphatidylinositol 3-phosphate (PI3P) during the maturation of lysosomes along the endocytic pathway. Transient receptor potential mucolipin1 (TRPML1) is the major lysosomal Ca^{2+} channel, which mediates Ca^{2+} release from the lysosome lumen to the cytosol. Two-pore channels (TPCs) are lysosomal Na^+ channels. Compartmentalized $PI(3,5)P_2$ serves as an endogenous activator for TRPML1 and TPCs. Niemann-Pick type C proteins (NPC1&2) bind with free cholesterol and mediate the export of cholesterol from the lysosomes to diverse cellular compartments. Dysfunctions in NPCs result in cholesterol accumulation inside lysosomes and a lipid storage disorder, Niemann-Pick type C.

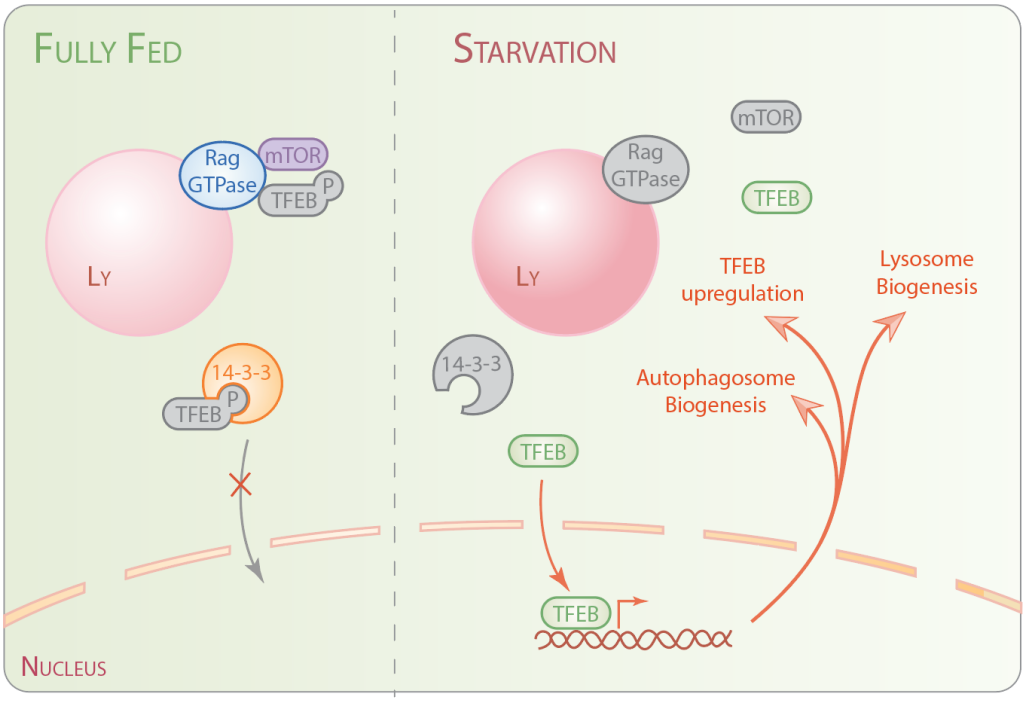

Figure 1.3 The lysosome is a cell signaling center.

The lysosome membrane is home to the lysosomal nutrient sensing machinery, which integrates metabolic signals with intracellular changes. One major component is Rag GTPases. When amino acids are present, active Rag GTPases recruit mTOR to the lysosomal surface and facilitate its activation. Meanwhile, TFEB is recruited to the lysosomal membrane via active Rag GTPases for phosphorylation by mTOR. Phosphorylated TFEB binds with adaptor protein 14-3-3, and is retained in the cytosol. Under starvation, mTOR is inhibited, and TFEB rapidly gets dephosphorylated and translocates to the nucleus, inducing autophagosome and lysosome biogenesis. Up-regulation of TFEB itself is also induced by TFEB activation, forming a positive transcriptional auto-regulatory feedback loop for a sustained response.

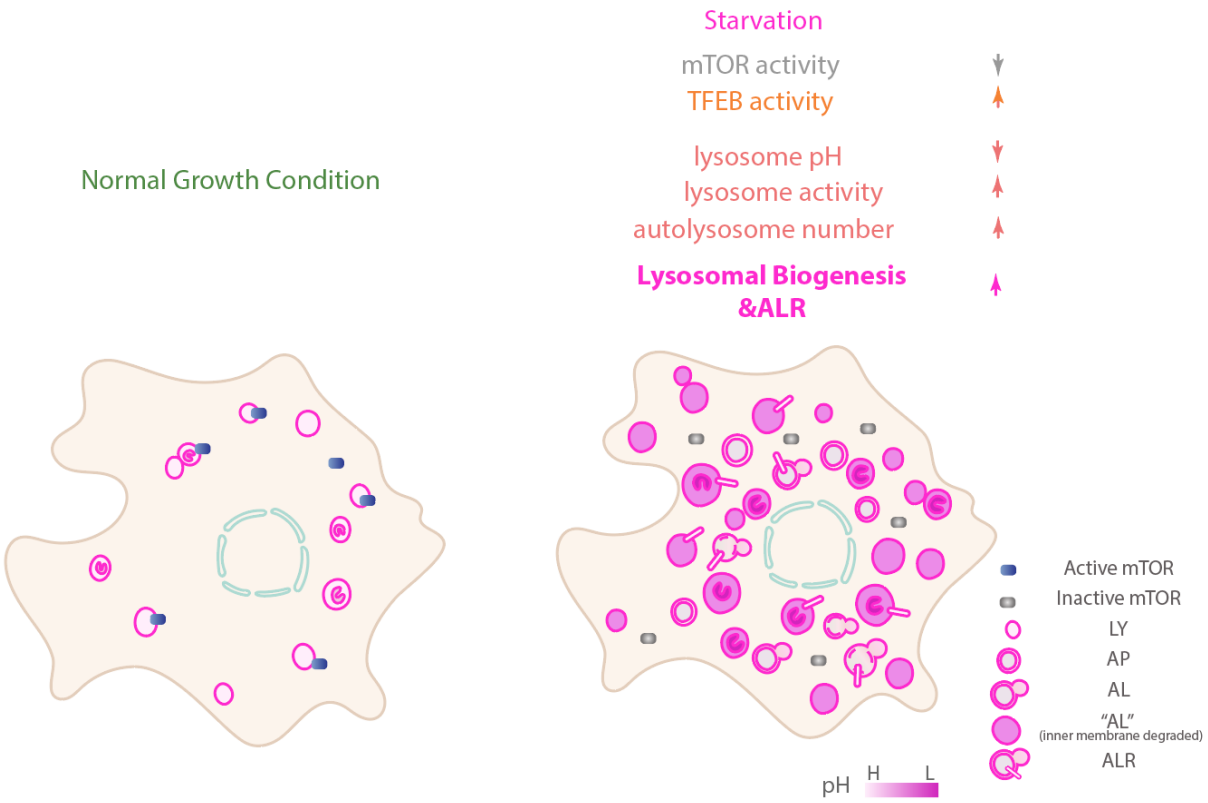

Figure 1.4 Lysosomal adaptations in response to starvation.

Lysosomes undergo multifaceted changes in the enzyme activity, ionic composition, lipid composition, position, size, and number to provide the optimal lysosome conditions for cell function in response to environmental changes, which are referred to as "lysosomal adaptation". Under nutrient deprivation, mTOR is inhibited and TFEB is activated. Autophagy is then induced and greatly enhanced. To complete autophagy, lysosomes (LYs) are transported to the perinuclear region to fuse with autophagosomes (APs). Lysosomal pH becomes more acidic and activities of hydrolases are promoted to enhance lysosomal degradation capabilities. Due to increased fusion between autophagosomes and lysosomes, the number of autolysosomes (ALs) is dramatically increased and lysosome number is reduced after a short period of starvation time (0-2 hr). To adapt, lysosome number and size are recovered via lysosomal biogenesis and autophagic lysosome reformation (ALR) after a longer starvation time.

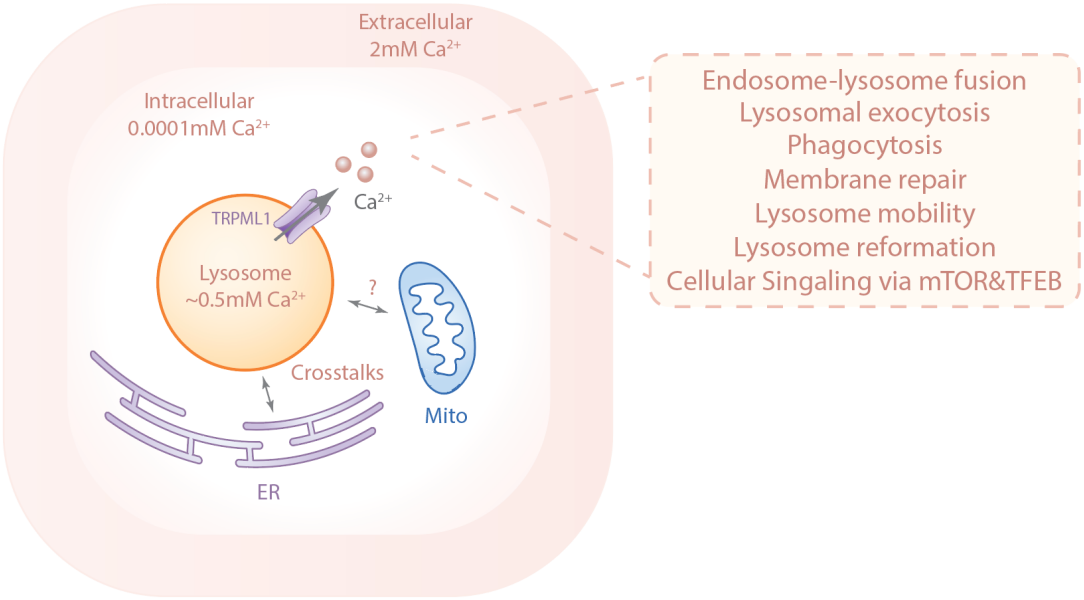

Figure 1.5 The lysosome is an important intracellular Ca^{2+} store.

The lysosome is a newly characterized and significant Ca^{2+} store in the cell, with ~0.5 mM Ca^{2+} within the lysosome lumen, 5,000 times more concentrated than in the cytosol. Upon physiological stimuli, specific proteins and channels are regulated. Ca^{2+} is released from the lysosome lumen to the cytosol, regulating endosome-lysosome fusion, lysosomal exocytosis, phagocytosis, membrane repair, lysosomal transportation, lysosomal reformation and signal transduction. Lysosomal Ca^{2+} also cross-talks with ER. Lysosomal Ca^{2+} is suggested to be refilled from the ER, which requires IP3 receptor and membrane contacts sites between lysosomes and ER.

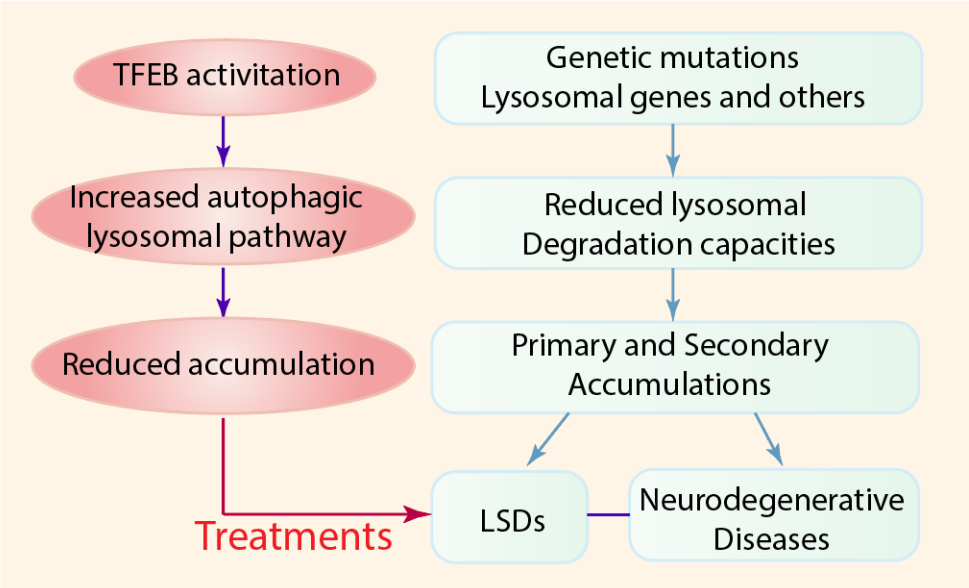

Figure 1.6 Activation of TFEB is a promising treatment for lysosome-related diseases.

Accumulation of substrates due to dysfunction of lysosomes is a hallmark of lysosomal storage disorders (LSDs) and many common neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's Diseases. Activation of TFEB enhances the autophagy-lysosomal pathway and facilitates clearance of primary and secondary accumulations in lysosomal storage diseases, neurodegenerative diseases, and other diseases. Development of small molecules that modulate TFEB to prime the autophagy-lysosomal pathway (ALP) for substance digestion is an attractive therapeutic strategy.

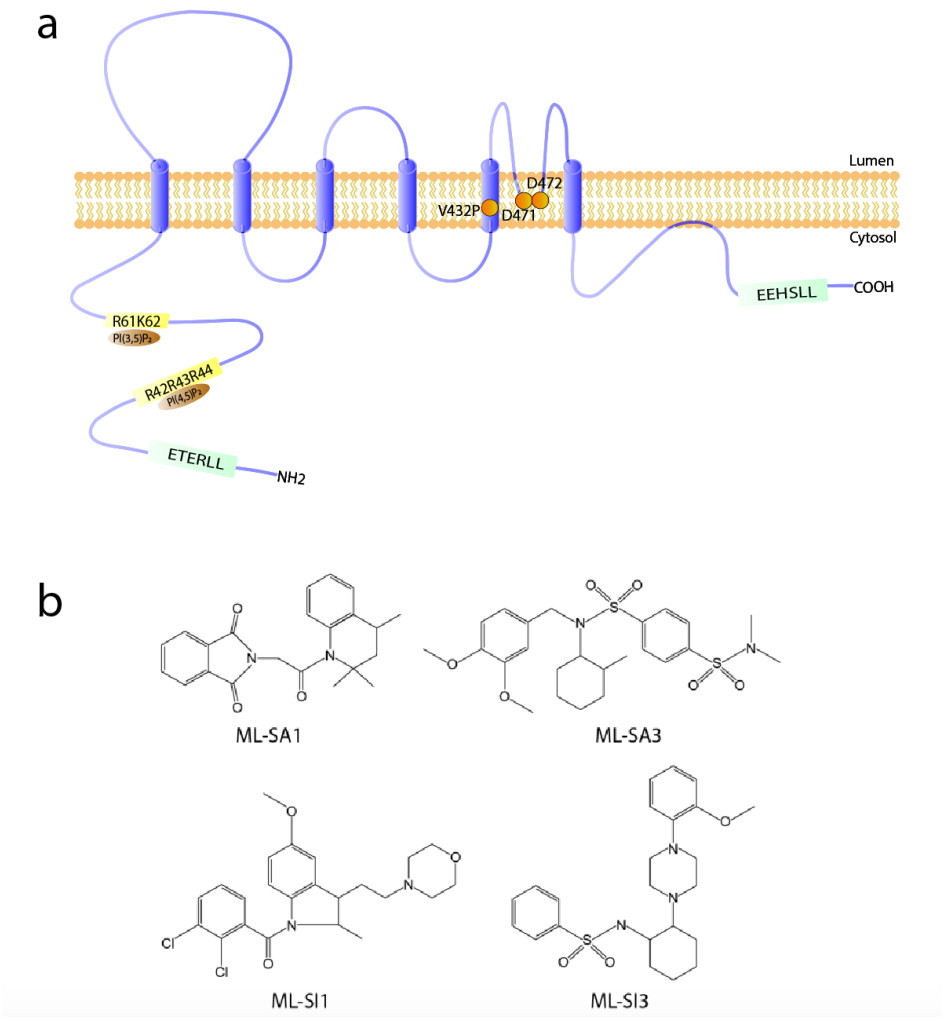

Figure 1.7 Structure and functional modulation of TRPML1.

(a) TRPML1 consists six trans-membrane (6TM) domains with the amino (NH_2)- and carboxyl (COOH)- terminal tails oriented within the cytosol. The putative pore of TRPML1 is formed by S5, S6 and the linker region between S5 and S6. Replacing two negatively-charged amino acid residues in the pore loop with positively-charged ones ($D^{471}D^{472}$-KK) results in a pore-dead non-conducting channel. The LEL localization of TRPML1 is instructed by two di-Leucine motifs located separately in the N-terminal and the C-terminal cytosolic tails. In addition, $TRPML1^{Va}$ with $V^{432}P$ has a dramatically-increased plasma membrane localization. Phosphoinositides have been shown to directly bind and regulate TRPML1 function in a compartment-specific manner. $PI(3,5)P_2$, a phosphoinositide mainly localized in the LEL, binds with R61K62 and potently activates TRPML1, while $PI(4,5)P_2$, a plasma membrane-specific phosphoinositide, binds with R42R43R44 and inhibits TRPML1. **(b)** To facilitate functional studies of TRPML1, synthetic agonists and antagonists of TRPML1 have been generated and optimized, including the first generation of TRPML1 synthetic agonist and antagonist (ML-SA1 and ML-SI1), as well as newer generations (ML-SA3 and ML-SI3).

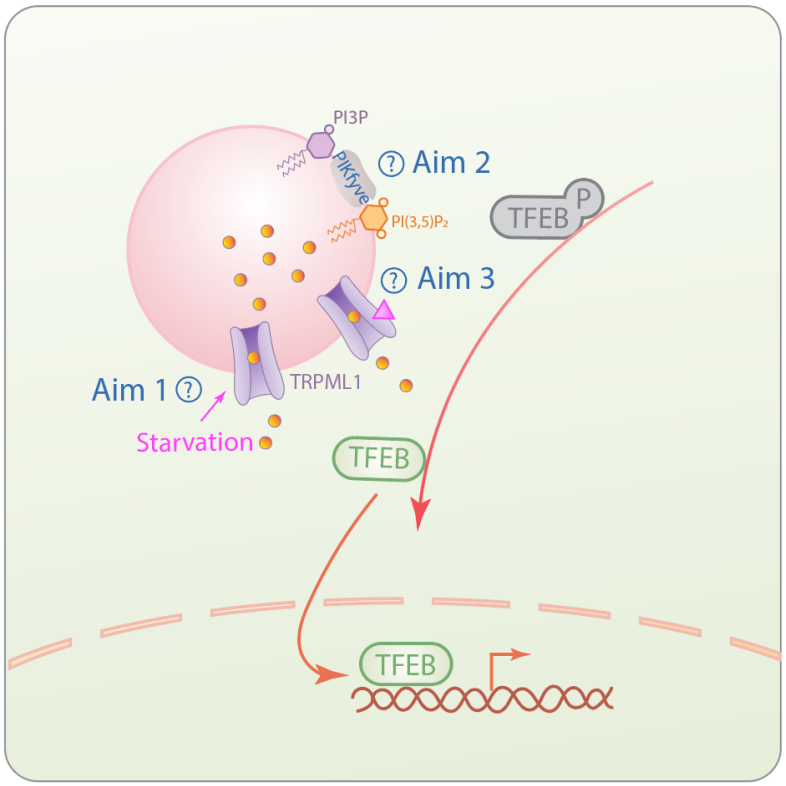

Figure 1.8 Aims of this thesis.

This thesis study aimed to understand the mechanisms by which lysosomes relay environmental signals and confer lysosomal changes and regulations. In particular, I intended to elucidate the relationship between TRPML1 (**Aim 1**), and PIKfyve (**Aim 2**) with TFEB. Meanwhile, development of effective treatments for lysosome-related diseases via screening for small molecules, TRPML or TFEB activators, is the other aim of my thesis (**Aim 3**).

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CHAPTER II [2]

Activation and Upregulation of Lysosomal TRPML1 Channels Are Essential for Lysosomal Adaptation to Nutrient Starvation via TFEB

II-1 Abstract

Upon nutrient starvation, autophagy digests unwanted cellular components to generate catabolites that are required for housekeeping biosynthesis processes. A complete execution of autophagy demands an enhancement in lysosome function and biogenesis to match the increase in autophagosome formation. Here, I report that mucolipin-1 (also known as TRPML1 or ML1), a Ca^{2+} channel in the lysosome that regulates many aspects of lysosomal trafficking, plays a central role in the autophagic-lysosomal pathway at two phases. Via Ca^{2+} imaging, a rapid lysosomal Ca^{2+} release largely mediated by TRPML1 was detected within minutes of nutrient starvation, which was required for initiating TFEB nuclear translocation. Furthermore, by Ca^{2+} imaging and whole-lysosome patch-clamping, lysosomal Ca^{2+} release and ML1 currents were detected within hours of nutrient starvation and were potently upregulated. In contrast, lysosomal Na^{+}-selective currents were not upregulated. Activation of TRPML1, inhibition of mechanistic target of rapamycin (mTOR) or activation of transcription factor EB (TFEB) mimicked a starvation effect in fed cells. The starvation effect also included an increase in lysosomal proteostasis and enhanced clearance of lysosomal storage, including cholesterol accumulation in Niemann-Pick disease type C (NPC) cells. However, this effect was not observed when ML1

[2] Materials presented in the chapter II are adapted with modifications from my co-first-authored paper published in PNAS Plus (*Wang, W.*, Q. Gao *, M. Yang, X. Zhang, L. Yu, M. Lawas, X. Li, M. Bryant-Genevier, N.T. Southall, J. Marugan, M. Ferrer, and H. Xu. 2015. Up-regulation of lysosomal TRPML1 channels is essential for lysosomal adaptation to nutrient starvation. Proc Natl Acad Sci U S A. 112: E1373-1381*) and co-authored paper in Nature Cell Biology (*Medina, D.L., S. Di Paola, I. Peluso, A. Armani, D. De Stefani, R. Venditti, S. Montefusco, A. Scotto-Rosato, C. Prezioso, A. Forrester, C. Settembre, W. Wang, Q. Gao, H. Xu, M. Sandri, R. Rizzuto, M.A. De Matteis, and A. Ballabio. 2015. Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. Nat Cell Biol. 17:288-299*). Electrophysiology recording experiments in Fig. 2.4, 2.6, 2.7, 2.8, 2.9, Filipin Staining in Fig. 2.10 and Ca^{2+} imaging experiments in Fig. 2.5 were performed by my collaborator Dr. Wuyang Wang. DQ-BSA experiments in Fig 2.11 were performed in collaboration with Dr. Wuyang Wang.

was pharmacologically inhibited or genetically deleted. Moreover, overexpression of ML1 mimicked the starvation effect. Hence, lysosomal adaptation to environmental cues such as nutrient deprivation requires lysosomal Ca^{2+} signaling, which is contributed by TRPML1 upon immediate starvation, and TFEB-dependent, lysosome-to-nucleus regulation of lysosomal ML1 channels after hours of starvation. Therefore, TRPML1 and TFEB constitute a positive feedback loop and play essential roles in lysosomal adaptation in response to nutrient deprivation.

II-2 Introduction

Macroautophagy (referred to as autophagy hereafter) is a cellular adaptation process that is essential for cell survival when nutrients (e.g., amino acids (AA) and growth factors) are limited (Rabinowitz and White, 2010). During this process, protein aggregates and damaged organelles are digested to generate basic building-block catabolites that can be utilized for "house-keeping" biosynthesis tasks (Mizushima and Komatsu, 2011). In the past few decades, autophagy research has mainly focused on the mechanisms that underlie the initial phase of autophagy: autophagosome formation (Rubinsztein et al., 2012). However, the entire autophagy process requires a sufficient and sustained supply of functional lysosomes to perform autophagosome-lysosome fusion continuously (Sardiello et al., 2009; Settembre et al., 2011; Shen and Mizushima, 2014; Zhou et al., 2013). Moreover, it remains unclear how environmental cues such as nutrient availability and regulation of lysosomal function and biogenesis (particularly lysosomal adaptation) contribute to cellular homeostasis. Temporal regulation of these processes is also of interest. For example, lysosome activation, manifested as increased acidification and delivery of hydrolases, may occur rapidly (within 2–3 h of starvation) during the initial phase of autophagy (Zhou et al., 2013). Lysosome reformation usually occurs 4–6 h after starvation (Yu et al., 2010).

The mechanistic Target of Rapamycin Complex 1 (mTORC1) is a master regulator of cell growth (Zoncu et al., 2011) and is localized on the lysosomal surface via a Rag GTPases-dependent mechanism when free AAs are abundant (Bar-Peled and Sabatini, 2014; Jewell et al., 2013). The activity of lysosome-localized mTORC1 is tightly controlled by Rheb GTPase, which in turn, is regulated by growth factors in the serum (Bar-Peled and Sabatini, 2014). Thus, upon AA withdrawal and/or serum starvation, mTORC1 activity is suppressed in the lysosome (Bar-Peled and Sabatini, 2014; Jewell et al., 2013), and the activities of mTORC1 effectors are

subsequently switched on or off. This includes S6K and 4E-BP1 which are responsible for protein synthesis, ATG13 and ULK1 which mediate autophagosomal biogenesis, and transcription factor EB (TFEB) which regulates lysosome function (Bar-Peled and Sabatini, 2014; Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). TFEB is a transcription factor that regulates both autophagy and lysosomal biogenesis via rapid translocation to the nucleus from the cytosol and lysosomes upon starvation (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Sardiello et al., 2009; Settembre et al., 2012). Correspondingly, overexpression of TFEB has been shown to affect the expression of a unique set of genes that are related to lysosome function and autophagy (Settembre et al., 2011). However, it remains to be determined what roles transcriptional and post-transcriptional regulation have in lysosome activation, consumption, and biogenesis during lysosomal adaptation to environmental changes.

Lysosomal ionic conductance regulates all aspects of lysosome function, including lysosomal degradation, catabolite export, and membrane trafficking (Samie and Xu, 2014). Hence, regulation of lysosomal conductance by environmental cues may serve as a primary mechanism for lysosome adaptation (Settembre and Ballabio, 2014; Settembre et al., 2013). Lysosomal trafficking (including membrane fusion and fission) supplies hydrolases for lysosome activation, provides autophagic substrates for degradation (autophagosome-lysosome fusion), and generates new lysosomes from autolysosomes (lysosome reformation). A key player in lysosomal trafficking is ML1, a cation channel on the lysosomal membrane that releases Ca^{2+} from the lumen into the cytosol in response to trafficking cues (Cheng et al., 2010; LaPlante et al., 2002; Li et al., 2013; Samie and Xu, 2014; Shen et al., 2012; Shen and Mizushima, 2014). ML1-mediated lysosomal Ca^{2+} release may regulate many aspects of lysosomal trafficking, including lysosome to trans-Golgi-network (TGN) retrograde trafficking, autophagosome-lysosome fusion, lysosome reformation, and lysosomal exocytosis (Cheng et al., 2014; Medina et al., 2011; Samie et al., 2013; Samie and Xu, 2014; Shen et al., 2012). Moreover, it has previously been demonstrated that nutrient starvation affects Ca^{2+} signaling (Li et al., 2013; Settembre et al., 2013; Zolov et al., 2012). Therefore, the objective of this study was to investigate nutrient regulation of endogenous ML1 channels, and the role of such regulation in lysosomal adaptation. Moreover, Ca^{2+} controls the activity of some transcriptional factors via regulating the kinases and phosphatases (Mellström and Naranjo, 2001). The localization and activity of TFEB are tightly controlled by its phosphorylation status (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre and

Ballabio, 2011). Considering that lysosomes are the signaling center that synchronizes nutrient availability with gene expression via TFEB regulation, it would be interesting to test the role of lysosomal Ca^{2+} in regulating TFEB activity upon starvation.

II-3 Results

3.1 Ca^{2+} is released from TRPML1 and induces TFEB translocation upon starvation.

To test the possibility that lysosome Ca^{2+} may regulate the lysosomal transcriptional regulator TFEB, TFEB localization was assayed in cells treated with mucolipin synthetic agonist (ML-SA), a TRPML1 specific agonist (Zhang et al., 2016). ML-SA treatment alone promoted TFEB translocation to the nucleus to a comparable level with amino acids and serum starvation **(Fig. 2.1 a)**. To examine the physiological relevance of this finding, Ca^{2+} imaging using HEK293 cells stably overexpressing ML1-GCaMP7, a lysosomal Ca^{2+} sensor, was employed (Zhang et al., 2016). An immediate Ca^{2+} release was detected within minutes of starvation **(Fig. 2.1 b, d)**, implying that lysosomal Ca^{2+} is released upon starvation. Consistently, pretreatment of glycyl-L-phenylalanine 2-naphthylamide (GPN), a lysosomotropic agent which depletes lysosomal Ca^{2+} (Berg et al., 1994), completely ablated Ca^{2+} release by starvation **(Fig. 2.1 d)**. Upon starvation, TFEB nuclear translocation was also completely blocked by BAPTA-AM, an intracellular Ca^{2+} chelator **(Fig. 2.3)** (Tsien, 1980).

As mentioned above, TRPML1 is the major Ca^{2+} channel in lysosomes and mediates Ca^{2+} release upon starvation, which is supported by the observation that starvation-induced Ca^{2+} release was potentiated by ML-SA **(Fig. 2.1 d)** and dramatically reduced by mucolipin synthetic inhibitor (ML-SI) **(Fig. 2.1 c, d)**, the potent TRPML1 specific antagonist. However, the residual Ca^{2+} signal indicated that starvation-induced Ca^{2+} release is not entirely dependent on TRPML1. In fact, the remaining Ca^{2+} signal is enough to activate TFEB. In cells treated with ML-SI and MLIV patient fibroblast with a mutation in the *Trpml1* gene and dysfunction of TRPML1, starvation-induced TFEB translocation to the nucleus was unaffected (**Fig. 2.2)**. Collectively, TRPML1 mediates partial Ca^{2+} release during starvation, which promotes TFEB translocation to the nucleus. In a high-content screening of a phosphatase short interfering RNA (siRNA) aiming to identify the phosphatases that dephosphorylate TFEB, the calcineurin catalytic subunit isoform beta (PPP3CB) was identified as the most significant hit by our collaborator. Together, we

demonstrate that Ca^{2+} release from TRPML1 activates Calcineurin, which dephosphorylates TFEB and promotes its nuclear translocation (Medina et al., 2015).

3.2 Lysosomal Ca^{2+} release, but not mTOR suppression, drives TFEB nuclear translocation upon starvation.

mTOR suppression has been considered to be the driving force for TFEB nuclear translocation. However, rapamycin, a mTOR inhibitor, only mildly induced TFEB nuclear translocation, implying that mTOR suppression may not be sufficient for TFEB nuclear translocation. **(Fig. 2.3)**. Moreover, BAPTA-AM, an intracellular Ca^{2+} chelator, blocked starvation-induced TFEB translocation, but did not block TFEB translocation induced by Torin-1, another mTOR inhibitor. Considering the essential role of lysosomal Ca^{2+} in mediating TFEB nuclear translocation, we speculate that Ca^{2+} release, instead of mTOR suppression upon starvation, plays a more active role in mediating TFEB translocation. Recently, several chemicals and physiological stimuli have been characterized to promote TFEB translocation in a mTOR-independent manner (Gayle et al., 2017; Li et al., 2016; Song et al., 2016; Zhang et al., 2016), which further supports our speculation. However, mTOR suppression during starvation may facilitate continuous translocation of TFEB to the nucleus by preventing the phosphorylation of TFEB.

3.3 Nutrient deprivation markedly increases lysosomal ML1 currents.

After the travel of TFEB to the nucleus, lysosomal and autophagic genes are up-regulated. We measured endogenous lysosomal TRPML1 currents using the whole-lysosome patch-clamp technique on enlarged vacuoles isolated from cells treated with vacuolin-1 (Dong et al., 2008; Dong et al., 2010; Wang et al., 2012). In most mammalian cell lines (HEK293, CHO, and Cos-1), as well as a Raw 264.7 macrophage cell line and various primary cells (mouse embryonic fibroblasts (MEFs), bone-marrow-derived macrophage (BMM), neurons, and myocytes), small ML1 currents were activated by ML-SA.

For Cos-1 cells grown in complete medium, only small ML-SA1-activated, inwardly rectifying, whole-endolysosome ML1 currents (I_{ML1}) were observed in most of the enlarged vacuoles **(Fig. 2.4 a,e)**. For Cos-1 cells that were serum-starved for 4 h, however, up to a 10-fold

increase in whole-endolysosome I_{ML1} was observed. Large increases in I_{ML1} were also observed at higher concentrations of ML-SA1 (20–50 μM), and in cells that were AA-starved or completely starved (AA-free + serum-free) **(Fig. 2.4 b,e)**. These results suggest that lysosomal ML1 channels are potently upregulated by nutrient starvation. This phenomenon also exists in CHO cells **(Fig. 2.4 f).** Since Cos-1 cells have a large cytoplasm which typically contains 200–400 lysosomes, these cells were selected for subsequent lysosomal trafficking and physiological studies.

3.4 Upregulation of lysosomal ML1 channels by pharmacological inhibition of mTOR.

mTORC1 is the primary nutrient sensor in the lysosome (Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Following nutrient starvation of Cos-1 cells, mTOR is inhibited. To investigate whether mTORC1 is involved in ML1 upregulation, two selective mTOR inhibitors were used to treat cells. While rapamycin is an allosteric inhibitor of mTOR that only partially decreases mTOR activity, Torin-1 is a catalytic inhibitor that completely suppresses the functions of mTORC1 (Thoreen et al., 2009; Thoreen and Sabatini, 2009; Zhou et al., 2013). Following a 12 h treatment with each of these mTOR inhibitors, whole-endolysosome I_{ML1} was found to markedly increased by Torin-1 (**Fig. 2.4 c,e)** but remained unchanged by rapamycin (**Fig. 2.4 d,e)**. In contrast, both Torin-1 and rapamycin completely suppressed levels of p-S6K and strongly induced autophagosome formation **(Fig. 2.4 g)** (Nyfeler et al., 2011). Thus, starvation-induced ML1 upregulation may occur via a mechanism distinct from the initiation of autophagy.

3.5 Activation of ML1 upon starvation induces Ca^{2+} release.

Next, ML1-mediated lysosomal Ca^{2+} release in intact cells was measured using Fura-2 Ca^{2+} imaging. Lysosomal Ca^{2+} release was induced in a zero Ca^{2+} (free $[Ca^{2+}] < 10$ nM) external solution using ML-SA compounds as reported previously (Shen et al., 2012). No measurable Ca^{2+} release was detected in Cos-1 cells treated with up to 50 μM ML-SA3 **(Fig. 2.5 a,d)**, and these results are consistent with the small amplitude of I_{ML1} that were recorded for these cells. In contrast, for Cos-1 cells that were serum-starved for 4 h, ML-SA3 induced a robust Ca^{2+} response **(Fig. 2.5 b,d)**, while smaller responses were induced by ML-SA1 (data not shown). ML-SA3 responses were completely abolished in the presence of the TRPML-specific synthetic inhibitor, ML-SI3 **(Fig. 2.5 c,d)** (Samie et al., 2013), thereby supporting the specificity of the responses

observed. Moreover, large ML-SA3 responses were observed in Cos-1 cells that were treated with Torin-1, and not in Cos-1 cells treated with rapamycin. Collectively, these results are in general agreement with the electrophysiological analyses of vacuolin-enlarged vacuoles, and demonstrate that nutrient deprivation dramatically increases ML1-mediated lysosomal Ca^{2+} release in intact Cos-1 cells.

3.6 Activating mutation of TFEB is sufficient to cause ML1 upregulation.

In Hela cells, complete starvation resulted in a rapid translocation of endogenous TFEB from the cytoplasm to the nucleus **(Fig. 2.6 a)**. Similarly, Cos-1 cells transfected with wild-type (WT) TFEB-mCherry and treated with Torin-1 exhibited a marked increase in the nuclear localization of TFEB. In contrast, constitutively active TFEB-S211A-mCherry was associated with predominantly nuclear localization of TFEB in the presence or absence of starvation or Torin-1. However, when mutations in TFEB included the removal of four charged residues within the putative nuclear localization motif (e.g., Arg^{245}-Arg^{248} to Ala^{245}-Ala^{248}; TFEB-S211A) (Roczniak-Ferguson et al., 2012), nuclear localization was completely abolished **(Fig. 2.6 b)**. Endogenous whole-endolysosome I_{ML1} dramatically increased in cells transfected with TFEB-S211A, but not in cells transfected with WT TFEB or TFEB-4A-S211A **(Fig. 2.7 a-d)**. These results suggest that activation of TFEB underlies the effect of starvation on ML1.

3.7 Nutrient-sensitive regulation of ML1 depends on gene expression and protein synthesis.

ML1 upregulation may result from a post-translational modification or an increase in mRNA/protein expression. Based on the observation that starvation-induced increases in I_{ML1} occurred to a similar degree for all of the ML-SA1 concentrations tested, an increase in agonist potency is unlikely to account for the upregulation. Instead, it is hypothesized that increased expression of ML1 proteins plays a key role, since Torin-1 treatment and starvation only modestly increased *ML1* mRNA levels (< 2-fold) **(Fig. 2.8 f)**. Notably, when a transcription or protein synthesis was blocked using actinomycin D (Arnold et al., 2005) or cycloheximide (An et al., 2008), respectively, starvation-induced I_{ML1} increases were almost abolished **(Fig. 2.8 a-d)**. In contrast, treatment with actinomycin D did not affect TFEB nuclear translocation, while treatment with cycloheximide partially blocked translocation **(Fig. 2.8 e)**. Lysosome-resident membrane proteins are reported to have an extremely slow turnover rate with $t_{1/2} > 3$ days (Wang and Touster,

1975), suggesting that degradation of TRPML1 proteins is likely negligible over the time course of starvation. Because increases in transcription and translation of ML1 proteins and transcripts cannot fully account for the large increase in currents, it is possible that unidentified post-translational modification mechanisms may increase agonist efficacy. Taken together, these results suggest that starvation-induced ML1 upregulation involves the synthesis of ML1 proteins or auxiliary proteins that modulate TRPML1 channel function.

3.8 Lysosomal Na^+-selective currents are not affected by nutrient starvation.

Two-pore (TPC) Na^+-selective channels have recently been proposed to be components of nutrient-sensing machinery in the cell (Cang et al., 2013). Both ML1 and TPC channels are activated by $PI(3,5)P_2$ (Dong et al., 2010; Wang et al., 2012), and upon $PI(3,5)P_2$ activation, TPC currents were isolated using MI-SI1 to block ML1 currents (Samie et al., 2013). Compared with control Cos-1 cells, neither TFEB-S211A nor starvation increased whole-endolysosome TPC currents. Likewise, lysosomal K^+ currents were not elevated in starved cells **(Fig. 2.9 a-d)**. Hence, starvation may only selectively upregulate certain lysosomal channels.

3.9 ML1 is required for the clearance of cholesterol accumulation from lysosomes in NPC cells.

Lysosomal Ca^{2+} may regulate cellular clearance and cholesterol export in NPC cells (Shen et al., 2012). To investigate whether ML1 upregulation by nutrient deprivation reduces cholesterol accumulation in NPC cells, Filipin staining was used to evaluate free cholesterol levels (Shen et al., 2012). Both starvation conditions and Torin-1 treatment dramatically reduced cholesterol accumulation in NPC1 knockout (KO) macrophage **(Fig. 2.10 a,b)** and macrophage cells treated with U18666A, a blocker of cholesterol transport (Shen et al., 2012) **(Fig. 2.10 c,e).** These results suggest that ML1 upregulation may play a critical role in starvation-induced cellular clearance.

In the presence of MI-SI3, cholesterol accumulation in NPC cells was not reduced by starvation or Torin-1 treatment **(Fig. 2.10 a,b)**. Similarly, starvation or Torin-1 treatment did not reduce cholesterol accumulation in ML1 KO macrophage treated with U18666A **(Fig. 2.10 d,f)**.

Hence, the channel activity of ML1 is required for starvation- or Torin-1-induced reductions in cholesterol accumulation in NPC cells.

3.10 ML1 is required for starvation-induced enhancement of lysosomal proteolytic function.

To further investigate the role of ML1 in cellular adaptation, lysosomal proteolytic activity was measured using an assay that yields red fluorescence according to the rate of proteolytic degradation of DQ-red-BSA (Yue et al., 2013). Consistent with previous studies (Vazquez and Colombo, 2009; Yue et al., 2013), DQ-BSA degradation was found to be enhanced following starvation of Cos-1 cells. ML-SA1 treatment to activate TRPML1 led to a small increase in proteolytic activity, the starvation effect was completely abolished by MI-SI3, the TRPML1 inhibitor **(Fig. 2.11)**. Therefore, ML1 may have a general role in regulating the adaptative responses of a cell to changes in nutrient availability.

II-4 Discussion

In this chapter, novel roles of lysosomal Ca^{2+} in regulating lysosomal functions were elucidated. Our work leads to a model that, upon starvation, Ca^{2+} is readily released via TRPML1 and mediates calcineurin-dependent dephosphorylation of TFEB, resulting in TFEB activation and further up-regulation of TRPML1. TRPML1 regulates the autophagic-lysosomal pathway at two steps and forms a positive feedback loop with TFEB, providing a signaling pathway with global transcriptional lysosomal regulation that originates from the lysosomal surface **(Fig. 2.12)**. Furthermore, activation of TRPML1, inhibition of mammalian target of rapamycin (mTOR) or activation of transcription factor EB (TFEB) mimicked the effect of starvation. The starvation effect also includes an increase in lysosomal proteostasis and an enhanced clearance of lysosomal storage, including cholesterol accumulation in Niemann-Pick disease type C (NPC) cells. However, this effect was not observed when ML1 was pharmacologically inhibited or genetically deleted. Consistently, overexpression of ML1 mimicked the starvation effect.

Interestingly, under starvation, Ca^{2+} from TRPML1 only plays a permissive role in mediating starvation-induced TFEB translocation, implying the existence of TRPML1-independent mechanisms. However, under other environmental cues, such as oxidative stress, TRPML1 may supply all the Ca^{2+} that is required for the cell to adapt to stress and induce TFEB

translocation. When TRPML1 is genetically inactivated or pharmacologically inhibited, TFEB translocation to the nucleus is blocked under oxidative stress and clearance of damaged mitochondria and removal of excess ROS is also blocked (Zhang et al., 2016). Cells that respond to different stress have evolved distinct survival pathways (Fulda et al., 2010) and starvation may require several parallel pathways to ensure TFEB activation for necessary lysosomal adaptation, which may be essential for cell survival. Further studies are in needs to identify these TRPML1-independent pathways.

One intriguing question left is the molecular mechanism underlying the initial acute activation of TRPML1 (timescale of seconds) in response to environmental cues. It has been noticed that protein phosphorylation by kinases and phosphatases often serves as a rapid molecular switch (Humphrey et al., 2015). Indeed, the phosphorylations of TRPML1 by mTOR and protein kinase A (PKA), which are inhibited upon starvation (Barbet et al., 1996; Gomes et al., 2011), and have been shown to negatively regulate TRPML1 activity (Onyenwoke et al., 2015). One possibility is that mTOR or PKA may release the suppression of ML1 upon nutrient deprivation or other stress., resulting in an acute Ca^{2+} release.

By directly patch-clamping lysosomal membranes, we demonstrated that the current density of lysosomal ML1 channels is selectively and dramatically upregulated within hours of nutrient starvation. This upregulation was also mimicked with pharmacological inhibition by mTORC1 with TFEB activation (and nuclear translocation). Hence, nutrient-sensitive regulation of ML1 channels may link lysosome function with nutrient availability via mTORC1 and TFEB. While our study has focused on the effect of mTORC1 inhibition on upregulation of TRPML1, it has recently been demonstrated that the *Drosophila* homolog of TRPML1, TRPML, regulates the activity of TORC1 *in vivo* (Wong et al., 2012). Hence, TRPML1 and TORC1 may constitute a feedback loop to regulate amino acid homeostasis *in vivo*. Although TFEB activation is known to trigger the expression of many lysosomal genes required for lysosome biogenesis (Sardiello et al., 2009; Settembre et al., 2011), the large increase in current density for ML1, and not for lysosomal TPC Na^+ channels, suggests that ML1 upregulation plays an active role in lysosomal adaptation. Thus, ML1 upregulation may represent one of the key functional changes that occur in a lysosome, and this may be required for lysosomal adaptation. Consistent with this hypothesis, ML1 was found to be required for starvation-induced enhancement of lysosomal proteolytic

activity and cholesterol export. It is possible that starvation-induced enhancement of ML1-mediated lysosomal Ca^{2+} release may also facilitate lysosomal trafficking (Shen et al., 2012) for the following reasons. Given the timeframe for autophagosome-lysosome fusion (0.5–4 h after starvation) (Yu et al., 2010), ML1 upregulation may promote Ca^{2+}-dependent fusion of autophagosomes and lysosomes for the autophagy process (Luzio et al., 2007; Samie and Xu, 2014). ML1 channels may also be directly sensitized via a post-translational mechanism, thereby increasing lysosomal activity and proteolytic function. Further studies are needed to confirm and elucidate these mechanisms. Secondly, during the next phase of lysosomal adaptation (2–6 h after starvation), ML1 upregulation may promote lysosomal reformation and biogenesis (Pryor et al., 2006; Treusch et al., 2004; Li et al., 2016) required for sustained autophagy (Yu et al., 2010). Therefore, during prolonged starvation, an increase in lysosomal reformation and biogenesis could also indirectly increase autophagosome-lysosome fusion to boost lysosome function.

ML1 has been recently implicated in regulating lysosomal cholesterol export in NPC cells (Shen et al., 2012). However, while starvation and mTOR inhibition were sufficient to reduce cholesterol accumulation in NPC cells in the present study, such effects were not seen when ML1 was genetically deleted or pharmacologically inhibited. Conversely, overexpression of ML1 was found to mimic the effect of starvation on reducing cholesterol accumulation. Proteolytic analyses also support the observation that nutrient starvation stimulates lysosomal activity, and ML1 channel activity is required for nutrient-sensitive regulation of proteostasis. Collectively, ML1 may play an essential role in lysosomal adaptation during normal physiology and disease. Hence, upregulation of ML1 expression may provide an opportunity to protect NPC and other lysosomal storage diseases (LSDs). Further research is needed to identify new reagents that can specifically activate TFEB and induce ML1 expression without affecting other cellular processes. However, the capacity for ML-SA compounds to potently up-regulate ML1 channel activity, in combination with strategies to upregulate the TFEB-ML1 pathway, may represent a treatment strategy applicable to both LSDs and metabolic diseases.

II-5 Methods

Molecular biology. Human TFEB (from Drs. Rosa Puertollano and Dr. Andrea Ballabio) was cloned into EcoRI and SacII sites of pmCherry to generate a TFEB-mCherry fusion construct. Arg^{245} to Arg^{248} residues were mutated to alanine to generate TFEB-4A-mCherry using

overlapping PCR. The single mutation for S211A at TFEB was generated using the QuickChange Lightning Site-Direct Mutagenesis Kit (Agilent Technologies). All constructs were confirmed by DNA sequencing.

Quantitative PCR. RNA was isolated from cells using TRIzol (Invitrogen) and was reverse-transcribed into cDNA using Superscript III RT (Invitrogen). Q-PCR was performed using Absolute Blue qPCR SYBR Green Mix (Fisher Scientific). mRNA levels of *ML1* were normalized to mRNA levels of *L32*, a housekeeping gene. The primers used are as follows:

ML1: 5'-AAACACCCCAGTGTCTCCAG-3' (forward)
5'-GAATGACACCGACCCAGACT-3' (reverse);
L32: 5'-TGGTGAAGCCCAAGATCGTC-3' (forward)
5'-CTTCTCCGCACCCTGTTGTC-3' (reverse)

Mouse lines. NPC1 KO mice (BALB/cNctr-Npc1m1N/J) and wildtype (WT) littermates were ordered from Jackson Laboratories. ML1-KO mice were kindly provided by Dr. Susan Slaugenhaupt (Harvard Medical School) and Dr. Jim Pickel (NIH). Animal experiments were conducted using an approved animal protocol (#4280) and Institutional Animal Care Guidelines of the University of Michigan.

Mammalian Cell Culture. HEK293 cells stably expressing TFEB-mCherry were generated using the Flip-In T-Rex 293 cell line (Invitrogen). HEK-293T was cultured at 37 ^{o}C in a 1:1 mixture of DMEM and Ham's F12 media supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO_2 incubator. Cos-1 was maintained in DMEM with 10% FBS. Human skin fibroblast cell lines from a mucolipidosis IV patient (clone GM02048) and a healthy control (clone GM05659) were obtained from the Coriell Institue for Medical Research and cultured in 1:1 mixture of DMEM and Ham's F12 media with 15% FBS. Cos1 cells were transfected using Lipofectamine 2000 (Invitrogen).

Bone marrow cells were harvested from femurs and tibias and were cultured in macrophage differentiation medium (DMEM, Gibco) with 10% FBS and 100 U/ml recombinant murine GM-CSF (PeproTech) (Samie et al., 2013). After 4-5 d in culture at 37 ^{o}C with 5% CO_2, adherent cells (> 95% were expected to be macrophage) were harvested for assays (Samie et al., 2013).

Western blotting. Cells were lysed with ice-cold RIPA buffer (Boston BioProducts) in the presence of 1X protease inhibitor cocktail (Sigma), 1 mM NaF, and 1mM Na_3VO_4. Total cell lysates were mixed with 2X SDS loading buffer and were boiled at 95 ^oC for 10 min. Protein samples were then loaded and separated on 4–12% gradient SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes. The membranes were blocked for 1 h with 1% BSA in PBST and were incubated with various antibodies against p-S6K1, S6K1 (all at 1:1,000 and were purchased from Cell Signaling) in PBST. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:5000) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). Band intensities were quantified using Image J software.

Whole-endolysosome electrophysiology. Endolysosomal electrophysiology was performed in isolated endolysosomes using a modified patch-clamp method. Cells were treated with 1 μM vacuolin-1 overnight (O/N), a lipid-soluble polycyclic triazine that can selectively increase the size of endosomes and lysosomes. A patch pipette (electrode) was then pressed against individual cells and then was quickly pulled away to slice the cell membrane. Whole-endolysosome recordings were then performed for enlarged vacuoles from Cos-1 cells that were released into a dish. Bath (internal/cytoplasmic) solution contained 140 mM K^+-Gluconate, 4 mM NaCl, 1 mM EGTA, 2 mM Na_2-ATP, 2 mM $MgCl_2$, 0.39 mM $CaCl_2$, 0.1 mM GTP, and 10 mM HEPES (pH adjusted with KOH to 7.2; free $[Ca^{2+}]_i \sim 100$ nM). The pipette (luminal) solution contained 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM glucose (pH adjusted to 4.6 with NaOH). All bath solutions were applied using a fast perfusion system to achieve a complete solution exchange within a few seconds. Data were collected using an Axopatch 2A patch clamp amplifier, Digidata 1440, and pClamp 10.0 software (Axon Instruments). Whole-endolysosome currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at room temperature (21–23 ^oC) and all recordings were analyzed using pCLAMP10 (Axon Instruments) and Origin 8.0 (OriginLab).

Fura-2 Ca^{2+} imaging. Ca^{2+} imaging was carried out within 2–3 h after plating while cells exhibited a round morphology. Cells were loaded with 5 μM Fura-2 AM in the culture medium at 37 ^oC for 1 h. Fluorescence was recorded at different excitation wavelengths using an EasyRatioPro system (PTI). Fura-2 ratios (F_{340}/F_{380}) were used to monitor changes in intracellular

[Ca^{2+}] upon stimulation. Lysosomal Ca^{2+} release was measured under a 'zero' Ca^{2+} external solution (Shen et al., 2012), which contained 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM glucose, 1 mM EGTA, and 20 mM HEPES (pH 7.4). Ca^{2+} concentration in the nominally free Ca^{2+} solution was estimated to be 1–10 μM. With 1 mM EGTA, the free Ca^{2+} concentration was estimated to be < 10 nM [based on calculations performed using Maxchelator software.

GCaMP7-TRPML1 Ca^{2+} imaging. GCaMP7 imaging was performed in HEK cells stably expressing GCaMP7–TRPML1, a lysosome-targeted genetically-encoded Ca^{2+} sensor. The fluorescence intensity at 488 nm (F488) was recorded at 37 °C with the spinning-disk confocal live-imaging system, which included an Olympus IX81 inverted microscope, a × 60 or × 100 objective (Olympus), a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor) and MetaMorph Advanced Imaging acquisition software v.7.7.8.0 (Molecular Devices).

Filipin staining. Cells were fixed in 4% paraformaldehyde (PFA) for 1 h, were washed 3x with PBS, then were incubated with 1.5 mg/ml glycine in PBS for 10 min to quench the PFA. Cells were then stained for 2 h with 0.05 mg/ml Filipin in PBS supplemented with 10% FBS. All procedures were conducted at room temperature (21–23 ^{o}C). Images were obtained using a fluorescence microscope with a UV filter. Filipin intensity was calculated using Image J software.

DQ-BSA proteolytic assay. DQ-red-BSA was used as an artificial substrate to evaluate lysosomal proteolytic degradation (Yue et al., 2013). Briefly, cells were treated with DQ-red-BSA (10 μg/ml) at 37 °C for 2 h. After the extracellular DQ-red-BSA was removed, cells were incubated with AA-free and serum-free media for 4 h. Upon cleavage, DQ-red-BSA exhibited red fluorescence that was detected by confocal microscopy and quantified using Image J software.

TFEB immunofluorescence. Cells were grown on glass coverslips and then fixed with 4% PFA and permeabilized with 0.3% Triton X-100 after treatments. And then the cells are blocked with the immunofluorescence buffer with 1% BSA in PBS. Endogenous TFEB was recognized by incubating cells with anti-TFEB antibody (1:1000; Cell Signaling) for 1h. Then cells were washed 4-5 times with PBS and incubated with anti-rabbit secondary antibodies conjugated to Alexa Fluor 568 (A10042, Invitrogen) for 1h. After three washes with PBS, the coverslips were mounted on slides with Fluoromount-G (Southern Biotech). Then the cells were imaging using a Leica confocal microscope.

Cell treatments. For serum starvation, cells were washed three times in Hank's balanced salt solution (Invitrogen) and incubated for 2-4h at 37°C in DMEM without 10% FBS. For complete starvation experiments, cells were washed three times in Hank's balanced salt solution (Invitrogen) and incubated for 2–4 h at 37°C in either a 1:1 mixture of DMEM and Ham's F12 media without amino acids (US Biological). Recovery after starvation was achieved by the addition of normal culture medium.

Reagents. The following reagents were purchased: U-18666A (Enzo life Sciences), ML-SA1 (Princeton BioMolecular Research Inc), Torin-1 (Tocris), Rapamycin (LC Laboratories), DQ-BSA-red, Fura-2 AM (Life Technologies), Vacuolin-1 (Calbiochem), Actinomycin-D, Cycloheximide, Filipin, and Ionomycin (Sigma).

Data analysis. Data are presented as the mean ± standard error of the mean (SEM) from at least three independent experimental replicates. Statistical comparisons were performed using Student's *t*-test and ANOVA test. A P value < 0.05 was considered statistically significant.

II-6 Figures

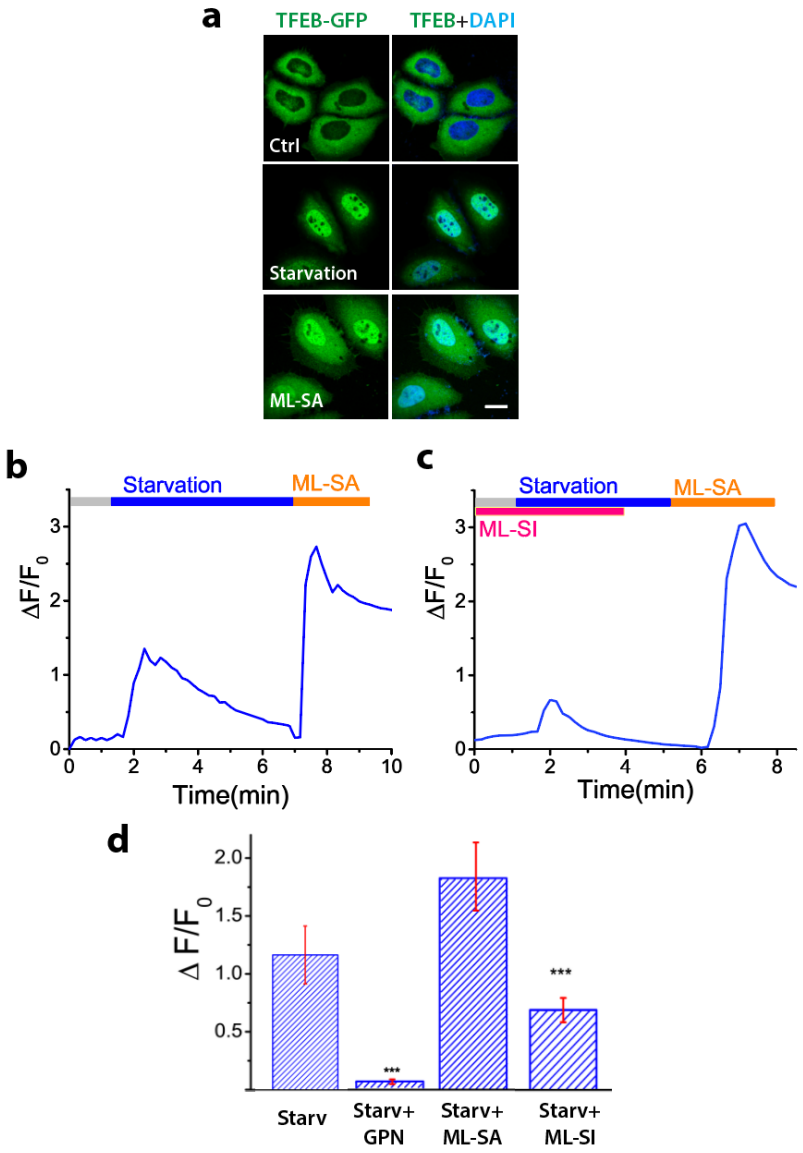


Figure 2.1 Starvation induces Ca^{2+} release via TRPML1.

(a) ML-SA promoted TFEB translocation to the nucleus to a similar level as starvation in Hela cells stably expressing TFEB-GFP (Green signal represents TFEB; blue signal represents DAPI stained nuclei). **(b)** Starvation elicited rapid Ca^{2+} release (Based on the GCaMP7 signal, $\Delta F/F_0$) in HEK cells stably expressing GCaMP7-TRPML1. ML-SA was added at the conclusion of all experiments to induce a maximal intracellular release for comparison. Shown are selected traces from the same coverslip that typically contained 10-20 cells. **(c)** Starvation-induced Ca^{2+} release was dramatically reduced by ML-SI; **(d)** Average starvation-induced Ca^{2+} release in control cells, GPN-treated, ML-SA treated, and ML-SI treated cells. Data are presented as the mean ± SEM. Statistical comparisons were made using ANOVA. *** $P < 0.001$. Scale bar = 10 μm.

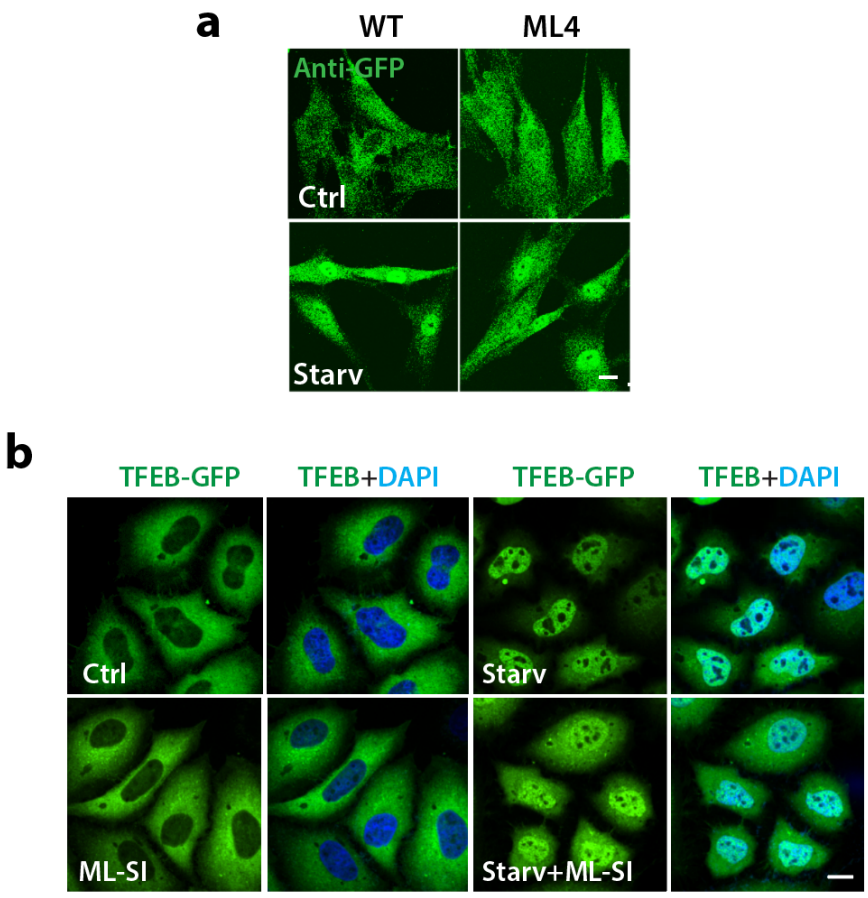


Figure 2.2 Starvation-induced TFEB translocation does not require TRPML1 activity.

(a) Starvation-induced TFEB nuclear translocation was unaffected in ML4 patient fibroblast cells with ML1 dysfunction. **(b)** Starvation-induced TFEB nuclear translocation was not prevented by ML-SI. Scale bar = 10 μm

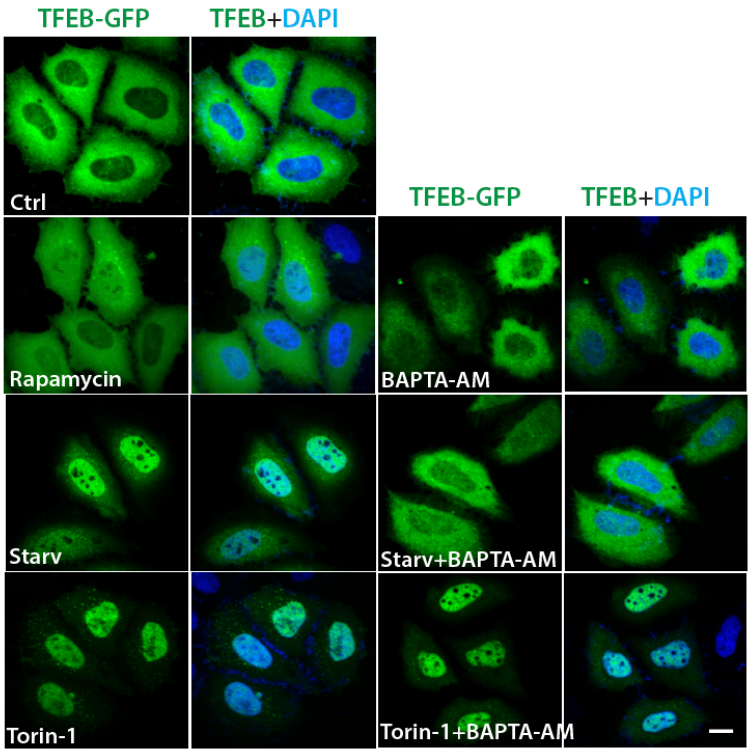


Figure 2.3 Ca^{2+} release, not mTOR inhibition plays a major role in mediating TFEB translocation under starvation.

Rapamycin (1 μM), an allosteric mTOR inhibitor, only mildly induced TFEB nuclear translocation in Hela cells stably expressing TFEB-GFP. Torin-1 (50 nM), a catalytic mTOR inhibitor, promoted TFEB nuclear translocation. BAPTA-AM prevented starvation, but not Torin-1-induced TFEB translocation. Scale bar = 10 μm.

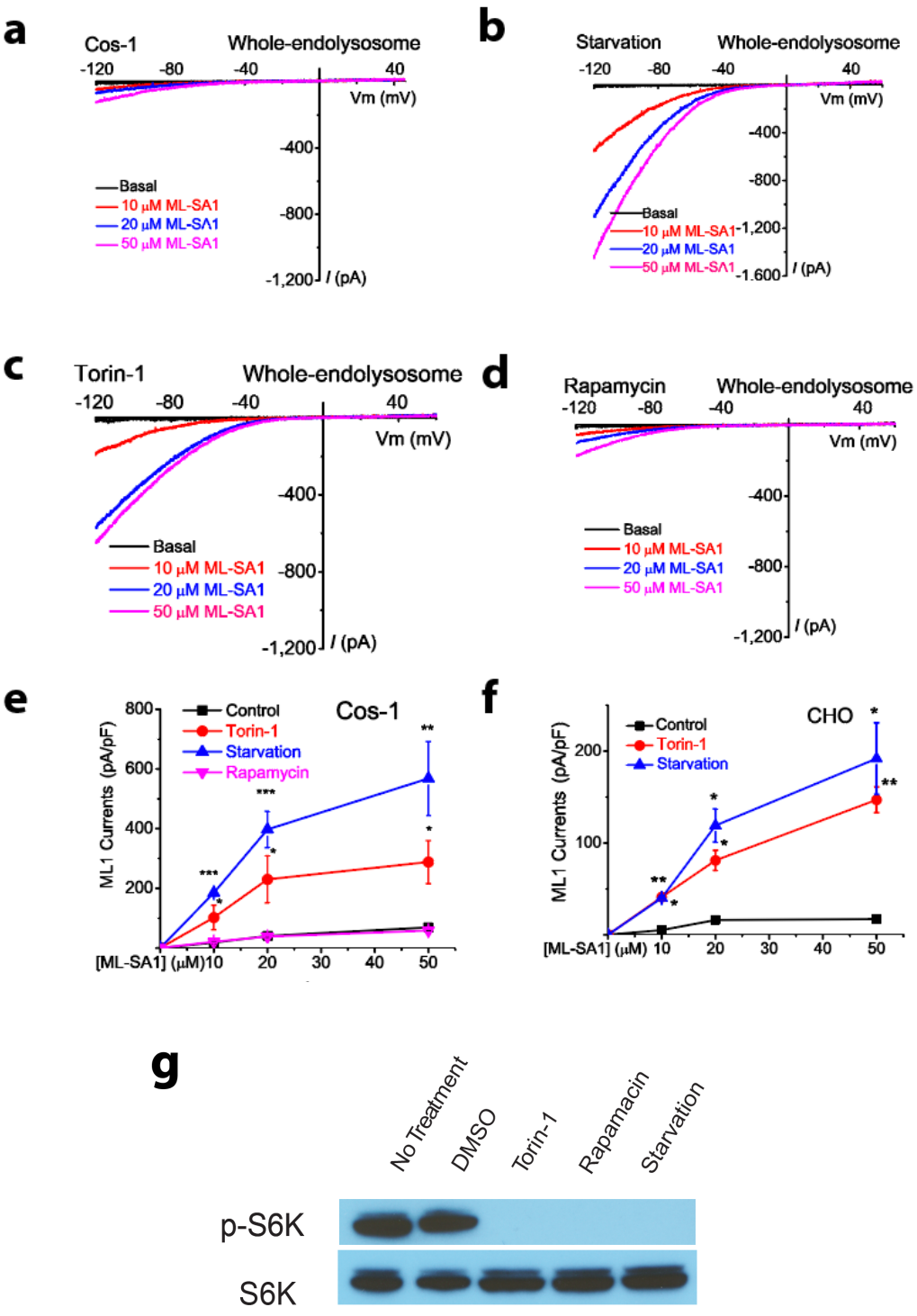

a
Cos-1
Whole-endolysosome
-120
-80
-40
40
Vm (mV)
-400
-800
-1,200
I (pA)
Basal
10 μM ML-SA1
20 μM ML-SA1
50 μM ML-SA1
b
Starvation
Whole-endolysosome
-120
-80
-40
40
Vm (mV)
-400
-800
-1,200
-1,600
I (pA)
Basal
10 μM ML-SA1
20 μM ML-SA1
50 μM ML-SA1
c
Torin-1
Whole-endolysosome
-120
-80
-40
40
Vm (mV)
-400
-800
-1,200
I (pA)
Basal
10 μM ML-SA1
20 μM ML-SA1
50 μM ML-SA1
d
Rapamycin
Whole-endolysosome
-120
-80
-40
40
Vm (mV)
-400
-800
-1,200
I (pA)
Basal
10 μM ML-SA1
20 μM ML-SA1
50 μM ML-SA1
e
Cos-1
Control
Torin-1
Starvation
Rapamycin
ML1 Currents (pA/pF)
800
600
400
200
[ML-SA1] (μM)
10
20
30
40
50
f
CHO
Control
Torin-1
Starvation
ML1 Currents (pA/pF)
200
100
[ML-SA1] (μM)
10
20
30
40
50
g
No Treatment
DMSO
Torin-1
Rapamacin
Starvation
p-S6K
S6K

Figure 2.4 Starvation and mTOR inhibition dramatically increase endogenous lysosomal ML1 currents.

(a) Representative traces of endogenous I_{ML1} for an enlarged vacuole isolated from vacuolin-1-treated Cos-1 cells grown in complete media. I_{ML1} was activated by three different concentrations of ML-SA1 (10, 20, and 50 μM) using a voltage protocol from -140 to +100 mV (only partial voltage ranges are shown). Pipette (luminal) solution was a standard external (Tyrode's) solution adjusted to pH 4.6 to mimic the acidic environment of the lysosome lumen. Bath (internal/cytoplasmic) solution was a K^+-based solution (140 mM K^+-gluconate). Note that the inward currents indicate that cations are flowing out of the endolysosome. **(b)** Whole-endolysosome I_{ML1} for Cos-1 cells after 4 h of serum starvation (DMEM/F-12 medium without FBS). **(c)**) I_{ML1} for Cos-1 cells treated with Torin-1 (2 μM) for 12 h **(d)** I_{ML1} for Cos-1 cells treated with rapamycin (2 μM) for 12 h. **(e)** Mean current densities (the current amplitude normalized to the capacitance of the vacuole) for I_{ML1} in non-treated (black), starved (blue), Torin-1-treated (red), and Rapamycin-treated (pink) Cos-1 cells. **(f)** Summary of I_{ML1} for CHO cells under the different experimental conditions indicated. **(g)** The effects of serum starvation, Torin-1 (1 μM), and rapamycin (1 μM) on the levels of phosphorylated S6K kinase (p-S6K) that were detected by Western blotting in extracts from Cos-1cells as a readout for mTOR kinase activity.

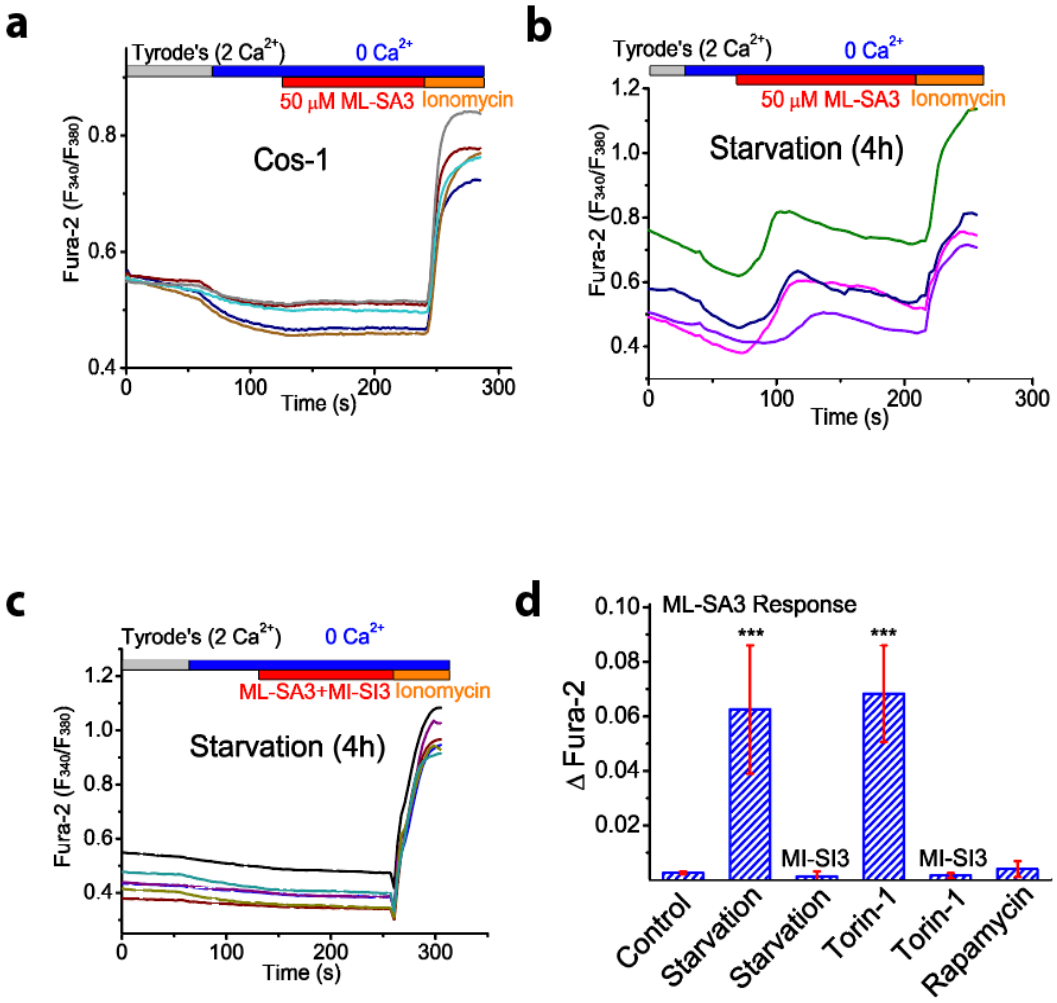


Figure 2.5 Starvation and mTOR inhibition increase lysosomal Ca^{2+} release from TRPML1.

(a) ML-SA3 (50 μM) did not induce any obvious Ca^{2+} release (based on the Fura-2 ratio, F_{340}/F_{380}) in Cos-1 cells grown in complete media. Ionomycin (5 μM) was added at the conclusion of all experiments to induce a maximal intracellular release for comparison. Shown are selected traces from the same coverslip that typically contained 15-30 cells. **(b)** ML-SA3-induced Ca^{2+} release in starved Cos-1 cells. **(c)** ML-SA3-induced Ca^{2+} release in starved cells in the presence of the synthetic TRPML inhibitors, MI-SI3 (20 μM). **(d)** Average ML-SA3-induced Ca^{2+} release in control, starved, Torin-1-treated, and rapamycin-treated Cos-1 cells. The results are the mean of 40–100 cells from n=4 independent experiments. Data are presented as the mean ± SEM. Statistical comparisons were made using variance analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

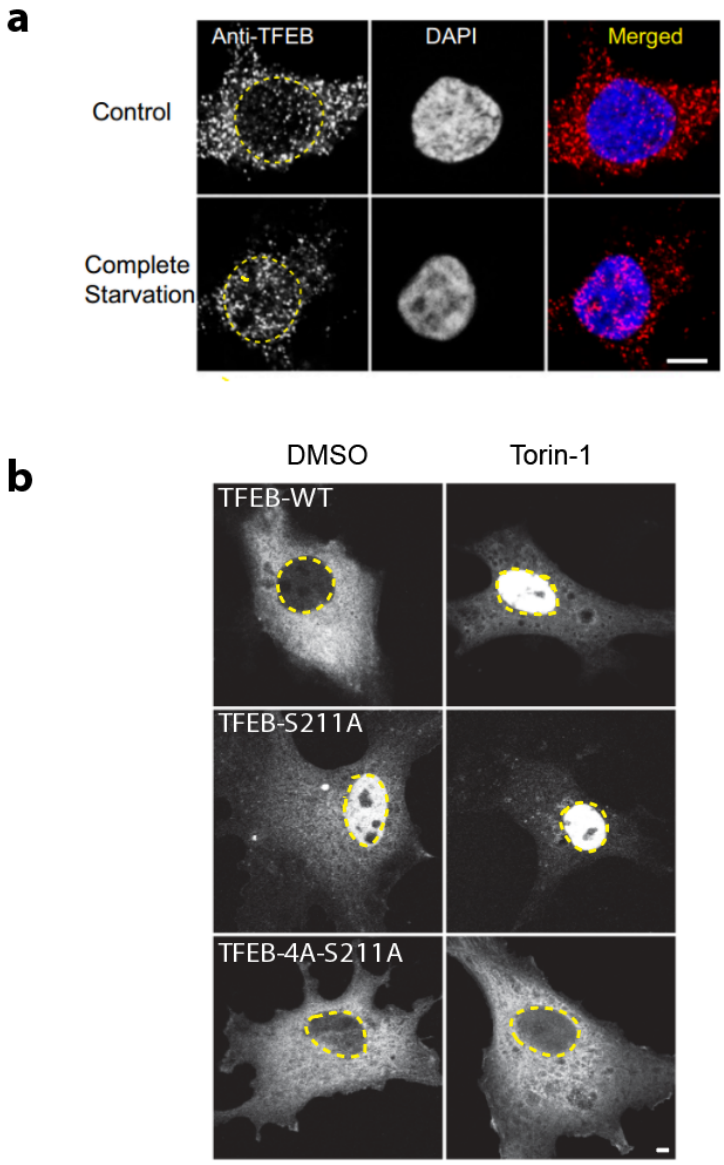


Figure 2.6 Characterization of endogenous TFEB and different TFEB constructs.

(a) Complete starvation (AA-free + serum-free) promoted endogenous TFEB nuclear translocation in Hela cells. Endogenous TFEB was recognized by an anti-human TFEB antibody. Nuclei were stained with DAPI and indicted by yellow dotted lines. The effects of Torin-1-treatment (4 h) on the subcellular localization of over-expressed TFEB- mCherry, TFEB-S211A-mCherry, and TFEB-4A-S211A-mCherry were shown. Scale bar = 5 μm **(b)** The effects of Torin-1-treatment (4 h) on the subcellular localization of over-expressed TFEB- mCherry, TFEB-S211A-mCherry, and TFEB-4A-S211A-mCherry. Scale bar = 5 μm.

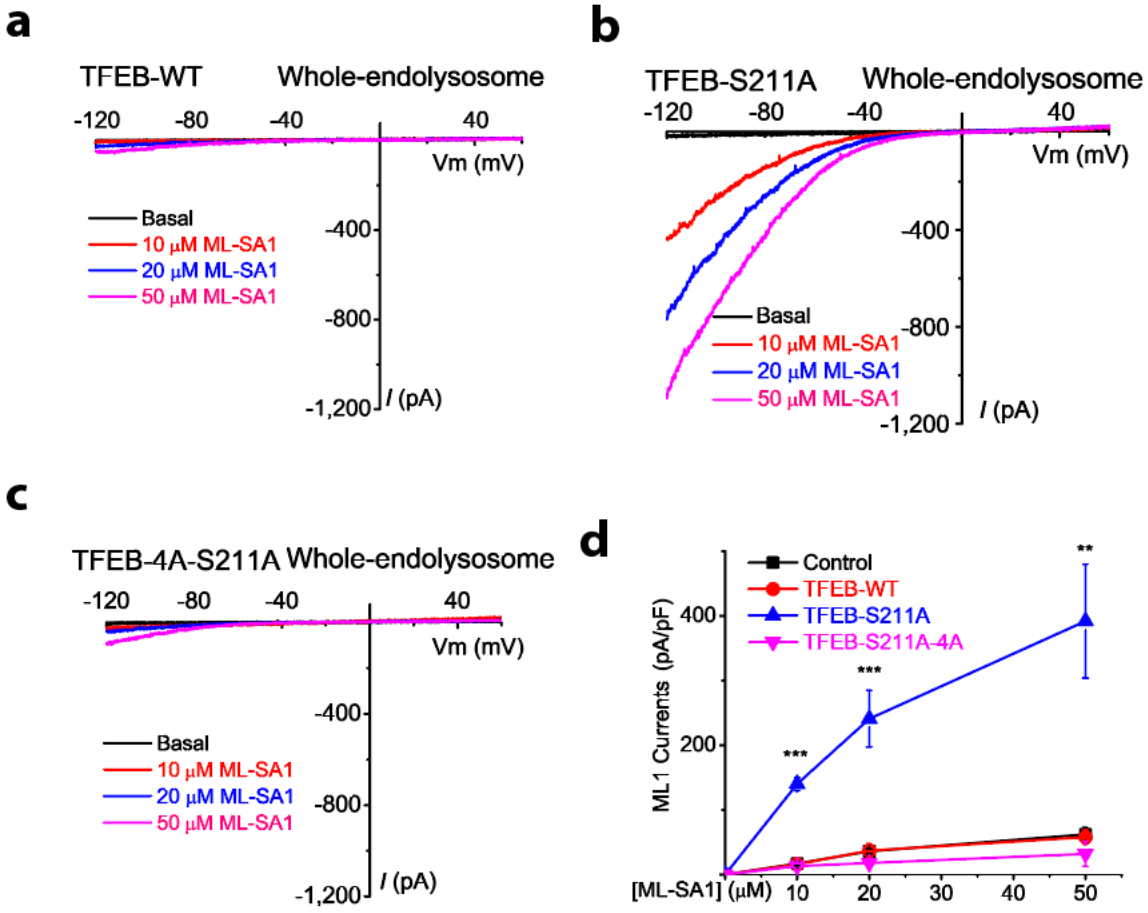


Figure 2.7 Activating mutation of TFEB is sufficient to upregulate lysosomal ML1 currents.

Representative traces of whole-endolysosome I_{ML1} for Cos-1 cells transfected with TFEB-WT **(a)**, TFEB-S211A **(b)**, and TFEB-4A-S211A **(c)**. (**d**) Mean current densities for I_{ML1} in non-transfected (black; n=14 vacuoles), TFEB-WT (red; n=4), TFEB-S211A (blue; n=13), and TFEB-S211A-4A (pink; n=3)-transfected Cos-1 cells. Data are presented as the mean ± SEM. Statistical comparisons were made using variance analysis (*t*-test). ** $P < 0.01$; *** $P < 0.001$

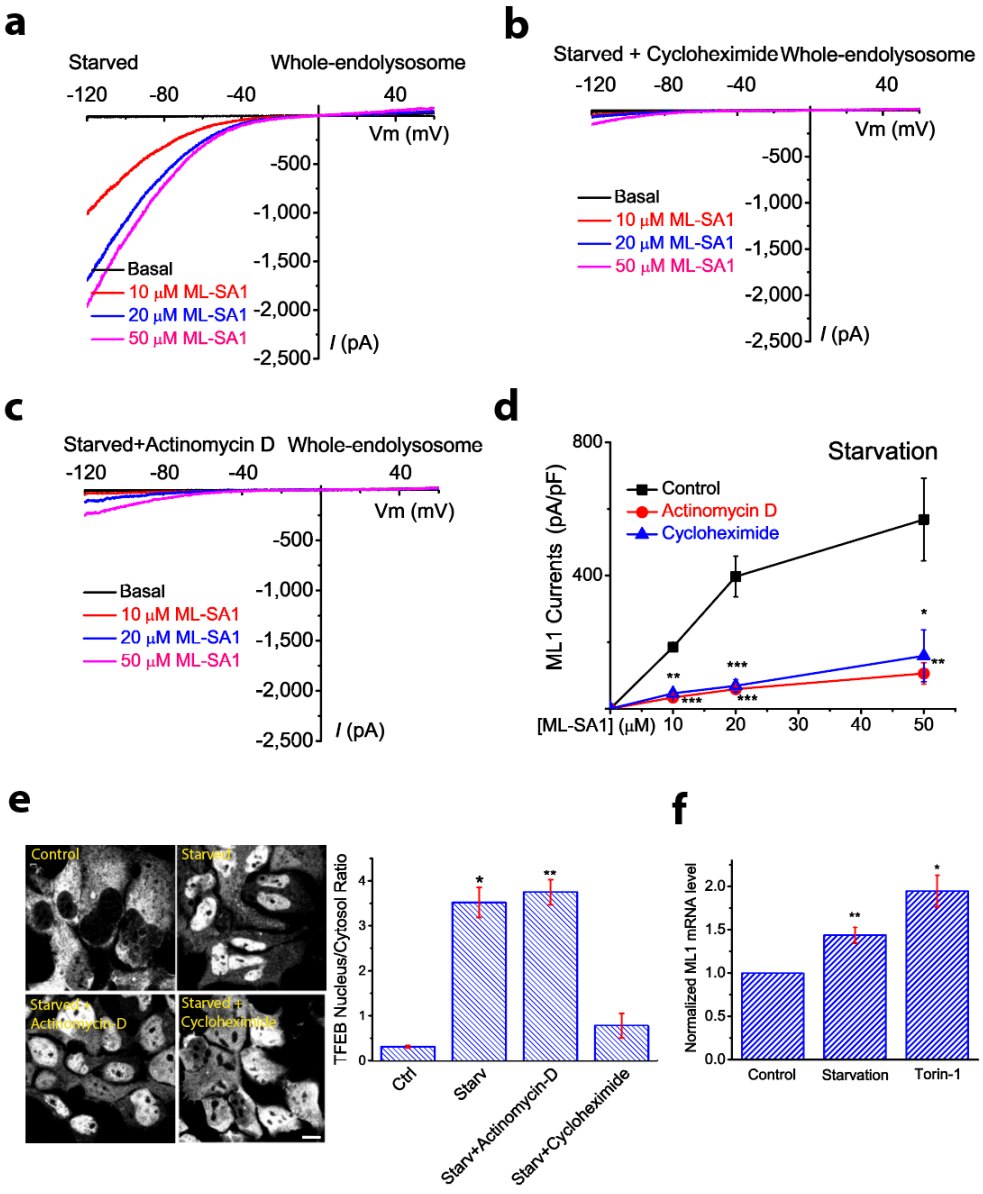


Figure 2.8 ML1 upregulation by starvation via transcription- and translation-dependent mechanisms.

(a) Representative traces of whole-endolysosome I_{ML1} for starved cells. **(b,c)** The effects of cycloheximide (2 μg/ml (b) and Actinomycin D (10 μg/ml (c) on I_{ML1} for starved Cos-1 cells **(d)** Mean densities for I_{ML1} in starved cells treated with cycloheximide (blue; n=3) and Actinomycin D (red; n=3). **(e)** The effects of Actinomycin D or cycloheximide on TFEB localization in HEK293 cells stably expressing TFEB-mCherry. Scale bar = 5 μm **(f)** Normalized expression levels of *ML1* mRNA were assessed by quantitative RT-PCR of RAW macrophage cells under different experimental conditions as indicated (n=5). Expression of the housekeeping gene, *L32*, served as a control, Data are presented as the mean ± SEM. Statistical comparisons were made using variance analysis (t-test). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

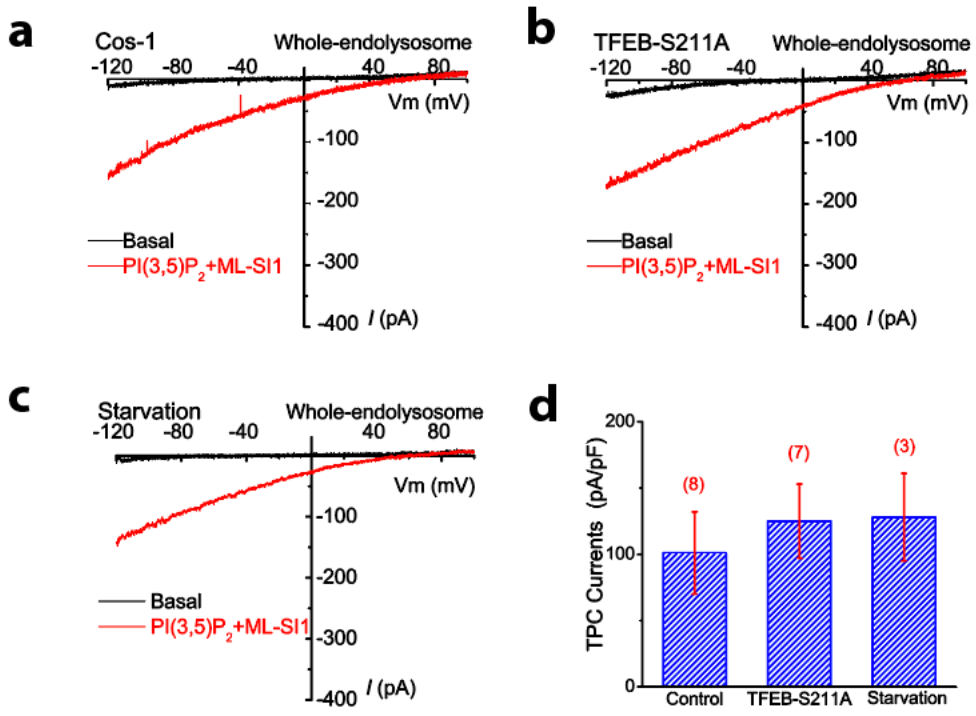
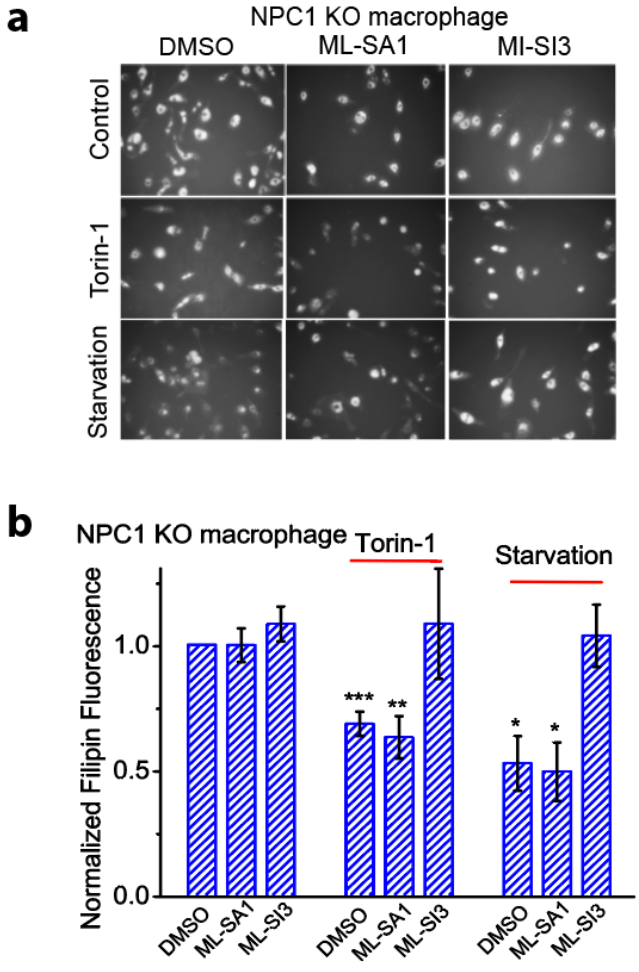


Figure 2.9 Starvation or TFEB activation does not increase the endogenous TPC currents in the lysosome.

(a) Representative traces of endogenous whole-endolysosome, Na^+-selective TPC currents (I_{TPC}) activated by $PI(3,5)P_2$ (100 nM) in the presence of the TRPML inhibitor, ML-SI1 (10 μM). **(b)** Whole-endolysosome I_{TPC} for a TFEB-S211A-transfected Cos-1 cell **(c)** Whole-endolysosome I_{TPC} for a serum-starved Cos-1 cell. **(d)** Mean current densities for I_{TPC} of control, TFEB-S211A-transfected, and starved Cos-1 cells. The number of cells being recorded was indicated in parentheses. Data are presented as the mean ± SEM. Statistical comparisons were made using variance analysis (*t*-test).


a
NPC1 KO macrophage
DMSO
ML-SA1
MI-SI3
Control
Torin-1
Starvation
b
NPC1 KO macrophage
Torin-1
Starvation
Normalized Filipin Fluorescence
1.0
0.5
0.0
***
**
*
*
DMSO
ML-SA1
ML-SI3
DMSO
ML-SA1
ML-SI3
DMSO
ML-SA1
ML-SI3

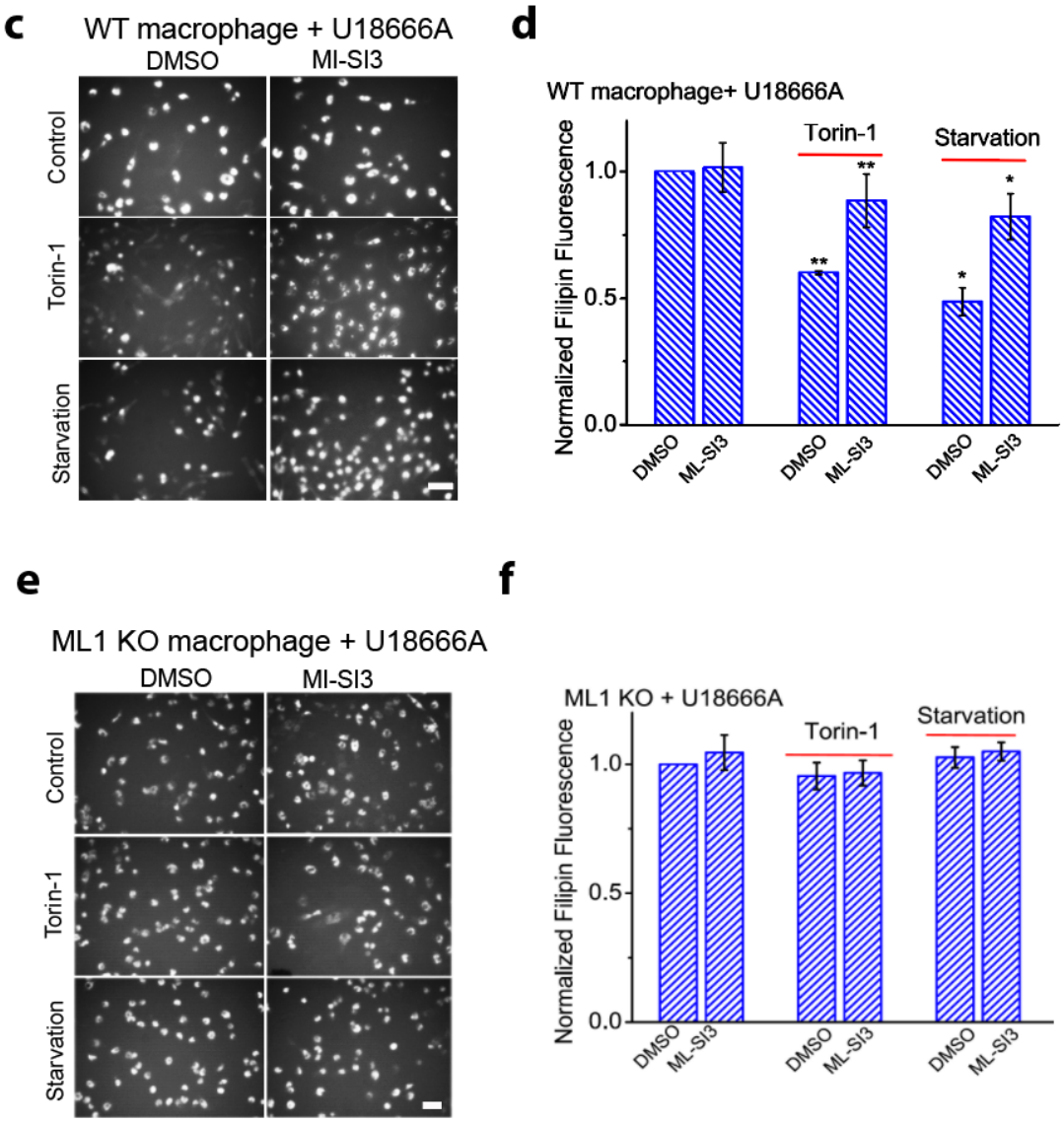


Figure 2.10 Reduction of lysosome storage by starvation or mTOR inhibition requires ML1.

(a) The effects of starvation (4 h), Torin-1 treatment (12 h), and TRPML synthetic modulator treatment (20 μM ML-SA1 or 20 μM ML-SI3) on cholesterol accumulation in NPC KO cells. Free cholesterol was detected using Filipin staining. **(b)** Normalized cholesterol levels in NPC1$^{-/-}$ primary macrophage upon starvation or mTOR inhibition in the presence of ML-SA1 and ML-SI3 as indicated. **(c,d)** Filipin staining of WT **(d)** and ML1 KO **(d)** macrophage cells treated with U18666A. **(e,f)** Torin-1 treatment or starvation reduced cholesterol levels in WT macrophage (**e**, n=3), but not in ML1$^{-/-}$ macrophage (**f**; n=3) treated with U18666A. Statistical comparisons were made using ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Scale bars = 100 μm

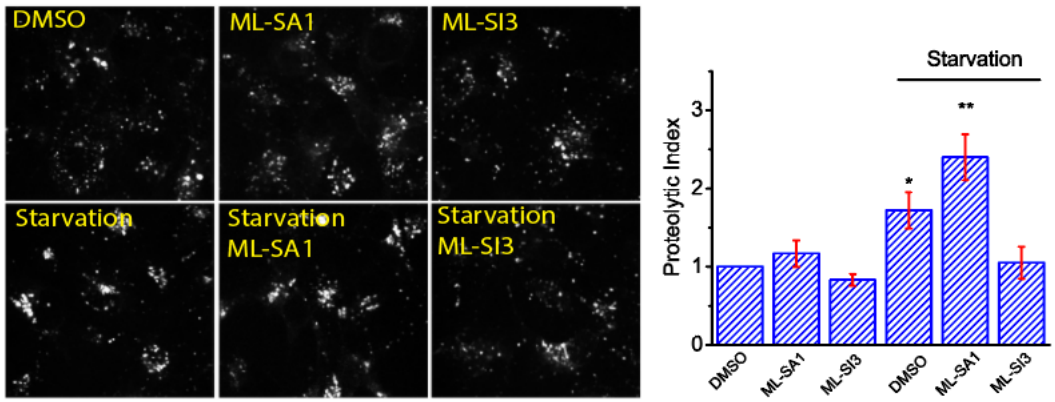


Figure 2.11 ML1 regulates starvation-induced lysosomal proteolysis.

(a) Confocal imaging of DQ-red-BSA Cos-1 cells grown in complete media versus AA-free + serum-free media (complete starvation) in the presence of ML-SA1 (20 μM) or MI-SI3 (20 μM). Scale bar = 5 μm **(b)** Normalized proteolytic index values for completely starved Cos-1 cells treated with ML-SA1 or MI-SI3. Data are presented as the mean ± SEM. Statistical comparisons were made using ANOVA (n=7). * $P < 0.05$; ** $P < 0.01$

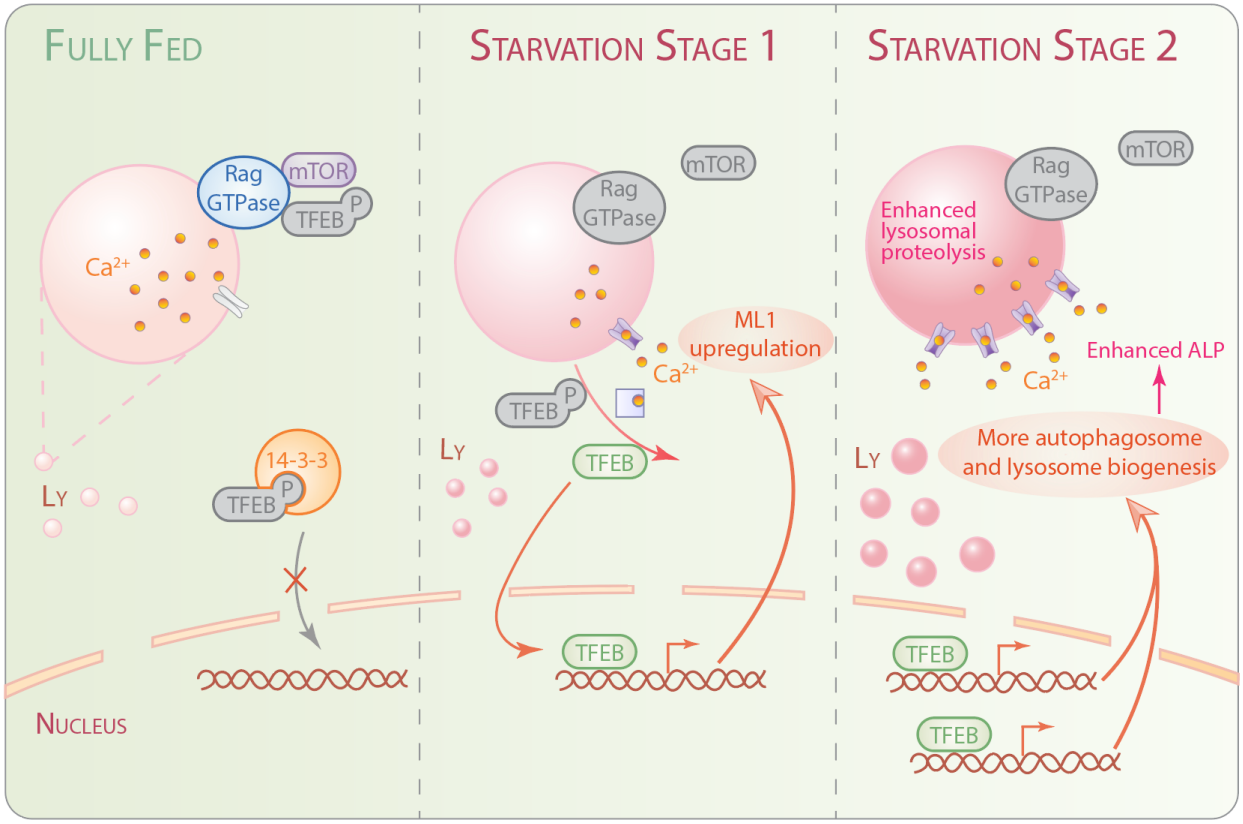


Figure 2.12 Reciprocal regulation between TRPML1 and TFEB in response to nutrient starvation.

Upon starvation (starvation stage one), TRPML1 mediates Ca^{2+} release, which further results in calcineurin-dependent dephosphorylation and nuclear translocation (~40min) of TFEB. Activated TFEB in the nucleus upregulates TRPML1 expression level (starvation stage two 2-4h), which enhances autophagic-lysosomal pathway (ALP) and lysosomal proteolysis. Thus, TRPML1 is essential for lysosomal adaptation in response to nutrient deprivation, and it constitutes a positive feedback loop with TFEB.

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CHAPTER III [3]

A Lysosomal Lipid Kinase PIKfyve Controls Lysosome Adaptation Independent of mTORC1

III-1 Abstract

Lipid kinases control a variety of cellular processes by controlling the dynamics of phosphoinositides. Here, I report that PIKfyve, a lysosomal lipid kinase, which converts phosphatidylinositol 3-phsphate [PI3P] to phosphatidylinositol 3,5-biphosphate [$PI(3,5)P_2$] on lysosomes, regulates transcriptional factor EB (TFEB), a major regulator of autophagy and lysosomal genes. Specific PIKfyve inhibition by two small molecule compounds (apilimod and YM201636) induced TFEB nuclear translocation under fed conditions and prevented the export of TFEB to the cytosol upon nutrient replenishment. Unexpectedly, basal mTOR activity and reactivation of mTOR upon nutrient replenishment was unaffected by PIKfyve inhibition, suggesting that PIKfyve regulates TFEB independently of mTOR. Similar to the regulation of TFEB by starvation, PIKfyve inhibition induced TFEB translocation in a lysosomal Ca^{2+}-dependent manner. This study demonstrates that PIKfyve may mediate lysosomal adaptation to extracellular cues and provides insights into the effects of PIKfyve on lysosome-related processes. Given the well-studied therapeutic role of activation of TFEB and side effects often accompanied by mTOR inhibition, apilimod, as a mTOR-independent activator of TFEB and a drug undergoing clinical trial, may serve as a potential treatment towards lysosome-related disorders.

[3] Partial materials presented in the chapter III are adapted with modifications from my co-first-authored paper published in PNAS Plus (*Wang, W.*, Q. Gao *, M. Yang, X. Zhang, L. Yu, M. Lawas, X. Li, M. Bryant-Genevier, N.T. Southall, J. Marugan, M. Ferrer, and H. Xu. 2015. Up-regulation of lysosomal TRPML1 channels is essential for lysosomal adaptation to nutrient starvation. Proc Natl Acad Sci U S A. 112: E1373-1381*). Ca^{2+} imaging experiments in Fig. 3.2 were performed by my collaborator Dr. Wuyang Wang.

III-2 Introduction

Transcriptional factor EB (TFEB), a key regulator of autophagy and lysosome genes, coordinates multiple steps in the autophagic-lysosomal pathway in response to environmental conditions (Palmieri et al., 2011; Sardiello et al., 2009). The lysosomal changes associated with this process are referred to as "lysosomal adaptation". The location of TFEB in the cell is tightly controlled by its phosphorylation status. Phosphorylated TFEB binds to cytosolic adaptor protein 14-3-3 and stays in the cytosol, while dephosphorylated TFEB translocates to the nucleus and becomes active (Martina et al., 2012; Medina et al., 2015; Roczniak-Ferguson et al., 2012; Settembre et al., 2012) TFEB phosphorylation is mediated by the mechanistic target of rapamycin complex 1 (mTORC1), while the Ca^{2+}-dependent phosphatase calcineurin is reported to dephosphorylate TFEB (Martina et al., 2012; Medina et al., 2015; Roczniak-Ferguson et al., 2012; Settembre et al., 2012a). Recruitment of TFEB and mTOR to lysosomes via Rag GTPases in an amino-acid dependent manner is essential for TFEB and mTOR regulation (Kim et al., 2008; Martina and Puertollano, 2013; Sancak et al., 2008). Rag GTPases function as heterodimers in which the active conformation consists of GTP-bound RagA/B and GDP-bound RagC/D. Amino acids promote the formation of the active complex and lead to recruitment of mTORC1 and TFEB to the lysosomes, so that TFEB gets phosphorylated and is retained in the cytosol. In the absence of amino acids, the Rag GTPases take on an inactivate conformation (GDP-bound Rag A/B and GTP-bound Rag C/D), so that TFEB falls off the lysosomal membrane, gets dephosphorylated via a Ca^{2+}-dependent signaling and translocates to the nucleus (Martina and Puertollano, 2013; Medina et al., 2015).

In addition to Rag GTPases, a lysosome-specific phosphatidylinositol 3,5-biphosphate [$PI(3,5)P_2$] is also implicated in regulating the localization and activity of mTORC1. $PI(3,5)P_2$ is sensitive to environmental changes, which coordinates mTORC1 activity. Nutrient deprivation results in a rapid decrease in lysosomal $PI(3,5)P_2$ levels and nutrient replenishment leads to an increase in $PI(3,5)P_2$ levels (Bridges et al., 2012; Zolov et al., 2012). In adipocytes, $PI(3,5)P_2$ levels correlate with insulin levels, and raptor, the major component of mTORC1, was shown to directly interact with $PI(3,5)P_2$ via its WD40 domain for mTORC1 activation (Bridges et al., 2012). In budding yeast, $PI(3,5)P_2$ was reported to be required for TORC1 activity and regulate TORC1 on

the vacuole (the lysosome equivalent) via directly recruiting Sch9 (a homolog of the well-characterized mTOR substrate S6 kinase) (Jin et al., 2014).

$PI(3,5)P_2$ is generated from phosphatidylinositol-3-phosphate (PI3P) by phosphatidylinositol-3-phosphate 5-kinase (Phosphoinositide kinase for five position containing a Five finger; PIKfyve), which is specifically localized on late endosome and lysosomes membrane (Ho et al., 2012; Sbrissa et al., 1999; Yamamoto et al., 1995). PIKfyve is part of a multimeric complex, which includes the scaffolding protein Vac14 and the $PI(3,5)P_2$ 5-phosphatase Fig 4 (Jin et al., 2008; Sbrissa et al., 2008). Despite the low abundance (only about 0.04% of total phosphatidylinositol in mouse fibroblasts), $PI(3,5)P_2$ regulates many cellular processes, including the endo-lysosomal system, autophagy, ion channel activation, and cellular signaling (Di Paolo and De Camilli, 2006; Ho et al., 2012; McCartney et al., 2014). Loss of function of PIKfyve induces the formation of large vacuoles that are characteristic of late endosomes and lysosomes, causing defects in many signaling pathways including retrograde trafficking and autophagy (de Lartigue et al., 2009). Similarly, in yeast, inactivation of Fab1, the yeast homologue of PIKfyve, causes an enlargement of the vacuole (Gary et al., 1998). $PI(3,5)P_2$ also plays a role in membrane trafficking by serving as an endogenous activator for both the transient receptor potential mucolipin1 (TRPML1), a lysosomal Ca^{2+} channel (Dong et al., 2010) and two-pore channels (TPCs), lysosomal Na^{+} channels (Wang et al., 2012). In mice, a whole-body knockout of *Pikfyve* results in embryonic death (Ikonomov et al., 2011), while mutations or ablations in *Fig4* and *Vac14* result in membrane trafficking defects and neurodegeneration (Chow et al., 2007; Ferguson et al., 2009; Jin et al., 2008; Lenk and Meisler, 2014; Zhang et al., 2007; Zolov et al., 2012). Homozygous Fig4 mutations have also been identified in human patients with the neurodegenerative diseases Charcot-Marie-Tooth syndrome 4J and amyotrophic lateral sclerosis (Chow et al., 2009; Chow et al., 2007; Osmanovic et al., 2017), demonstrating an important role of $PI(3,5)P_2$ in neuron development. As to the mechanism, autophagy is implicated to explain some of the phenotypes resulting from dysfunction of PIKfyve complex (Ferguson et al., 2009; Martin et al., 2013). However, the detailed mechanisms underlying neurodegeneration remain unclear. Given the reported role of mTOR in regulating the autophagic-lysosomal pathway via TFEB and the role of $PI(3,5)P_2$ on mTOR, I aimed to characterize the role of PIKfyve in the autophagic-lysosomal pathway.

III-3 Results

3.1 PIKfyve inhibition induces TFEB nuclear translocation.

To investigate the role of $PI(3,5)P_2$ in TFEB regulation, two different inhibitors of PIKfyve: YM201636 (Jefferies et al., 2008) and apilimod (Cai et al., 2013; Gayle et al., 2017) were employed. HEK293 cells that stably expressed TFEB were treated with YM201636 or apilimod, and TFEB nuclear translocation was observed in both experiments **(Fig. 3.1 a,b)**. The extent of translocation in each case was comparable to that observed with Torin-1 treatment **(Fig. 3.1 a,b)**. As previously reported (Settembre et al., 2011), re-stimulation of starved cells with amino acids drove TFEB out of the nucleus. In contrast, when YM201636 was added together with amino acids, TFEB re-translocation from the nucleus to the cytosol was blocked **(Fig. 3.1 c)**. Similar to the effect of starvation on the lysosomal Ca^{2+} channel TRPML1 (Wang et al., 2015), which has been shown to be essential for lysosomal adaptation, treatment of Cos-1 cells with apilimod dramatically increases the ML1 expression level and thus ML1-mediated lysosomal Ca^{2+} release **(Fig. 3.2)**, suggesting that PIKfyve may mediate lysosomal adaptation via TRPML1.

3.2 mTOR activity is not affected by PIKfyve inhibition.

Upon PIKfyve inhibition, $PI(3,5)P_2$ level is reduced (Cai et al., 2013; Zolov et al., 2012). $PI(3,5)P_2$ levels have been reported to affect mTORC1 localization and activity in budding yeast and adipocytes (Bridges et al., 2012; Jin et al., 2014). However, I found that p-S6K levels were only slightly reduced following YM201636 treatment, and remained unchanged following apilimod treatment **(Fig. 3.3 a)**. In sharp contrast, rapamycin, which was unable to induce TFEB nuclear translocation (Settembre et al., 2012b), completely suppressed the level of p-S6K. The re-activation of mTOR upon re-addition of amino acids in starved cells was also unchanged following apilimod treatment **(Fig. 3.3 b)**. The drug activity was confirmed by the precense of enlarged lysosomes under YM and apilimod treatment **(Fig. 3.3 c)**. Since phosphoinositie levels, including $PI(3,5)P_2$, PI3P and PI5P, are altered by both compounds (Cai et al., 2013; Zolov et al., 2012), these results suggest that regulation of TFEB nuclear translocation during starvation may be phosphoinositide-dependent but mTOR-independent.

3.3 PIKfyve may function upstream of Rag GTPases to regulate TFEB.

$PI(3,5)P_2$ has been shown to regulate mTORC1 activity via recruiting cytosolic raptor (a mTORC1 component) and Sch9 (yeast homology of S6K), in adipocytes and yeast respectively, to lysosomal/vacuole membrane (Bridges et al., 2012; Jin et al., 2014). PI5P and PI3P have been shown to recruit specific downstream effector proteins. We hypothesized that phosphoinositides with altered levels upon PIKfyve inhibition might regulate TFEB via facilitating its recruitment to lysosomes. TFEB has been reported to be recruited to the lysosomal membrane via active Rag GTPases under nutrient fed condition, and fall off lysosomal membrane due to inactive Rag GTPases under starvation. Interestingly, overexpression of constitutive active Rag GTPases (FLAG-$RagB^{Q99L}$ and GST-$RagD^{Q121L}$) (Kim et al., 2008) in HEK cells stably expressing TFEB prevented apilimod-induced TFEB translocation **(Fig. 3.4)**, suggesting that PIKfyve may regulate TFEB activity via controlling Rag GTPases activity. Consistent with a previous report, constitutively active Rag GTPases prevented starvation-induced TFEB translocation, but not Torin-1 induced TFEB translocation (Martina and Puertollano, 2013). Hence, PIKfyve may regulate Rag GTPases by altering the level of phosphoinositides, but the mechanisms are not clear.

3.4 PIKfyve inhibition-induced TFEB translocation depends on intracellular Ca^{2+}, but not TRPML1 and calcineurin.

To further elucidate the mechanism of action of apilimod on TFEB, the role of known regulators of TFEB were tested. Similar to the regulation of TFEB by nutrient availability, apilimod-induced TFEB translocation required intracellular Ca^{2+} since BAPTA-AM, the intracellular Ca^{2+} chelator, blocked apilimod-induced TFEB translocation **(Fig. 3.5 a)**. This Ca^{2+} mediated regulation does not depend on the major lysosomal Ca^{2+} channel TRPML1. In cells treated with the ML1 inhibitor ML-SI and MLIV patient fibroblast with a mutation in the *Trpml1* gene and dysfunction of TRPML1, apilimod- or starvation- induced TFEB nuclear translocation was unaffected **(Fig. 3.5 a,b)**. The currently known downstream effector of Ca^{2+} during starvation is calcineurin (Medina et al., 2015). TFEB translocation to the nucleus is mildly reduced under starvation. However, TFEB translocation to the nucleus under apilimod remained unaffected in cells treated with the potent calcineurin inhibitors, FK506 and CysA **(Fig. 3.6 a)**, which completely prevented the nuclear translocation of another well-characterized calcineurin effector nuclear factor of activated T cells (NFAT) in response to thapsigargin (TG) treatment **(Fig. 3.6 b)**

(Marangoni et al., 2013), suggesting a novel Ca^{2+}-dependent mechanism in TFEB regulation under PIKfyve inhibition.

III-4 Discussion

4.1 PIKfyve plays a role in lysosomal adaptation to nutrient deprivation at two distinct steps.

In this study, I present PIKfyve as a novel regulator of lysosomal adaptation via TFEB, which establishes a previously unrecognized link between PIKfyve and TFEB in regulating lysosomal adaptation. This further demonstrates that lysosomes are tightly regulated by self-provided signals. Upon PIKfyve inhibition, lysosomal compartments are expanded due to the up-regulation of lysosomal genes (such as TRPML1) under activation of TFEB, mediating necessary lysosomal adaptations **(Fig. 3.8)**.

The PIKfyve activity is implicated to be inhibited upon starvation (Zolov et al., 2012). Apilimod treatment mimics starvation condition and may activate an additional and parallel pathway besides mTOR inhibition under starvation to induce TFEB translocation to provide lysosomal adaptation for cell survival. Moreover, Vac14, the interacting partner of PIKfyve, is suggested to be a downstream target of TFEB (Sardiello et al., 2009). Upregulation of TFEB may result in upregulation of Vac14, which may relieve the inhibition effect of PIKfyve, constituting a negative feedback regulation to finely tune the optimal activity of TFEB at different stages of starvation. Thus, upon prolonged starvation (> 2-4 hrs), the efflux of lysosomal AAs that are produced during the course of lysosomal degradation and TFEB activation may readily trigger re-synthesis of $PI(3,5)P_2$, thereby causing mTOR reactivation and TFEB inactivation (Settembre et al., 2013; Yu et al., 2010; Zolov et al., 2012). Hence, $PI(3,5)P_2$ may be involved in nutrient-regulation of lysosomal functions in at least two distinct steps.

4.2. $PI(3,5)P_2$ mediates lysosomal adaptation by up-regulating and activating TRPML1

$PI(3,5)P_2$ is an endogenous agonist of lysosomal Ca^{2+} channel TRPML1, which plays an essential role in lysosomal adaptation to starvation (Dong et al., 2010; Wang et al., 2015). In this study, ML1 was up-regulated by PIKfyve inhibition, which may occur as an adaptive response to $PI(3,5)P_2$ reduction. In the initial phase, $PI(3,5)P_2$ reduction may serve as a trigger to activate TFEB, and this function may be TRPML1-independent or require partial a Ca^{2+} release from

TRPML1. In the late phase of starvation, $PI(3,5)P_2$ re-elevation, which is triggered by lysosomal AA efflux and up-regulation of PIKfyve functional complex, may activate TRPML1 to regulate lysosome reformation. Both $PI(3,5)P_2$ and TRPML1 are shown to be required for lysosome reformation and tubulation (Li et al., 2016; Li et al., 2013). Moreover, TRPML1 agonists were sufficient to "rescue" the lysosome reformation defects in $PI(3,5)P_2$-deficient cells (Zou et al., 2015).). In addition, AA-independent local and transient activation of PIKfyve may directly regulate lysosomal membrane trafficking and reformation, which is also likely mediated by TRPML1. Putting all evidence together, starvation may trigger a cascade of signaling to regulate both autophagosomal biogenesis and lysosome biogenesis, during which $PI(3,5)P_2$ reduction and re-synthesis regulate corresponding responses to different starvation stages.

4.3 Role of phosphoinositides in regulating lysosomal adaptation

Upon PIKfyve inhibition by apilimod/YM or starvation, levels of different phosphoinositides are changed. In addition to reduction in $PI(3,5)P_2$ level, there is a marked increase in the PI3P level due to the blockage of the conversion of PI3P to $PI(3,5)P_2$ (Cai et al., 2013). Furthermore, PIKfyve is required to generate most of the PI5P pool. Upon PIKfyve inhibition, PI5P level is also rapidly reduced (Zolov et al., 2012). These phosphoinositides with changes in level may provide lysosomal and temporal regulation of downstream signaling pathways. In this study, TFEB nuclear translocation by PIKfyve inhibition may result from decrease of $PI(3,5)P_2$, PI5P or both lipids, or increase of PI3P. The interconversion between these lipids makes it very difficult to elucidate the regulatory lipid (s) in TFEB regulation. It requires fine manipulation of a specific phosphoinositide.

To examine whether PI3P increase may induce TFEB nuclear translocation, we may employ an inducible-dimerization system to deplete PI3P at lysosomes with EGFP-FKBP-Rab7 targeting construct and an RFP-FRB-MTM1 cytosolic construct. MTM 1 (a myotubularin phosphatase) is recruited to lysosomes upon rapamycin treatment and converts $PI(3,5)P_2$ into PI5P (Stahelin et al., 2013; Dong et al., 2010). Together with apilimod treatment, we may dissect the role of PI3P increase upon PIKfyve inhibition in TFEB regulation. And this regulation system may compensate for the decrease of PI5P at the same time. Furthermore, exogenous application of phosphoinositides may help dissect the effect of a specific phosphoinositide (Chang-Ileto et al., 2012). We may examine the effect of increase of PI3P in TFEB regulation under nutrient fed condition by exogenously applying PI3P (Subramanian et al., 2010) and study the effect of

decrease of $PI(3,5)P_2$ or PI5P in TFEB regulation under starvation condition by exogenously applying $PI(3,5)P_2$ (Silswal et al., 2011) and PI5P (Vicinanza et al., 2015).

Furthermore, characterization of protein effectors in TFEB regulation pathway may help us understand which phosphoinositide(s) regulates TFEB . It's possible these phosphoinositides may direct bind and modulate the activity of Rag GTPase to regulate TFEB activity. We may perform a lipid overlay assay to test the direct binding between Rag GTPases and different phosphoinositides (Hong et al., 2015). Besides, we could perform liposome binding assay with Rag GTPases and possibly other lysosomal proteins utilizing liposomes containing $PI(3,5)P_2$, PI3P or PI5P (Hong et al., 2015). We may also directly test whether different phosphoinositides may directly affect the GAP activity of Rag GTPases (Kam et al., 2000).

4.4 Molecular mechanisms underlying TFEB activation by PIKfyve inhibition

In search of a specific mechanism that couples PIP_2 and TFEB, a pathway which is Ca^{2+} dependent, but TRPML1- and calcineurin- independent, was revealed. Similar to starvation-induced TFEB nuclear translocation, PIKfyve inhibition results in dephosphorylation of TFEB (Gayle et al., 2017) via Ca^{2+} release. The underlying mechanisms of Ca^{2+} release upon PIKfyve inhibition is unclear.. Interestingly, in a recent study by Gayle et al, apilimod was shown to mediate apilimod-induced expansion of the acidified compartment and vacuolization by a mechanism requiring lysosome-related genes *CLCN7*, *OSTM1* (chloride transporter) and *SNX10*, in addition to *TFEB*, in a genome-wide CRISPR screen (Gayle et al., 2017). Loss of either CLCN7 or OSTM1, but not TFEB, blocked apilimod-induced vacuolization (Gayle et al., 2017). It would be interesting to test whether PIP2 may couple with TFEB via these candidates. Furthermore, Rag GTPases, another well-characterized regulator of TFEB, seems to function downstream of PIKfyve. However, it remains elusive how PIKfyve may affect the Rag GTPases.

4.5 The Role of $PI(3,5)P_2$ in mTOR regulation

mTOR signaling was unaffected by Apilimod treatment, which has been confirmed by other researchers in MCF10A cells, J774.1 macrophages (Krishna et al., 2016) and Non-Hodgkin lymphoma (B-NHL) cell (Gayle et al., 2017). These findings cast doubt as the previous studies establishing the role of $PI(3,5)P_2$ in regulating TORC1 activity. However, no consensus could be reached since the regulatory mechanisms have been examined in different cell types

(Epithelial/macrophage/B cells vs. adipocytes) and different organisms (Human vs. yeast). Besides, in Krishna's study, regulation of vacuole maturation and nutrient recovery following engulfment in starved cells by PIKfyve is largely independently of mTORC1 except when cells are dependent on nutrients supplied in the form of engulfed apoptotic corpses (Krishna et al., 2016), suggesting that specific pathway may mediate the differential effects of apilimod in different cell conditions, cell types, and organisms.

4.6 Evaluation of apilimod as a drug to promote cellular clearance via TFEB

Drugs, such as apilimod, that potently increase TFEB activity without inhibiting mTOR are clinically promising drugs considering that mTOR inhibition has resulted in a lot of unfavorably side effects (Pallet and Legendre, 2013, Gayle et al., 2017) However, it would be necessary to cautiously implement the regimens for apilimod and relevant drugs. Apilimod may block lysosome biogenesis, and apilimod-induced vacuole formation may be harmful to cells (Cai et al., 2013; Gayle et al., 2017). Simply enhancing autophagic and lysosomal compartments when endolysosomal membrane traffic is impaired, may further stress the cells and contribute to cell death (Gayle et al., 2017). Due to TFEB activation, vacuole enlargement and trafficking defects resulting from dysfunction of PIKfyve, excessively lysosomal components are accumulated (Min et al., 2014). Besides, although PIKfyve inhibition can mimic starvation regarding TFEB activation and TRPML1 upregulation, it also blocks $PI(3,5)P_2$ re-synthesis and subsequent lysosome reformation, implying that PIKfyve inhibition is unlikely to promote cellular clearance. Further research is needed to identify new reagents that can specifically activate TFEB without affecting other cellular processes. With the identified downstream targets (CLCN7 or OSTM1) involved vacuole formation, it's possible a combined therapy could be adopted. Inhibiting CLCN7 or OSTM1 while inhibiting PIKfyve may provide a scenario where the autophagic-lysosomal pathway is upregulated and completed due to reduced vacuole formation. In summary, this finding provides us new insights on the role of PIKfyve in regulating lysosomal functions, which may lead to promising drug development directions for lysosome-related diseases.

III-5 Materials and Methods

Molecular biology. Constitutive Rag constructs (FLAG-RagB^{Q99L} and GST-RagD^{Q121L}) are generous gifts from Dr. Ken Inoki at University of Michigan; Hela cells stably expressing TFEB-GFP is kindly provided by Dr. Shawn Ferguson at Yale University.

Mammalian Cell Culture. HEK293 cells stably expressing TFEB-mCherry were generated using the Flip-In T-Rex 293 cell line (Invitrogen). Unless otherwise indicated, Cos-1, HEK-293T, and Hela cells were grown at 37 ^{o}C in a 1:1 mixture of DMEM and Ham's F12 media supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO_2 incubator. Human skin fibroblast cell lines from a mucolipidosis IV patient (clone GM02048) and a healthy control (clone GM05659) were obtained from the Coriell Institue for Medical Research and cultured in 1:1 mixture of DMEM and Ham's F12 media with 15% FBS. Cos1 cells were transfected using Lipofectamine 2000 (Invitrogen). Cells were transfected using Lipofectamine 2000 (Invitrogen).

Western blotting. Cells were lysed with ice-cold RIPA buffer (Boston BioProducts) in the presence of 1X protease inhibitor cocktail (Sigma), 1 mM NaF, and 1mM Na_3VO_4. Total cell lysates were mixed with 2X SDS loading buffer and were boiled at 95 ^{o}C for 10 min. Protein samples were then loaded and separated on 4–12% gradient SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes. The membranes were blocked for 1 h with 1% BSA in PBST and were incubated with various antibodies against p70 p-S6 kinase (Thr389), S6 kinase (all at 1:1,000 and were purchased from Cell Signaling) in PBST. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:5000) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). Band intensities were quantified using Image J software.

Fura-2 Ca^{2+} imaging. Ca^{2+} imaging was carried out within 2–3 h after plating while cells exhibited a round morphology. Cells were loaded with 5 μM Fura-2 AM in the culture medium at 37 ^{o}C for 1 h. Fluorescence was recorded at different excitation wavelengths using an EasyRatioPro system (PTI). Fura-2 ratios (F340/F380) were used to monitor changes in intracellular [Ca^{2+}] upon stimulation. Lysosomal Ca^{2+} release was measured under a 'zero' Ca^{2+} external solution (Shen et al., 2012), which contained 145 mM NaCl, 5 mM KCl, 3 mM MgCl2, 10 mM glucose, 1 mM EGTA, and 20 mM HEPES (pH 7.4).

TFEB and NFAT Immunofluorescence. Cells were grown on glass coverslips and then fixed with 4% PFA and permeabilized with 0.3% Triton X-100 after treatments. And then the cells are blocked with an immunofluorescence buffer with 1% BSA in PBS. Endogenous TFEB/NFAT was recognized by incubating cells with anti-TFEB antibody (1:1000; Cell Signaling) or anti-NFAT antibody (1:1000; Cell Signaling) for 1h. Then cells were washed 4-5 times with PBS and incubated with anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or 568 for 1h. After three washes with PBS, the coverslips were mounted with Fluoromount-G (Southern Biotech).

Confocal Imaging. Prepared coverslips were imaged using a Leica confocal microscope or Spin-Disk confocal. The live imaging was performed at 37 °C with the spinning-disk confocal live-imaging system, which included an Olympus IX81 inverted microscope, a × 60 or × 100 objective (Olympus), a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor) and MetaMorph Advanced Imaging acquisition software v.7.7.8.0 (Molecular Devices) and data were analyzed using MetaMorph. Perfusion system was used to exchange solutions during imaging.

Cell Treatments. For serum starvation, cells were washed three times in Hank's balanced salt solution (Invitrogen) and incubated for 2-4h at 37°C in DMEM without 10% FBS. For complete starvation experiments, cells were washed three times in Hank's balanced salt solution (Invitrogen) and incubated for 2–4 h at 37°C in either a 1:1 mixture of DMEM and Ham's F12 media without amino acids (US Biological). Recovery after starvation was achieved by the addition of normal culture medium or starvation solution added with 4 X MEM Amino Acids solution from Thermo Fisher with adjusted pH to 7.4.

Reagents. The following reagents were purchased: Torin-1 (Tocris), Rapamycin (LC Laboratories), YM201636 (Symansis), Apilimod (Axon Medchem), Concanamycin A and Ionomycin (Sigma), Acridine Orange and Fura-2 AM (Life Technologies)

Data analysis. Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using Student's *t*-test and ANOVA test. A P value < 0.05 was considered statistically significant.

III-6 Figures

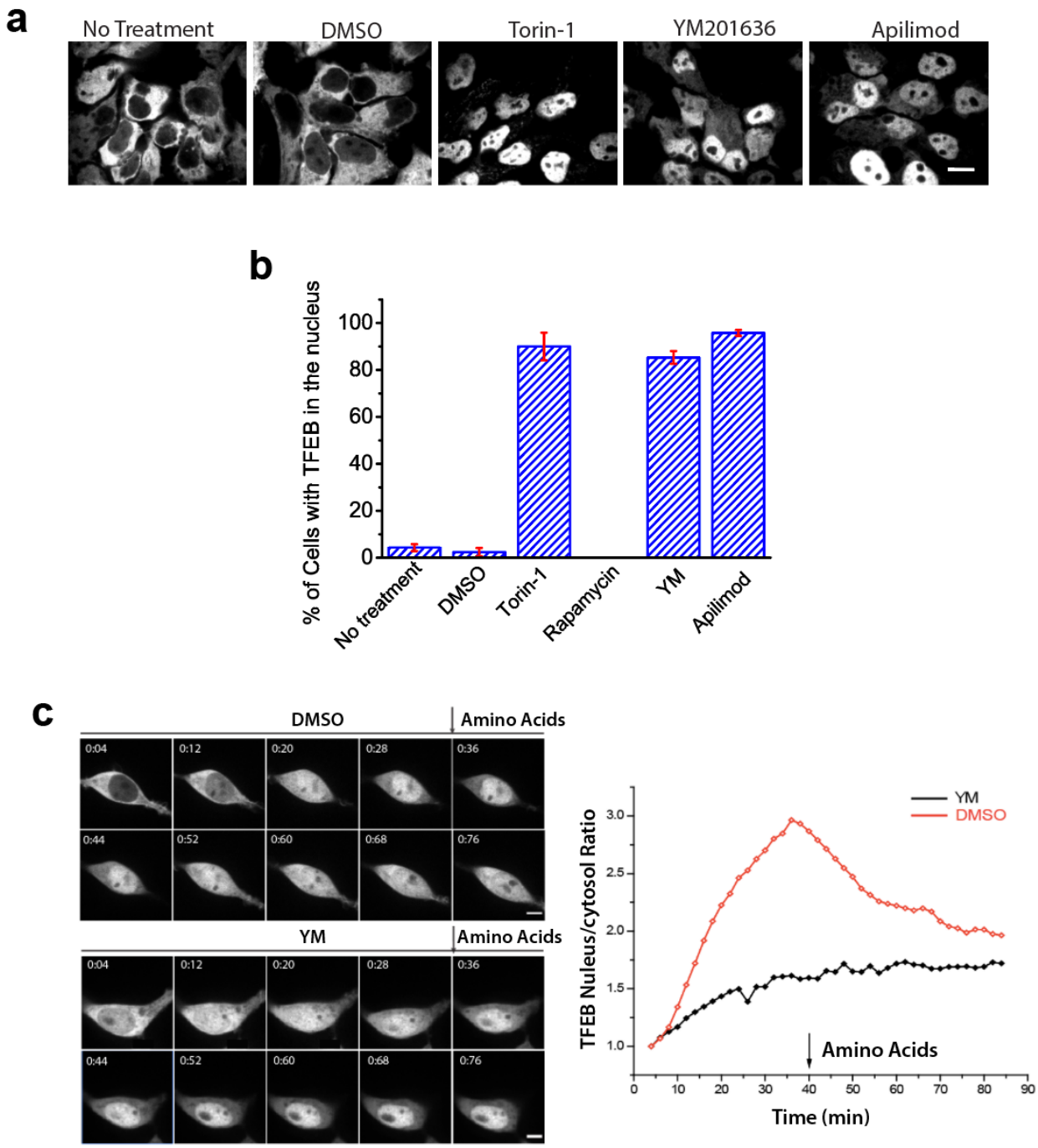

Figure 3.1 PIKfyve inhibition induces TFEB translocation to the nucleus.

(a) Confocal imaging of TFEB localization in cells treated with DMSO, Torin-1 (1 μM), YM201636 (YM, 1 μM), or apilimod (1 μM). Scale bar = 5 μm **(b)** Quantitation of data from panel A (n=6). Nuclear localization was determined using an arbitrary criterion of the fluorescent intensity of TFEB-mCherry in the nucleus being > 150% of the cytoplasmic signal. **(c)** Live-imaging of TFEB translocation with or without YM treatment upon the addition of amino acids after starvation. The TFEB nucleus to cytosol ratios in the cells imaged in the videos were quantified. Scale bar = 5 μm

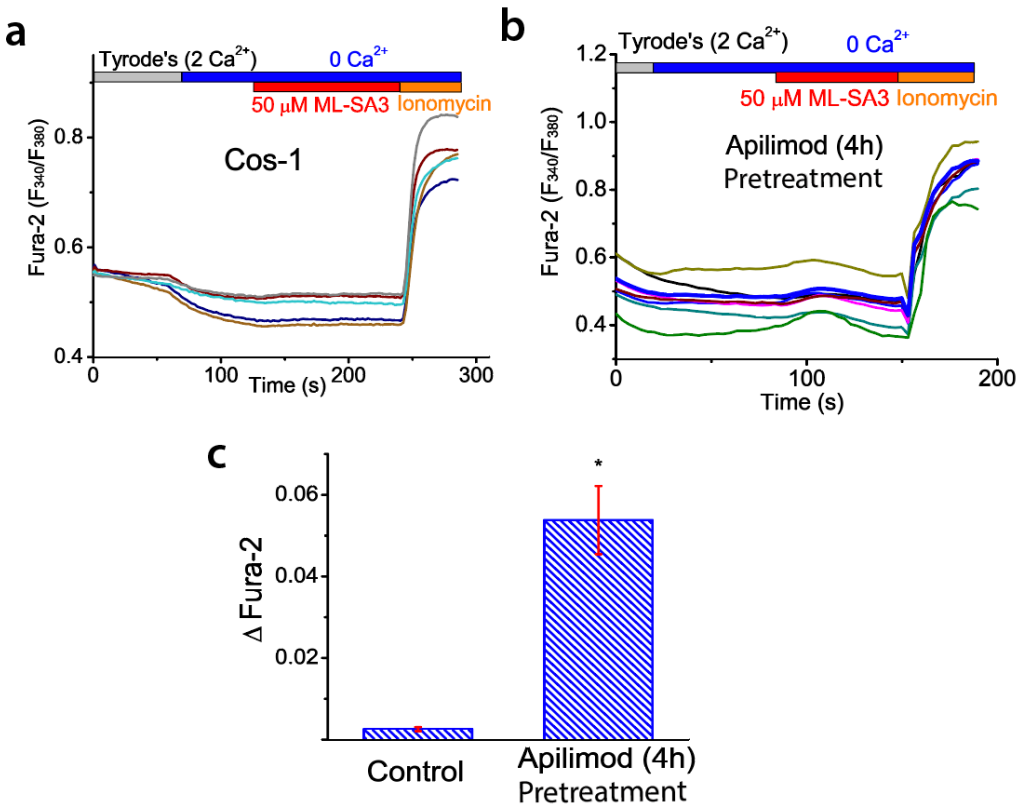

Figure 3.2 PIKfvye inhibition induces up-regulation of TRPML1.

(a) ML-SA3 (50 μM) did not induce any obvious Ca^{2+} release (according to the Fura-2 ratio, F340/F380) in Cos-1 cells grown in complete media. Ionomycin (5 μM) was added at the conclusion of all experiments to induce a maximal intracellular release for comparison. Shown are selected traces from the same coverslip that typically contained. **(b)** Cos-1 cells with Apilimod (1 μM) for 4 h pretreatment resulted in an increase in lysosomal Ca^{2+} release under same concentration of ML-SA. **(c)** The average TRPML1-mediated lysosomal Ca^{2+} release in Apilimod-pretreated Cos-1 cells. The results were averaged from 40–100 cells from n=3 independent experiments. Data are presented as the mean ± SEM. Statistical comparisons were made using variance analysis (t-test). * $P < 0.05$

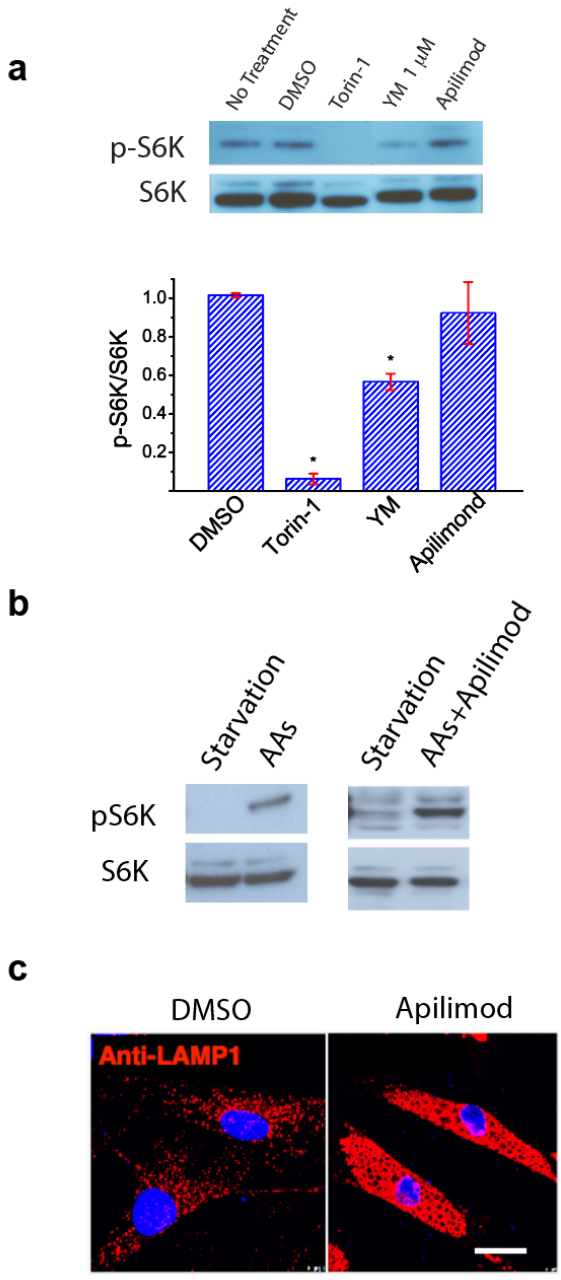

Figure 3.3 PIKfyve inhibition does not inhibit mTOR activity.

(a) Western blot analysis of phosphorylated S6K (p-S6K) levels following treatment with DMSO, Torin-1 (1 μM), YM (1&2 μM), and Apilimod (1 μM)-treated cells. Ratios of p-S6K to total S6K under different experimental conditions are shown in the lower panel (n=4). **(b)** Western blot analysis of phosphorylated S6K (p-S6K) levels upon addition of nutrients together with DMSO or Apilimomd after starvation. **(c)** Lysosomes were enlarged by apilimod treatment for 1h under the concentration used for western blot indicated by LAMP1 immunostaining. Scale bar = 25 μm

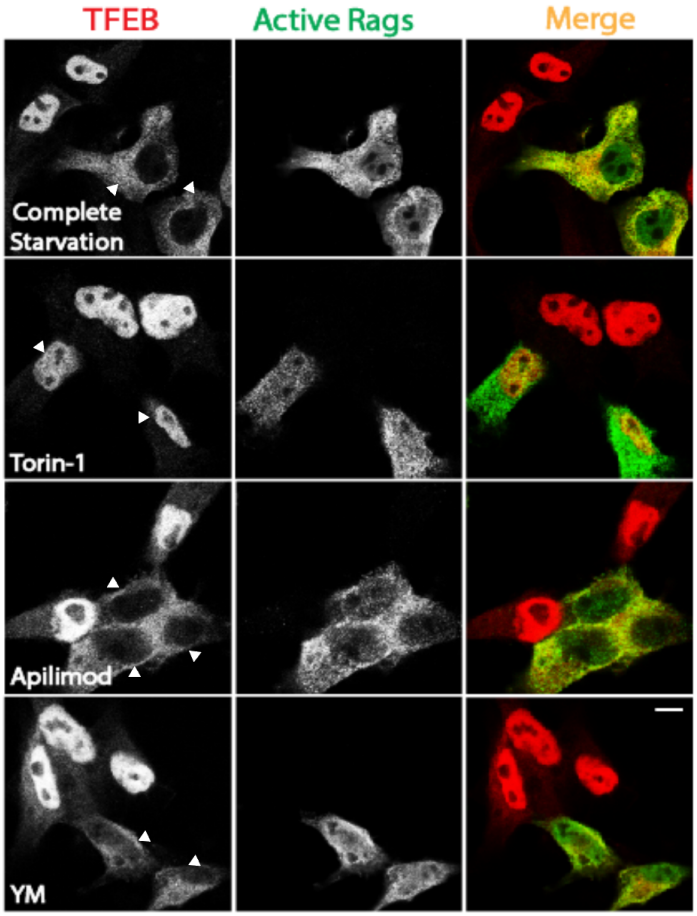

Figure 3.4 Overexpression of constitutive Rags GTPases prevents Apilimod or YM induced TFEB translocation.

In TFEB-mCherry HEK stable cells transfected with constitutive Rag GTPases (FLAG-RagB^{Q99L} and GST-RagD^{Q121L}; constitutive Rag B were labelled with anti-FLAG antibody and indicated with white arrowheads), starvation, apilimod and YM induced-TFEB nuclear translocation were completely blocked, while Torin-1-induced TFEB nuclear translocation was unaffected. Scale bar = 5 μm

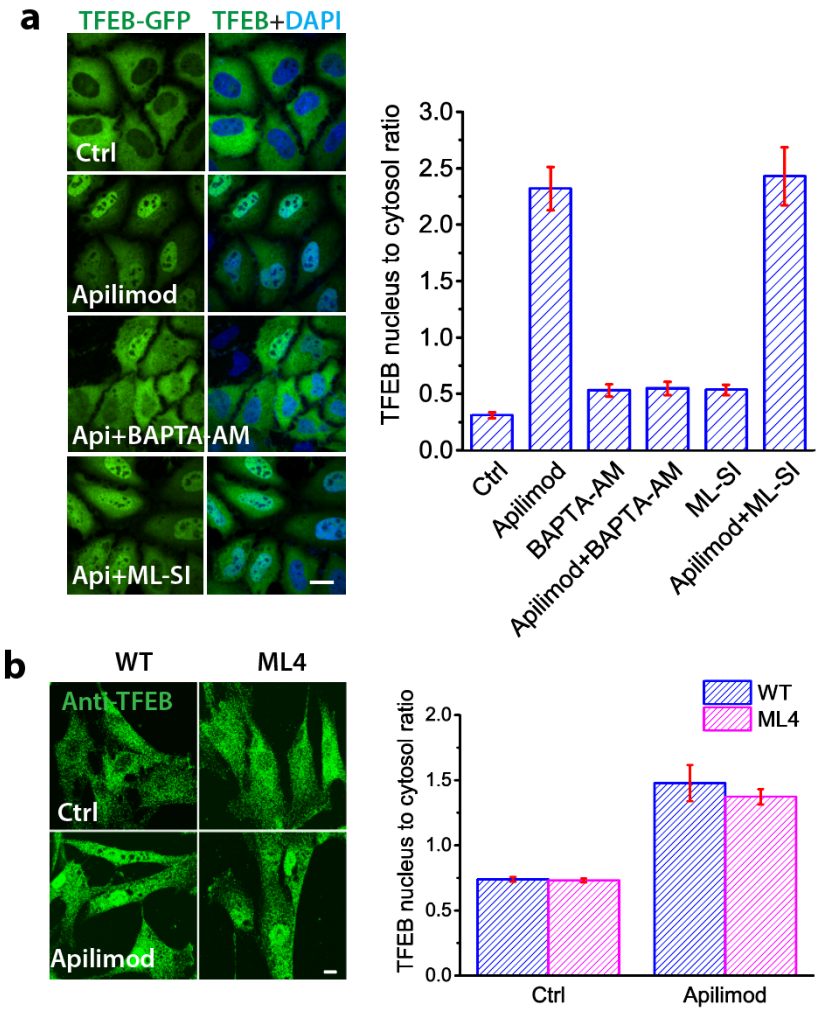

Figure 3.5 Apilimod-induced TFEB nuclear translocation is dependent on intracellular Ca^{2+}, but not TRPML1.

(a) BAPTA-AM (10 μM) treatment for 60 min blocked apilimod-induced TFEB translocation. Quantification data were obtained from more than 30 cells of each treatment from at least three replicates and shown as ±SEM **(b)** Apilimod-induced TFEB nuclear translocation was unaffected in ML-IV fibroblasts. Scale bar = 10 μm

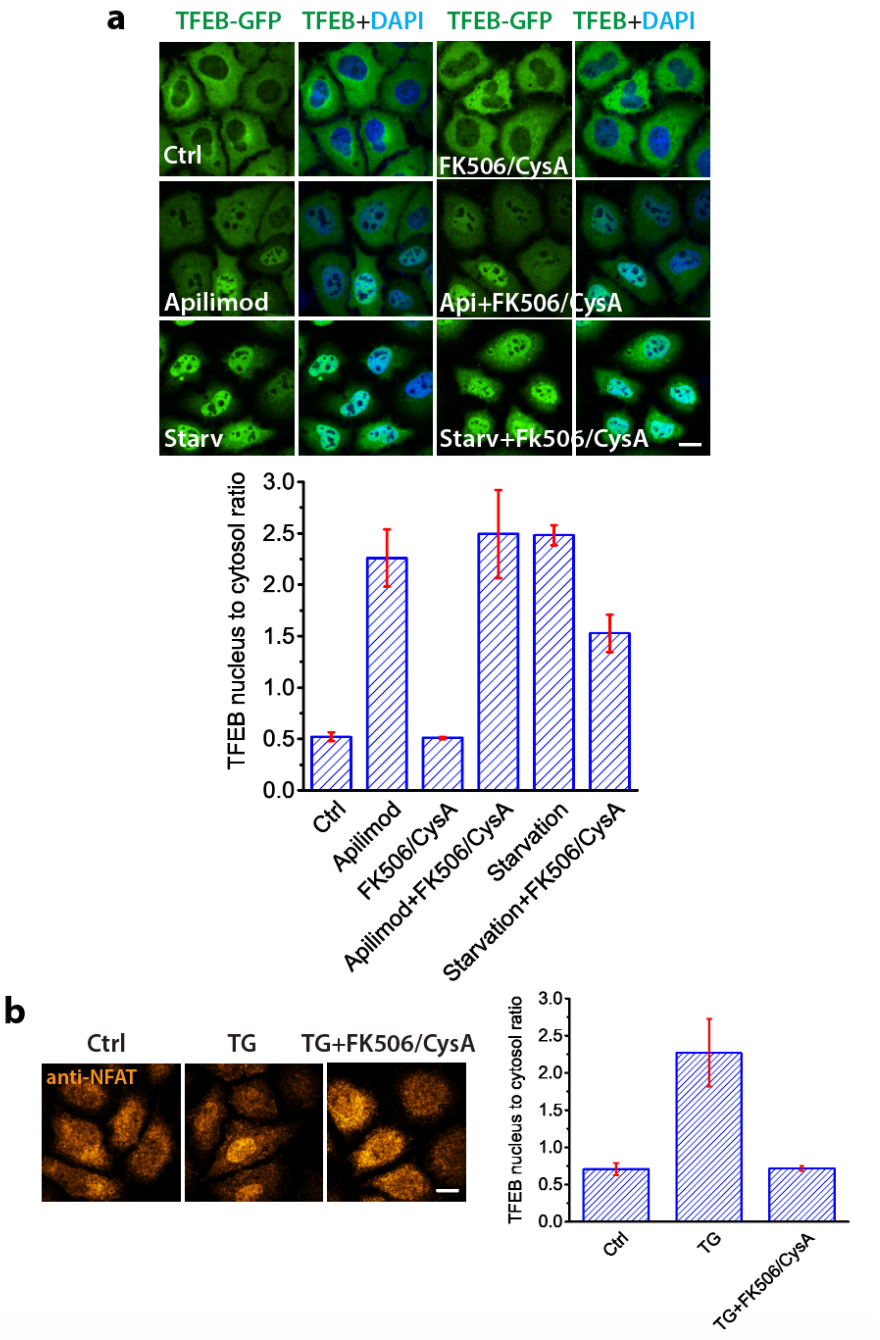

Figure 3.6 Apilimod-induced TFEB nuclear translocation is not dependent on calcineurin.

(a) Co-application of FK506 (a calcineurin inhibitor; 10 μM) and CysA (a calcineurin inhibitor; 10 μM) did not block apilimod-induced TFEB translocation. Quantification data were obtained from more than 30 cells of each treatment from at least three replicates and shown as ±SEM. **(b)** Co-application of FK506(10 μM) and CysA(10 μM) blocked thapsigargin (TG; 2μM)-induced nuclear translocation of nuclear factor of activated T-cells (NFAT), a well-characterized downstream target of calcineurin. Scale bar = 10 μm

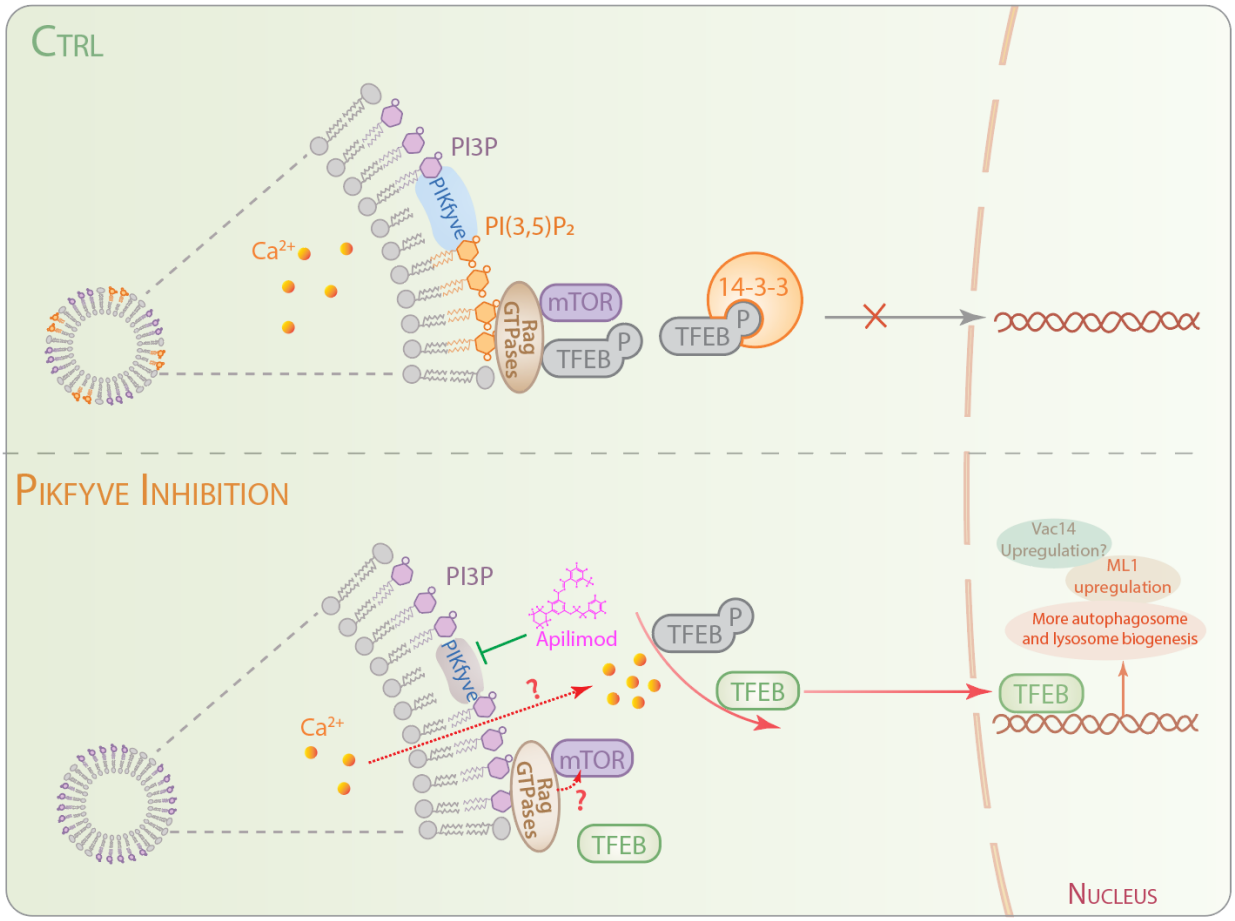

Figure 3.8 A working model to illustrate the role of PI(3,5)P_2 in lysosomal adaptation.

PIKfyve inhibition and PI(3,5)P_2 reduction trigger TFEB nuclear translocation and activation, inducing the expression of genes needed for autophagosome biogenesis and lysosome biogenesis, including upregulation of TRPML1 and possibly vac14, which may result in re-synthesis of PI(3,5)P_2 after a longer starvation time. PI(3,5)P_2 elevation may further increase the activity of lysosomal ML1 channels to enhance autophagic-lysosomal degradation via increased lysosomal biogenesis and trafficking.

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CHAPTER IV [4]

Rapamycin Directly Activates Lysosomal TRPML1 Channels Independent of mTOR

IV-1 Abstract

Rapamycin and its derivatives (rapalogs) are being actively pursued in a number of clinical trials targeting cancer, neurodegeneration, and aging. The underlying mechanisms of actions of rapamycin, however, are incompletely understood. The mechanistic target of rapamycin (mTOR), a lysosome-localized protein kinase that acts as a key regulator of cellular growth, is believed to mediate the most rapamycin actions. Here, we identified Mucolipin TRP channel 1 (TRPML1), the principle Ca^{2+} release channel in the lysosome, as another direct target of rapamycin. Patch-clamping isolated lysosomal membranes showed that micromolar concentrations of rapamycin and rapalogs directly and specifically activated TRPML1 and the related TRPML2 channels, but not TRPML3 or other lysosomal channels. The activation was independent of mTOR activity, as pharmacological inhibition or genetic inactivation of mTOR failed to mimic the rapamycin effect. In cells where the expression level of TRPML1 was high, rapamycin/rapalogs induced lysosomal Ca^{2+}-dependent nuclear translocation of transcriptional factor EB (TFEB), a master regulator of lysosome biogenesis and autophagy. Hence, rapamycin may promote autophagy via both mTOR-dependent and independent mechanisms. Given the demonstrated roles of TRPML1 and TFEB in cellular clearance, we propose that lysosomal TRPML1 may contribute to the *in vivo* neuroprotective effects of rapamycin, via an enhancement of autophagosome and lysosome biogenesis.

[4] The contents presented in chapter IV are unpublished data, which are under preparation for publication. The electrophysiology recording experiments in Fig. 4.2, 4.3, 4.4 were performed by my collaborator Dr. Xiaoli Zhang.

IV-2 Introduction

Rapamycin is a 31-membered macrocyclic natural product that was initially isolated from *Streptomyces hygroscopicus* as an antifungal medicine (Sehgal et al., 1975). Subsequently, a diverse range of cellular functions has been reported for rapamycin, with the most robust efficacies in immunosuppression and anti-proliferation (Thomson et al., 2009). Due to its promising therapeutic potentials, numerous rapamycin derivatives (**Fig. 4.1**) with improved pharmacokinetic properties have then been developed in pharmaceutics, including temsirolimus, everolimus, deforolimus, zotarolimus, WYE-592, and ILS-920 (Park et al., 2010; Ruan et al., 2008). Since 1999, rapamycin, in its brand name sirolimus, and multiple rapalogs have been approved by FDA to be tested in multiple clinical trials aiming to treat cancers, metabolic diseases, and neurodegenerative diseases (Li et al., 2014). More recently, rapamycin has also been shown to extend lifespan across diverse organisms ranging from flies to mammals (Li et al., 2014). Hence, elucidating the molecular mechanisms of rapamycin would be of great interests to both basic and clinical researchers.

The first rapamycin target protein was discovered in yeast, which was subsequently named as target of rapamycin (TOR) (Heitman et al., 1991). TOR, now renamed as mechanistic target of rapamycin or mTOR, is a Ser/Thr protein kinase highly conserved in eukaryotes (Hall, 2008; Kennedy and Lamming, 2016). Although multiple cellular locations have been reported, the current consensus is that mTOR is localized predominantly on the membranes of lysosomes (Sancak et al., 2010). In response to changes in the environmental cues such as nutrient availability, the kinase activity of mTOR is switched on or off by forming two different complexes: mTORC1 and mTORC2 (Laplante and Sabatini, 2012). Known mTOR substrates include, but are not limited to, autophagy-initiating ATG1 kinase (ULK-1; ATG1 in yeast), S6 kinase beta-1 (S6K1), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), and transcriptional factor EB (TFEB) (Laplante and Sabatini, 2012b). By binding to mTOR via a FKBP-rapamycin binding domain (FRB) to form a ternary FKBP12-rapamycin-mTOR complex, rapamycin acts a high-affinity (nM ranges) allosteric inhibitor blocking substrate recruitment (Choi et al., 1996; Ruan et al., 2008; Yang et al., 2013). mTOR integrates a number of signaling pathways in the cell, and has emerged as the major regulator of cellular proliferation and growth (van Dam et al., 2011). Both the anti-cancer and immunosuppressive effects of rapamycin are likely due to the inhibition of cell

proliferation (Pollizzi and Powell, 2015). However, mTOR inhibition also induces autophagy, a lysosome-dependent cellular survival mechanism that provides recycled nutrients in need by degrading obsolete cellular components (Laplante and Sabatini, 2012a). Defective autophagy may underlie numerous diseases including cancer, neurodegeneration, and aging (Thomson et al., 2009). Hence, autophagy induction caused by mTOR inhibition may also explain many of the reported effects of rapamycin, especially neuroprotection and anti-aging (Bove et al., 2011; Thomson et al., 2009). The basic autophagic processes consist of autophagosome formation, autophagosome-lysosome fusion, and lysosomal degradation (Kaur and Debnath, 2015). Nutrient starvation is a potent inducer of autophagy (Takeshige et al., 1992), in which the loss of nutrients, e.g., amino acids, causes mTOR inhibition. Subsequently, de-phosphorylation of ULK-1 (ATG-1), a major mTOR target, primes phagophore initiation (Chan et al., 2007; Chan et al., 2009; Noda and Ohsumi, 1998). Not surprisingly, rapamycin is sufficient to mimic the effect of starvation on ULK1-mediated autophagy induction (Wu et al., 2013). Although all rapalogs potently inhibit mTOR, their clinical efficacies vary significantly (Benjamin et al., 2011; Rogers-Broadway et al., 2016). In addition, rapalogs with much reduced binding affinities to mTOR, e.g., WYE-592 and ILS-920, still exhibit comparable or even more potent neuroprotective effects (Ruan et al., 2008). Furthermore, although mTOR is much more potently inhibited by its catalytic inhibitors, for instance, Torin-1 (Thoreen et al., 2009), an in vivo protective effect of Torin1 has been difficult to prove (Malagelada et al., 2010). Hence, it is likely that rapamycin may have other targets in the autophagy pathway in addition to mTOR.

Sustained autophagy also requires lysosome activation, reformation, and biogenesis (Eskelinen and Saftig, 2009; Gordon et al., 1992; Lawrence and Brown, 1992; Liou et al., 1997; Punnonen et al., 1993; Settembre et al., 2011; Zhou et al., 2013). In conditions when lysosome function is compromised, e.g., in neurodegenerative diseases and lysosome storage diseases (LSDs), it is unlikely that an increase in autophagosome formation alone could exert the beneficial effects by promoting cellular clearance (Levine and Kroemer, 2008). Nutrient starvation, a physiological inducer of autophagy, promotes both autophagosome formation and lysosome biogenesis. Upon starvation-induced mTOR inhibition, transcription factor EB (TFEB), an important regulator of autophagy and lysosome biogenesis (Settembre et al., 2013), is rapidly dephosphorylated and translocated from the cytosol to the nucleus (i.e., activation of TFEB) (Martina et al., 2012; Pena-Llopis et al., 2011; Roczniak-Ferguson et al., 2012; Settembre et al.,

2012). Starvation may also activate lysosomal Ca^{2+} channel Mucolipin 1 (MCOLN1 or TRPML1), which is required for TFEB activation via calcineurin, a Ca^{2+}-dependent phosphatase (Medina et al., 2015b; Zhang et al., 2016). TRPML1 expression is in turn up-regulated by TFEB activation (Wang et al., 2015). Therefore, TRPML1 and TFEB constitute a positive-feedback loop to boost lysosomal biogenesis and autophagy under lysosome stress conditions. Indeed, up-modulation of either TFEB or TRPML1 reportedly show beneficial effects on several neurodegenerative diseases and LSDs, including Alzheimer's disease, Pompe disease, and Niemann-Pick C disease (Cao et al., 2015; Martini-Stoica et al., 2016; Pastore et al., 2013a; Pastore et al., 2013b; Shen et al., 2012; Zou et al., 2015).

In the present study, we made an unexpected observation that the TRPML1-TFEB-lysosome biogenesis arm of autophagy is also activated by rapamycin, but not Torin-1. Employing whole-endolysosome electrophysiology, we demonstrated that rapamycin specifically activates TRPML1 independent of mTOR.

IV-3 Results

3.1 Activation of lysosomal TRPML1 channel by Rapamycin.

Given TRPML1's proposed roles in lysosomal membrane trafficking and cellular clearance (Weiss, 2012), we used Ca^{2+} imaging and electrophysiological assays to screen a list of natural products, which are known to affect lysosome function, in a search for potential TRPML1 modulators. Unexpectedly, we found that rapamycin activated whole-endolysosomal TRPML1 current (I_{TRPML1}) in mechanically-isolated vacuoles isolated from EGFP-TRPML1-transfected COS-1 cells (**Fig. 4.2 a, b**). The activation had an IC50 of 12.8 ± 1.0 μM (n=4 patches; **Fig. 4.2 c**), which was less potent than the endogenous agonist $PI(3,5)P_2$, but comparable to the synthetic TRPML1 agonist ML-SA1 (Shen et al., 2012). Like the currents evoked by the known agonists, rapamycin-evoked I_{TRPML1} was also inhibited by synthetic inhibitors of TRPML1 (e.g., ML-SI3; **Fig. 4.2 b**). In contrast, whole-endolysosome recording of other lysosomal channels including I_{TRPML3} and I_{TPC2} were not activated by rapamycin at 20 μM (**Fig. 4.2 d, f, g**) or even higher concentration (50 μM; data not shown). A mild but significant activation was observed in TRPML2-expressing cells (**Fig. 4.2 e, g**). Together, these results suggest that rapamycin specifically and robustly activates TRPML1.

3.2 TRPML1 activation by rapamycin and rapalogs is independent of mTOR.

Lysosome-localized mTOR is the most well-established rapamycin target (Benjamin et al., 2011). mTOR inhibition has been reported to modulate the activities of lysosomal TPC Na^+ channels (Cang et al., 2013) and TRPML1 (Onyenwoke et al., 2015). To test the role of mTOR in rapamycin-induced activation of TRPML1, we examined whether Torin1, a catalytic mTOR inhibitor that is structurally different from rapamycin (Thoreen et al., 2009), could activate I_{TRPML1}. No noticeable activation was seen with a range of concentrations of Torin 1 (up to 2 μM; **Fig. 4.3 a, d**), which completely abolished mTOR activity in biochemical assays with S6 kinase (S6K) phosphorylation as a readout (**Fig. 4.3 e**). Taken together, these results suggest that mTOR inhibition alone is insufficient to activate TRPML1.

We next investigated the effects of several commercially-available rapalogs (**Fig. 4.1**), which all effectively inhibited mTOR (**Fig. 4.3 e**). However, these rapalogs differed drastically in term of TRPML1 activation. Whereas temsirolimus (Tem) and everolimus (Eve) readily activated I_{TRPML1} (**Fig. 4.3 b, d**), no obvious activation effect was seen with deforolimus (Defo) or zotarolimus (Zota) at the same concentration (**Fig. 4.3 c, d**) and higher concentrations (50 μM; data not shown). Furthermore, seco-rapamycin, an open-ring metabolite of rapamycin, failed to activate I_{TRPML1} at the same concentration (**Fig. 4.3 c, d**) and higher concentrations (50 μM; data not shown). Together, these results suggest that rapamycin and rapalogs activate TRPML1 independent of mTOR inhibition.

3.3 The kinase activity of mTOR is not required for rapamycin activation of TRPML1.

To further rule out the involvement of mTOR in rapamycin activation, we also adopted a genetic approach. Over-expressing of the kinase-dead D2357E mutation has been reported to abolish the catalytic activity of mTOR (Tabatabaian et al., 2010). Consistent with previous studies (Cang et al., 2013; Jha et al., 2014), we found that, in COS-1 cells transfected with $mTOR^{D2357E}$, ATP inhibition of TPC2 was much reduced compared with cells transfected with $mTOR^{WT}$ (**Fig. 4.4 a, b**). This positive control experiment suggested that mTOR was associated with enlarged vacuoles in our whole-endolysosome recordings. However, in COS-1 cells that were doubly transfected with kinase-dead $mTOR^{D2357E}$ and TRPML1, I_{TRPML1} exhibited no measurable basal activity, and was still robustly activated by rapamycin (**Fig. 4.4 c, d**).

3.4 Rapamycin and rapalogs induce TFEB nuclear translocation in a TRPML1-dependent manner.

We have recently shown that activation of TRPML1 by ML-SAs and ROS is sufficient to activate (via nuclear translocation) TFEB and enhance autophagy in a Ca^{2+}-dependent but mTOR-independent manner (Zhang et al., 2016). Unlike Torin-1, low concentrations of rapamycin failed to induce TFEB nuclear translocation in Hela cells stably expressing TFEB-GFP (TFEB stable cells; **Fig. 4.5 a, b**), suggesting that moderate inhibition of mTOR and activation of TRPML1 was not sufficient to induce TFEB nuclear translocation. However, in TFEB stable cells that were transfected with mCherry-TRPML1, rapamycin (5 μM) readily induced rapid and dramatic TFEB nuclear translocation (**Fig. 4.5 a, b**). Consistent with our electrophysiology findings, TRPML1-activating rapalogs, such as Tem and everolimus, also induced TFEB nuclear translocation at 5 μM (**Fig. 4.5 a, b**). In contrast, rapalogs that were incapable of activating TRPML1, for instance, deforolimus or zotarolimus, was incapable of inducing TFEB nuclear translocation at 10 μM (**Fig. 4.5 a, b**) and higher concentration (50 μM; data not shown). Likewise, endogenous TFEB, detected with an anti-TFEB antibody, was also activated by rapamycin or Tem, but not zotarolimus in TRPML1-overexpressing HeLa cells (**Fig. 4.5 c**). Rapalog-induced TFEB activation was abolished by co-application of ML-SI3 (**Fig. 4.6 a, b**). Consistent with the role of TRPML1 in rapamycin-induced TFEB activation, in cells that were transfected with $TRPML1^{DDKK}$ (a channel-dead pore mutant), rapamycin failed to induce TFEB nuclear translocation (**Fig. 4.7 a, c**), while in active $TRPML1^{Va}$-transfected cells TFEB accumulated in the nucleus with or without rapamycin (**Fig. 4.7 b, c**). Hence, rapamycin induced robust TFEB activation, but only when the expression level of TRPML1 was high.

The results presented above suggest that rapamycin activates TRPML1 to induce lysosomal Ca^{2+} release. We tested this possibility using Tem, which appears to have a membrane-permeability higher than rapamycin in our experiments by an unclear mechanism. Tem application readily increased cytosolic Ca^{2+} levels in HEK293 cells stably expressing GCaMP7-TRPML1, and the increase was blocked by ML-SI3 (**Fig. 4.8 a**). The membrane-permeable Ca^{2+} chelator, BAPTA-AM, also prevented Tem-induced TFEB nuclear translocation (**Fig. 4.8 b.c**). Tem also evoked TFEB nuclear translocation in cells that were transfected with TRPML2. But tem had no

effect on cells transfected with TRPML3 (**Fig. 4.9 a, b**). Collectively, these results suggest that rapamycin and rapalogs activate TFEB via a TRPML1- and Ca^{2+}-dependent mechanism.

IV-4 Discussion

mTOR, the key regulator of cell growth and metabolism, is a well studied protein target of rapamycin (Li et al., 2014). Not surprisingly, the broad utilities of rapamycin/rapalogs have been presumed to be mediated by mTOR inhibition. In the current study, we present a challenge to this assumption by demonstrating lysosome Ca^{2+}-permeable channels, TRPML1 and TRPML2, as unexpected targets of rapamycin/rapalogs. Rapamycin activates TRPML1 via a direct binding independent of its actions on mTOR. The resulting increases in per-lysosomal Ca^{2+} levels then promote TFEB translocation from the cytosol to the nucleus (**Fig. 4.10**). Activated TFEB then induces the expression of a unique set of genes involved in autophagosome formation and lysosome biogenesis (Napolitano and Ballabio, 2016), enhancing autophagy and cellular clearance (Bae et al., 2014; Ballabio, 2016; Shen et al., 2012; Zou et al., 2015). Rapamycin is a well-known potent inducer of autophagy, and the induction has thus far been attributed to be mTOR-dependent. Our study has provided an mTOR-independent mechanism that links rapamycin to autophagy.

Recent studies suggest that there exist crosstalk mechanisms between autophagy, mTOR, TFEB, and lysosomal Ca^{2+} (Li et al., 2016; Medina et al., 2015). Lysosomal Ca^{2+} release is proposed to be crucial for mTOR activation upon stimulation by amino acids or growth factor signaling (Li et al., 2016). It is possible that TRPML1-mediated lysosomal Ca^{2+} release may help relieve the inhibitory effect of rapamycin on mTOR. To dissect out the contribution of TRPML1 to the in vivo efficacies of rapamycin, it might be necessary to perform the studies in TRPML1 knockout and overexpression transgenic mice. Meanwhile, it might prove helpful to systematically compare the efficacy and potency of rapalogs on TRPML1 activation and compare the in vivo efficacies of rapalogs with differential effect on TRPML1. It is worth noting that the hydroxyl group(s) at C40, found in rapamycin, temsirolimus, and everolimus, is not present in TRPML1-nonactivating rapalogs. Although the activation mechanism of TRPML1 by rapamycin/ rapalogs is not known, resolving structures of TRPML1 at the atomic resolution might prove helpful. mTOR inhibition has lots of adverse effects clinically (Pallet et al., 2013) and TFEB activation is a promising tool for cellular clearance (Sardiello et al., 2016). Understanding the activation

mechanism of TRPML1 by rapamycin may help us to develop rapalogues with better potency and efficacy to activate TRPML1 but less potency and efficacy to inhibit mTOR. These rapalogues may be better compounds to induce cellular clearance and better treatments for lysosomal storage diseases and neurodegenerative diseases.

IV-5 Materials and Methods

Molecular biology. The WT mTOR construct (Plasmid #26603) was purchased from Addgene. Additional mTOR and TRPML1 mutants were generated with a quick-change lightning site-directed mutagenesis kit (Qiagen). All constructs were confirmed by DNA sequencing.

Mammalian cell culture. The TFEB-GFP stable cell line was kindly provided by Shawn M. Ferguson (Roczniak-Ferguson et al., 2012). Unless otherwise indicated, all cell lines were maintained in DMEM medium supplemented with 10% Tet-free FBS at 37 °C in a humidified 5% CO2 incubator.

Confocal imaging. For TFEB immunofluorescence detection, cells were grown on glass coverslips and then fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 after treatments. The cells were then blocked with 1% bovine serum albumin in phosphate buffered saline (PBS). Endogenous TFEB was recognized by incubating cells with anti-TFEB (1:200; Cell Signaling Technology) at 4 °C overnight. Cells were then washed 4–5 times with PBS and incubated with anti-rabbit secondary antibodies conjugated to Alexa Fluor 568 or 488 (Invitrogen) for 1 h. After three washes with PBS, coverslips were mounted on the slides with Fluoromount-G (Southern Biotech). Images were acquired with an Olympus Spinning-Disk Confocal microscope.

Western blotting. Cells were lysed with ice-cold RIPA buffer (Boston BioProducts) in the presence of 1× protease inhibitor cocktail (Sigma), 1 mM NaF, and 1 mM Na_3VO_4. Total cell samples (10–100 μg) were then loaded and separated on 4–12% gradient SDS-PAGE gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h with 1% bovine serum albumin in PBS supplemented with 0.1% Tween20 and were incubated with antibodies against γ-tubulin (1:4,000; Sigma), S6K (1:1,000; Cell Signaling Technology), pS6K (1:1,000; Cell Signaling). Bound antibodies were detected using horseradish

peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Ca^{2+} imaging. GCaMP7 imaging was performed in HEK cells stably expressing GCaMP7-TRPML1, a lysosome-targeted genetically-encoded Ca^{2+} sensor (Shen et al., 2012). The fluorescence intensity at 488 nm (F488) was recorded with an EasyRatioPro system (PTI).

Whole-endolysosome electrophysiology. Isolated endolysosomes were subjected to whole-endolysosomal electrophysiology by a modified patch-clamp method (Dong et al., 2010; Wang et al., 2012). Briefly, cells were treated with 1 μM vacuolin-1 overnight to selectively increase the size of late endosomes and lysosomes (Cerny et al., 2004). Enlarged vacuoles were released into the dish by mechanical disruption of the cell membrane with a fine-tip glass electrode. Unless otherwise indicated, vacuoles were bathed continuously in an internal (cytoplasmic) solution containing 140 mM K^+-Gluconate, 4 mM NaCl, 1 mM EGTA, 2 mM Na_2-ATP, 2 mM $MgCl_2$, 0.39 mM $CaCl_2$, 0.1 mM GTP, and 10 mM HEPES (pH adjusted with KOH to 7.2; free $[Ca^{2+}]_i \approx$ 100 nM). The pipette (luminal) solution contained 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM MES and 10 mM glucose (pH adjusted to 4.6 with NaOH). The whole-endolysosome conFig.uration was achieved as described previously (Wang et al., 2012). After formation of a gigaseal between the patch pipette and an enlarged endolysosome, voltage steps of several hundred millivolts with a millisecond duration were applied to break into the vacuolar membrane (Wang et al., 2012). All bath solutions were applied via a fast perfusion system that produced a complete solution exchange within a few seconds. Data were collected via an Axopatch 2A patch clamp amplifier, Digidata 1440, and processed with pClamp 10.0 software (Axon Instruments). All experiments were conducted at room temperature (21–23 °C) and all recordings were analyzed in pCLAMP10 (Axon Instruments) and Origin 8.0 (OriginLab).

Reagents. The following reagents were purchased: ML-SA1 (Princeton BioMolecular Research Inc), ML-SI3 (AKOS), Torin 1 (Tocris), BAPTA-AM (Invitrogen), vacuolin-1 (Calbiochem), rapamycin, temsirolimus, everolimus are from LC Laboratories. deforolimus (MK-86669) and zotarolimus (ABT-578) from selleckchem, seco rapamycin (148554-65-8) from Caymanchem.

Data analysis. Data are presented as mean ± standard errors of the mean (SEM).

IV-6 Figures

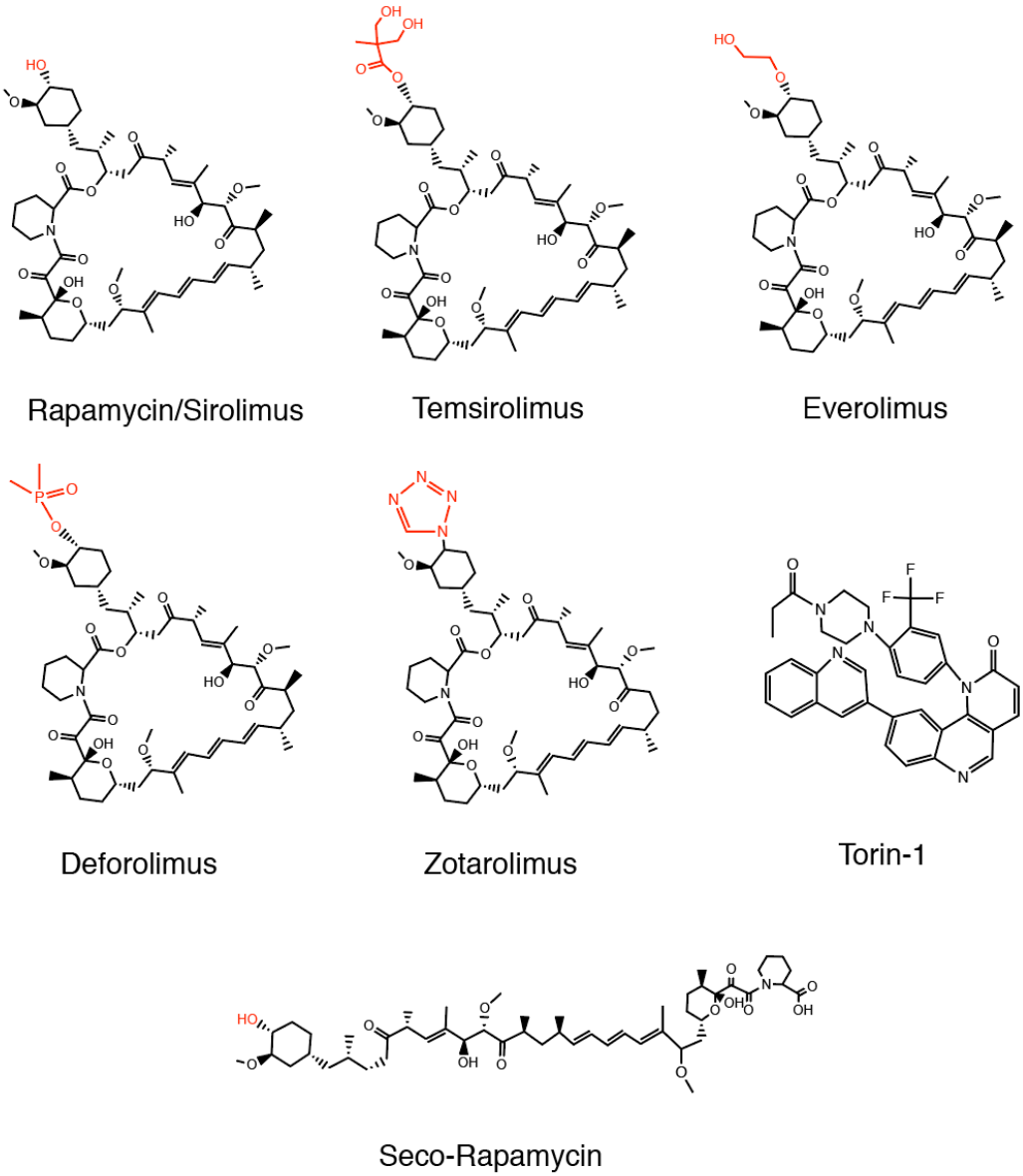

Figure 4.1 Structures of Torin-1, rapamycin, and rapalogs.

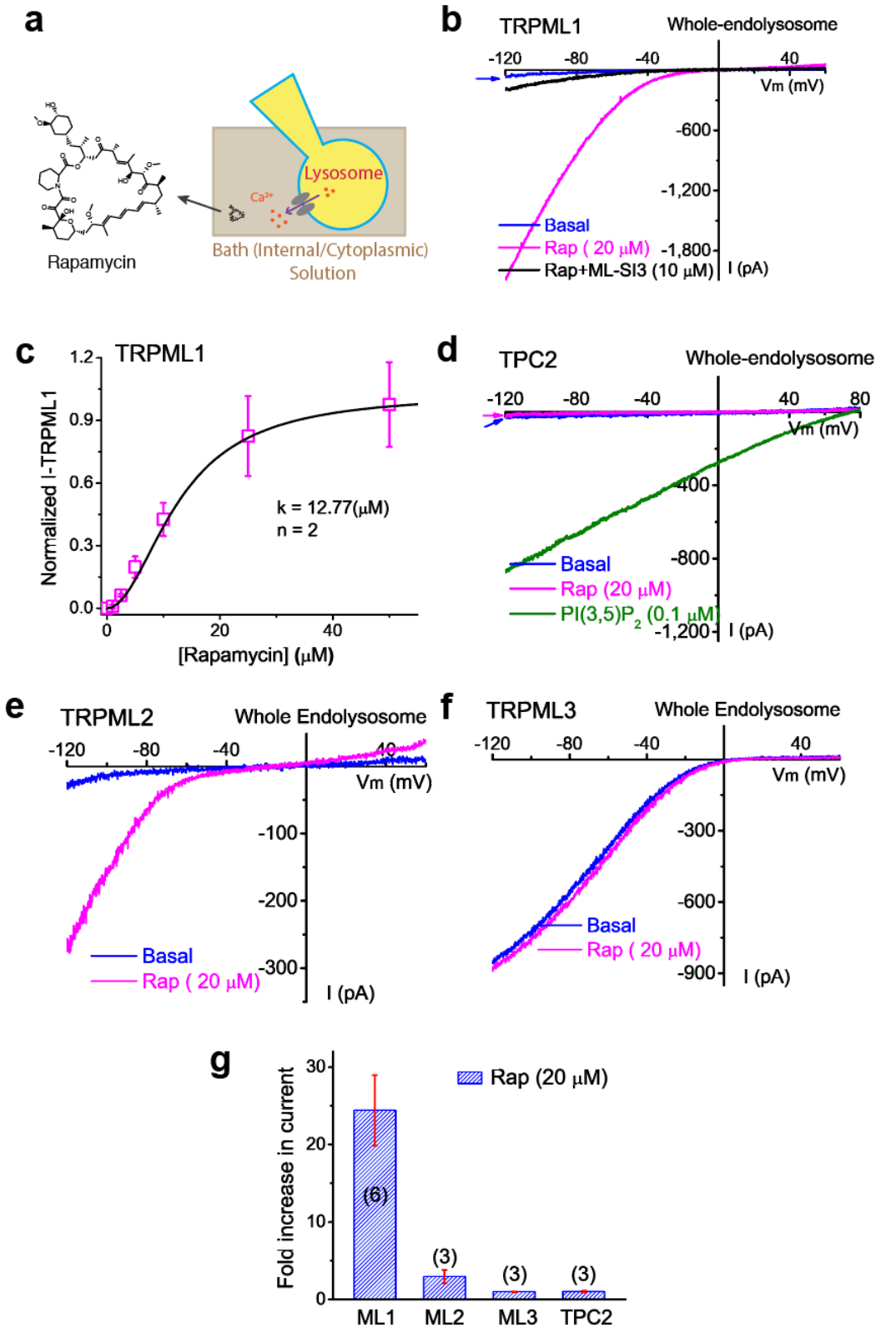
a
Rapamycin
Lysosome
Ca2+
Bath (Internal/Cytoplasmic) Solution
b
TRPML1
Whole-endolysosome
-120
-80
-40
40
Vm (mV)
-600
-1,200
-1,800
Basal
Rap (20 μM)
Rap+ML-SI3 (10 μM)
I (pA)
c
TRPML1
Normalized I-TRPML1
1.2
0.9
0.6
0.3
0.0
0
20
40
[Rapamycin] (μM)
k = 12.77(μM)
n = 2
d
TPC2
Whole-endolysosome
-120
-80
-40
40
80
Vm (mV)
400
-800
-1,200
Basal
Rap (20 μM)
PI(3,5)P2 (0.1 μM)
I (pA)
e
TRPML2
Whole Endolysosome
-120
-80
-40
Vm (mV)
-100
-200
-300
Basal
Rap (20 μM)
I (pA)
f
TRPML3
Whole Endolysosome
-120
-80
-40
40
Vm (mV)
-300
-600
-900
Basal
Rap (20 μM)
I (pA)
g
Rap (20 μM)
Fold increase in current
30
20
10
0
(6)
(3)
(3)
(3)
ML1
ML2
ML3
TPC2

Figure 4.2 Direct activation of lysosomal TRPML1 and TRPML2 channels by rapamycin.

(a) Illustration of a whole-endolysosome recording configuration. Pipette (luminal) solution was a standard Tyrode's solution with pH adjusted to 4.6 to mimic the acidic environment of lysosome lumen. Bath (internal) solution was a K^+-based solution (140mM K^+-gluconate). Inward currents indicate that cations were flowing out the lysosome. **(b)** Representative traces of basal (blue), rapamycin-activated I_{TRPML1}(magenta) by 20μM Rapamycin and I_{TRPML1} by rapamycin was specifically blocked by co-application of ML-SI3, a TRPML1 antagonist (black). **(c)** Dose-dependence of rapamycin activation of TRPML1 at -120mV. **(d)** Rapamycin failed to activate whole-endolysosome I_{TPC2} in EGFP-TPC2-transfected COS1 cells. **(e)** Whole-endolysosome I_{TRPML2} was similarly activated by rapamycin in mCherry-TRPML2-transfected COS1 cells. **(f)** Rapamycin had no effect on I_{TRPML3}. **(g)** Summary of rapamycin effects on TRPML1-3 and TPC2 channels at -120mV.

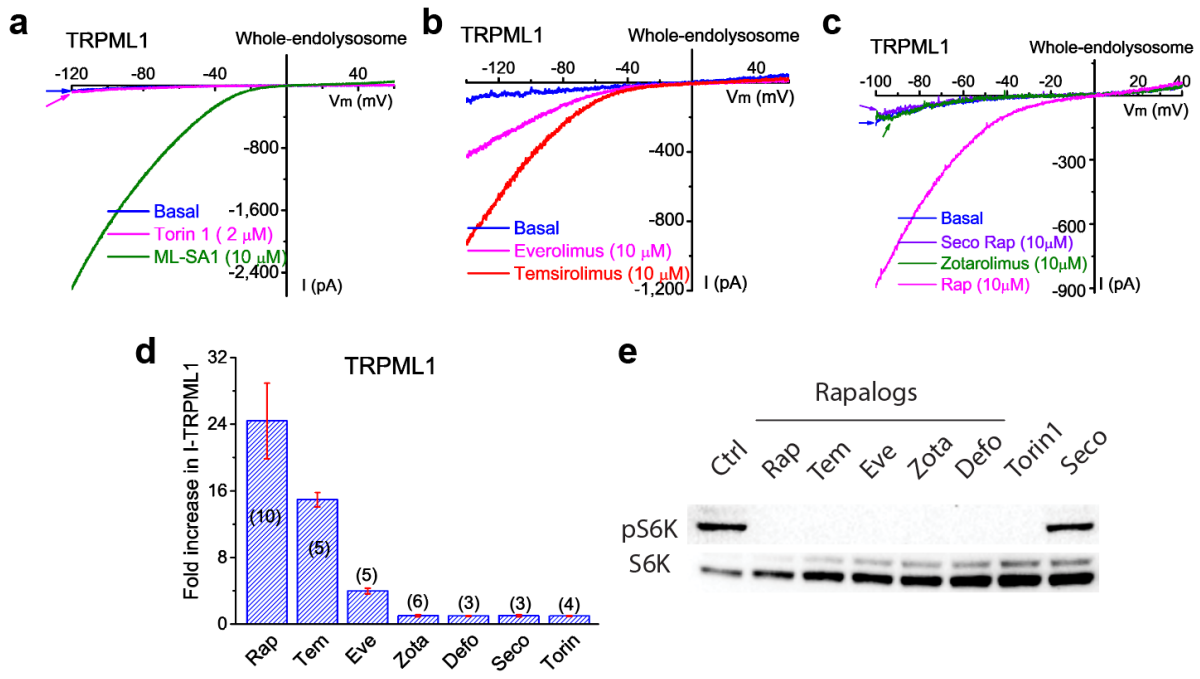

Figure 4.3 TRPML1 is differentially activated by rapalogs.

(a) The effect of Torin1, a potent ATP-competitive mTOR inhibitor, on whole-endolysosome I_{TRPML1}. **(b)** The effects of temsirolimus (Tem, 10 μM) and everolimus (Eve, 10 μM) on I_{TRPML1}. **(c)** The effects of deforolimus (Defo,10 μM), zotarolimus (Zota, 10 μM), and Seco rapamycin (a rapamycin metabolite,10 μM) on I_{TRPML1}. **(d)** Differential effects of rapalogs on I_{TRPML1} at -120mV. Data are presented as mean ± SEM. **(e)** Rapamycin and rapalogs inhibited mTOR activity, which was assayed by the phosphorylation of S6-K at Thr 389.

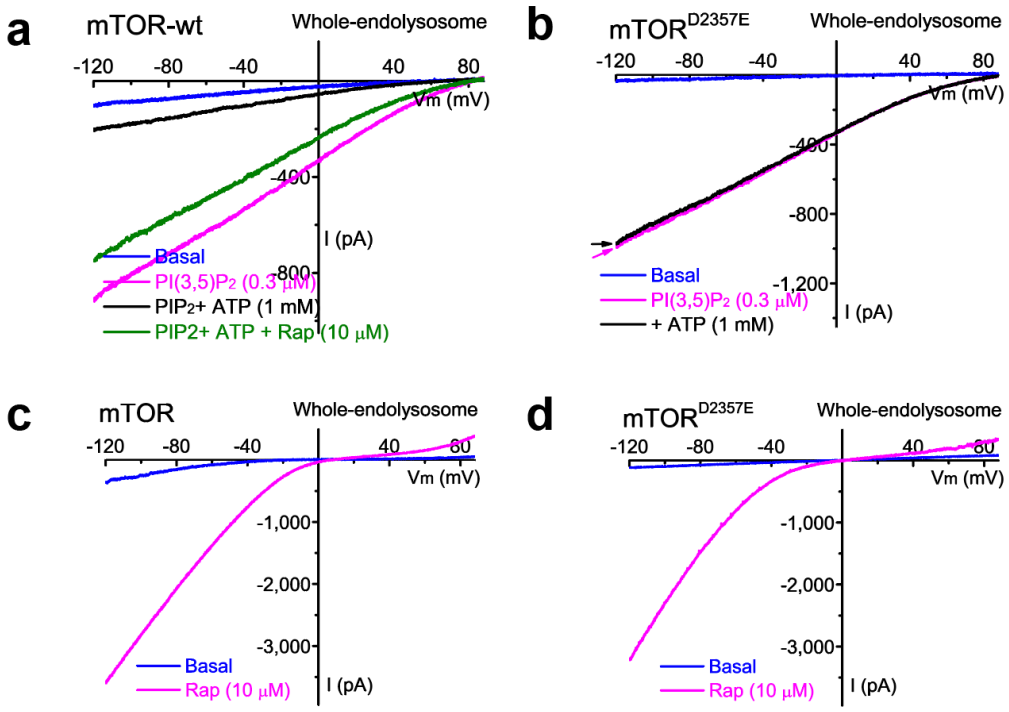

Figure 4.4 The kinase activity of mTOR is not required for rapamycin activation of TRPML1.

(a) ATP inhibition on I_{TPC2} in cells transfected with WT-mTOR **(b)** ATP failed to inhibit I_{TPC2} in cells transfected with kinase-dead mTOR^{D2357E} **(c)** Rapamycin consistently activated I_{TRPML1} in cells overexpressing mTOR. **(d)** The effect of rapamycin on I_{TRPML1} in cells transfected with a kinase-dead mTOR^{D2357E} mutant.

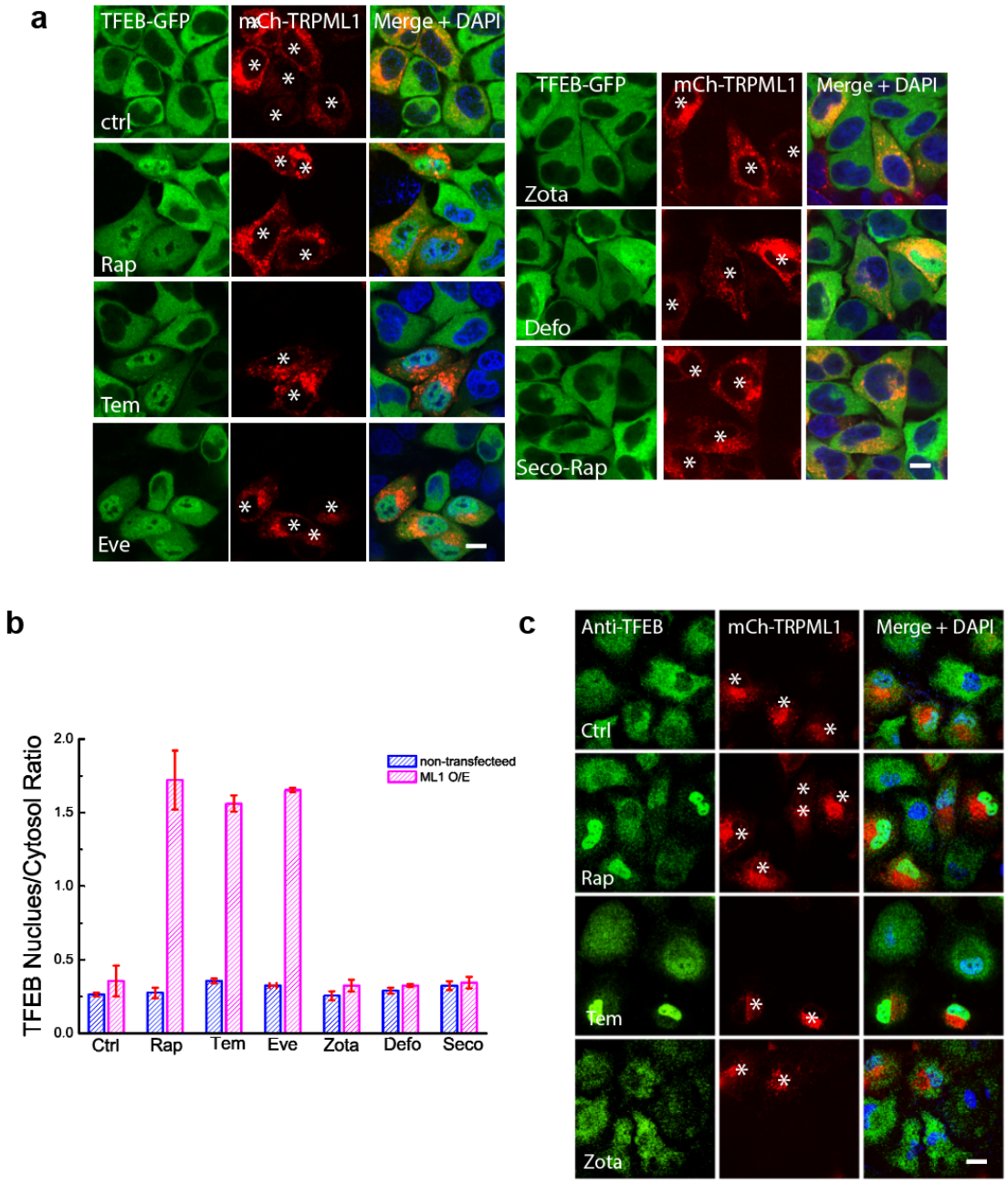

Figure 4.5 Rapamycin and rapalogs promote TFEB nuclear translocation.

(a) The effects of rapamycin/rapalogs on TFEB-GFP translocation in TFEB-GFP stable cells transfected with mCherry-TRPML1. Rapamycin (Rap; 5 μM), temsirolimus (Tem; 5 μM, below same, throughout figure legends), and everolimus (Eve; 5 μM) induced TFEB nuclear translocation (indicated by the asterisk). In contrast, zotarolimus (Zota; 10 μM), deforolimus (Defo; 10 μM), and seco-rapamycin (Seco; 10μM) did not activate TFEB. **(b)** Summary of rapamycin/rapalogs effects on TFEB activation. **(c)** Nuclear translocation of endogenous TFEB in response to rapamycin/rapalog stimulation. Means are shown with SEM. Scale bar = 10 μm

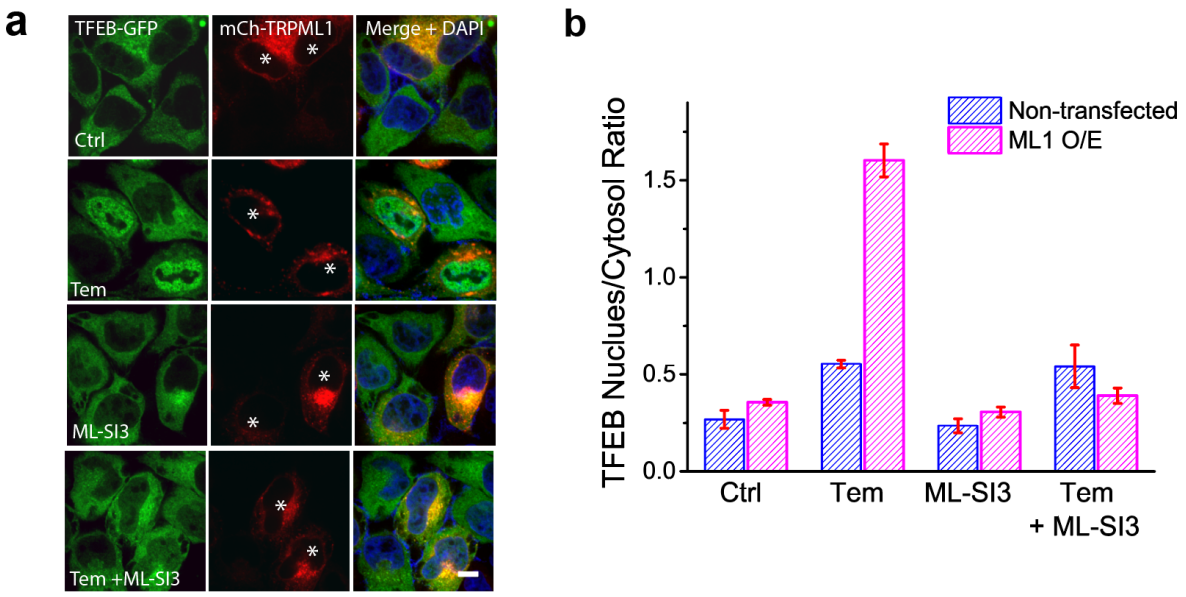

Figure 4.6 Temsirolimus promotes TFEB nuclear translocation in a TRPML1-dependent manner.

(a) Tem-induced TFEB translocation was blocked by ML-SI3 (10 μM) **(b)** Quantification of ML-SI3's effect on tem-induced TFEB activation. Means are shown with SEM. Scale bar = 10 μm

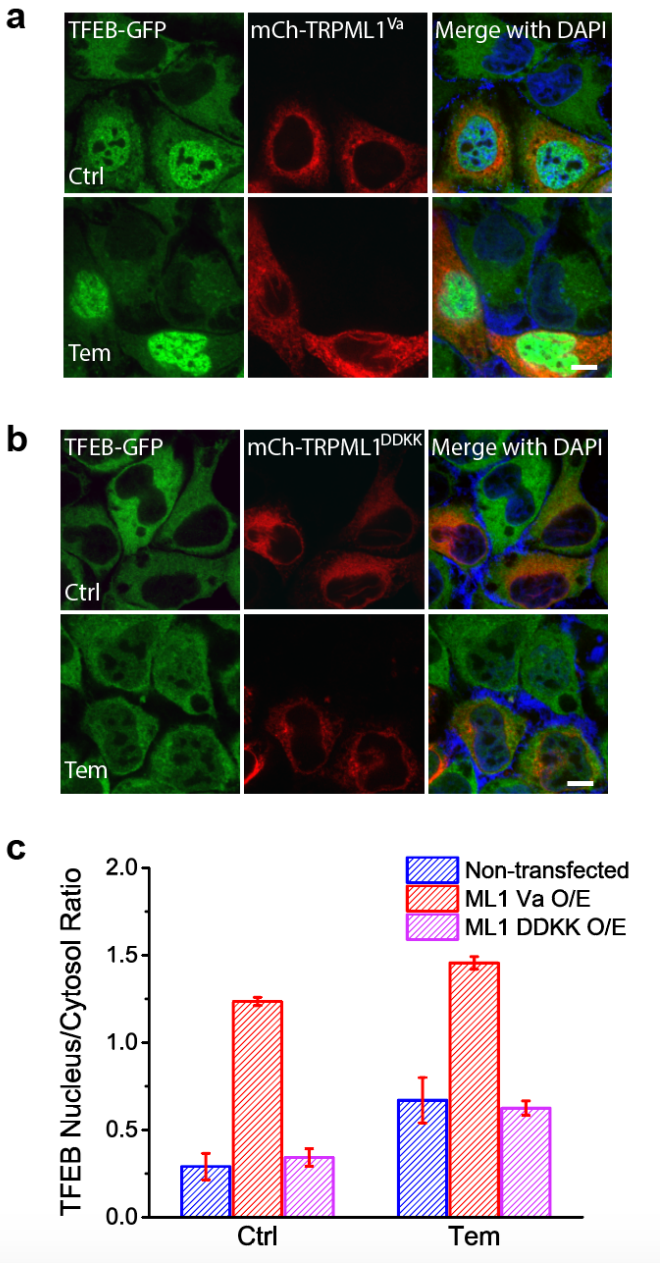

Figure 4.7 The effects of gain-of-function and loss-of-function mutations of TRPML1 on TFEB nuclear translocation.

(a) Overexpression of constitutively-active TRPML1^{Va} mutant resulted in nuclear accumulation of TFEB without Tem application. **(b)** Tem effect on TFEB nuclear translocation in cells transfected with channel-dead pore mutation TRPML1^{DDKK}. **(c)** Quantification on a and b. Means are shown with SEM. Scale bar = 10 μm

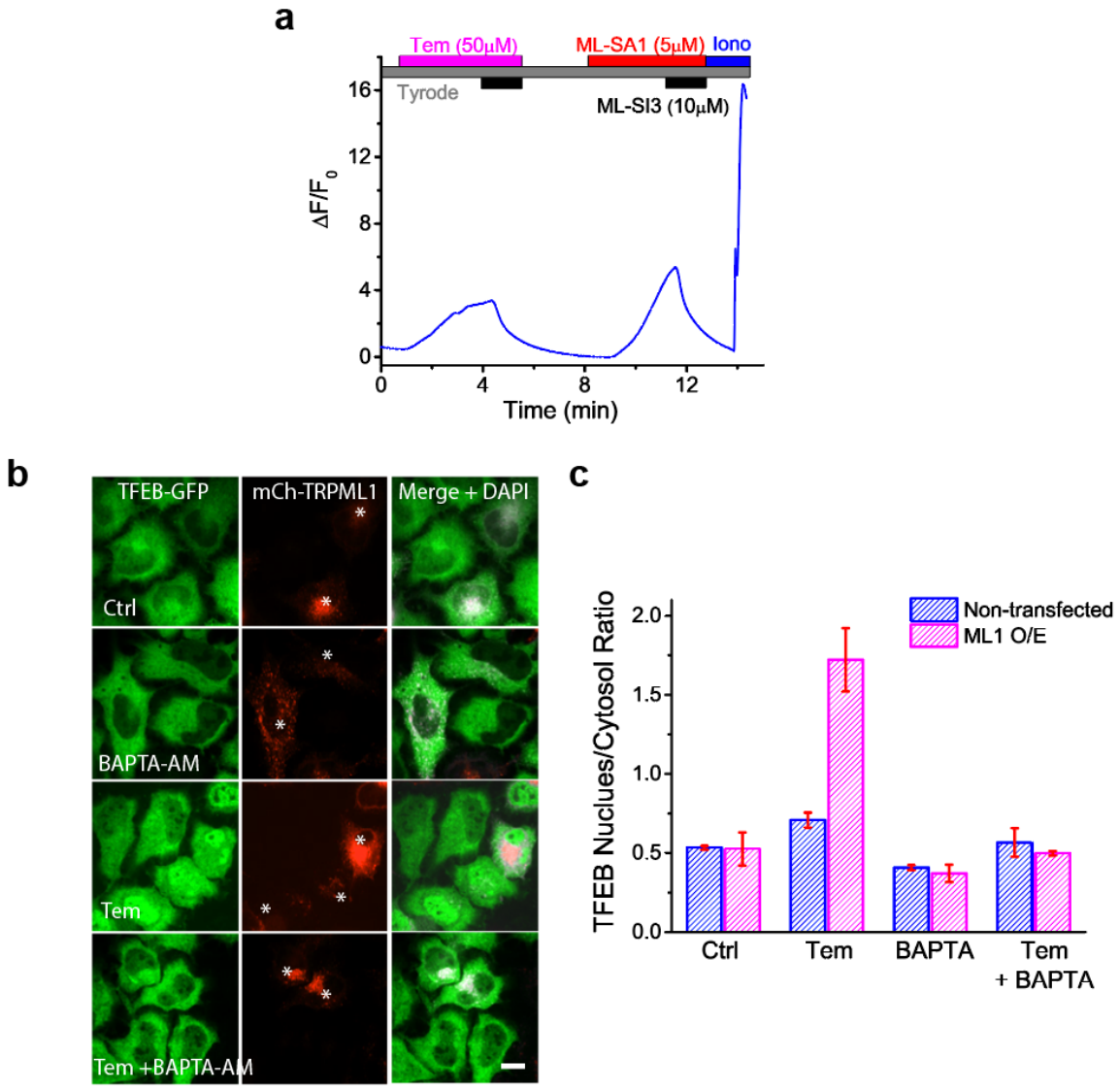

Figure 4.8 Temsirolimus induces TRPML1-mediated Ca^{2+} release, promoting TFEB nuclear translocation.

(a) In the cells stably expressing GCaMP7-ML1, Tem (50 μM) increased fluorescence intensity (ΔF; F470) that was blocked by ML-SI3 (10 μM). **(b)** BAPTA-AM (10 μM) treatment for 60 min blocked Tem(5 μM)-induced TFEB translocation. **(c)** Quantification on b. Means are shown with SEM. Scale bar = 10 μm

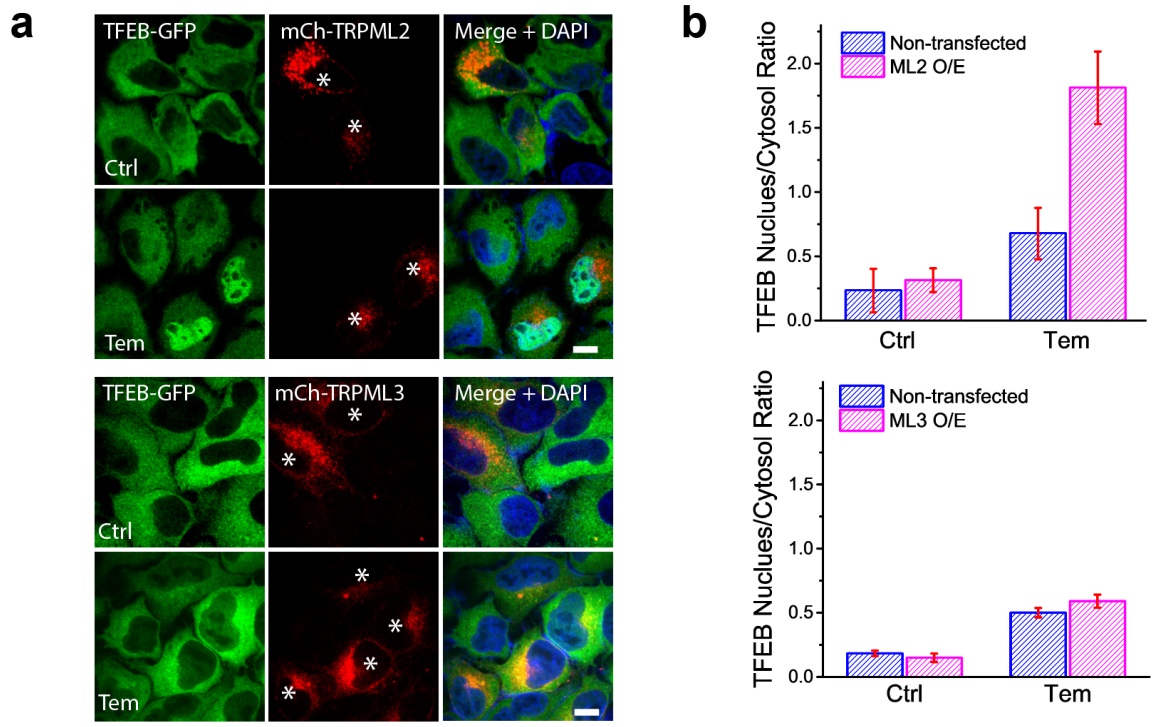

Figure 4.9 Temsirolimus induces TFEB translocation in TRPML2-expressing cells.

(a) Tem (5 μM) induced TFEB-GFP nuclear translocation in Hela cells transfected with mTRPML2 but not mTRPML3. **(b)** Statistics on a. Means are shown with SEM. Scale bar = 10 μm

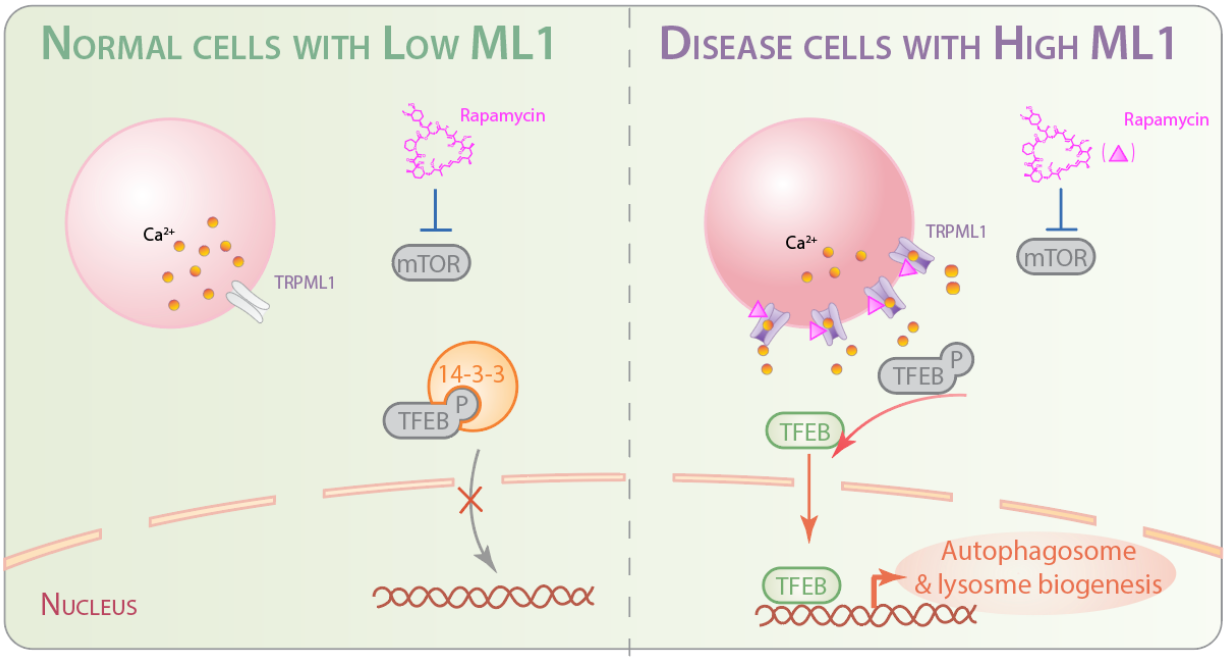

Figure 4.10 A working model to illustrate the effect of rapamycin activation on TRPML1/Ca^{2+}/TFEB pathway.

The effects of rapamycin are sensitive to the expression levels of TRPML1. Rapamycin directly binds and activates TRPML1 channels, inducing lysosomal Ca^{2+} release. Increases in peri-lysosomal Ca^{2+} levels cause TFEB translocation from the cytosol to the nucleus. Activated TFEB promotes the expression of autophagic and lysosomal genes, enhancing autophagic-lysosomal degradation pathway and cellular clearance.

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CHAPTER V

Conclusions, Discussions, and Future Directions

V-1 Conclusions

In my thesis work, I characterized new mechanisms of lysosomal adaptation to environmental changes. In chapter II, I demonstrated that upon acute starvation, TRPML1 mediates lysosomal Ca^{2+} release, which triggers TFEB nuclear translocation and activation. Activated TFEB, in turn, up-regulates TRPML1 expression level, which is essential for lysosomal adaptation including increased cellular clearance and enhanced lysosomal proteolytic function. In chapter III, I established that inhibition of the lysosomal lipid kinase PIKfyve activates TFEB in a similar Ca^{2+}-dependent manner and up-regulate TRPML1. Given that PIKfyve is acutely inhibited upon starvation (Li et al., 2013; Zolov et al., 2012), PIKfyve may function as a physiological regulator of TFEB with lysosomal Ca^{2+} to trigger TFEB nuclear translocation upon starvation.

Interestingly, Vac14, a scaffold protein for PIKfyve, was revealed as a downstream target of TFEB (Sardiello et al., 2009) and may be upregulated upon TFEB activation, leading to reactivation of PIKfyve and resynthesis of $PI(3,5)P_2$. $PI(3,5)P_2$ was shown to be required for autophagic-lysosomal reformation (ALR), which happens during prolonged starvation for recycling of the membrane components of lysosomes from autolysosomes, by activating TRPML1 (Li et al., 2016). Thus, we speculate that lysosomal Ca^{2+}/TRPML1 and PIKfyve function at two distinct steps in response to nutrient deprivation. Within minutes of nutrient withdrawal, lysosomal Ca^{2+} is released partially via TRPML1 and PIKfyve is inhibited, triggering a global transcriptional response via TFEB and inducing upregulation of ML1 and possibly Vac14. Upregulated Vac14 may trigger the re-synthesis of $PI(3,5)P_2$. After a few hours of nutrient deprivation, newly synthesized $PI(3,5)P_2$ will activate newly expressed TRPML1. We hypothesize that this coordinates lysosomal adaptation including enhanced lysosomal proteolytic function and autophagic-lysosomal reformation (**Fig. 5.1**).

In chapter IV, I made a quite unexpected finding that rapamycin, a specific mTOR inhibitor, activates TRPML1 in a mTOR-independent manner and triggers Ca^{2+}-dependent TFEB activation. Thus, rapamycin may promote autophagy via both mTOR-dependent and mTOR–independent mechanisms. This study provides us with a new direction for dissecting rapamycin's *in vivo* clinical effects. Given the demonstrated effects of TRPML1 and TFEB in cellular clearance, rapamycin may mediate the *in vivo* effects via TRPML1-TFEB-induced autophagic-lysosomal pathway, but not mTOR-mediated pathway. Besides, considering that mTOR inhibition may have lots of side effects, this study points out a potential new drug development direction by generating rapalogs with decreased mTOR inhibition but increased TRPML1 activation efficacy.

V-2 Discussions and Future Directions

2.1 The role of lysosomal Ca^{2+}, $PI(3,5)P_2$, and mTOR on starvation-induced TFEB activation

mTOR is by far the best-characterized regulator of TFEB (Settembre et al., 2013), however, unexpectedly, both lysosomal Ca^{2+} and $PI(3,5)P_2$ are likely to regulate TFEB in a mTOR-independent manner. Chelation of intracellular Ca^{2+} by BAPTA-AM completely blocked starvation-induced TFEB activation, while BAPTA-AM had no effect on Torin-1 induced TFEB activation. Besides, mTOR was in fact further inhibited by BAPTA-AM (10 μM) in addition to starvation during the experiment. (Gulati et al., 2008; Li et al., 2016). Thus, different from the conventional notion that mTOR inhibition triggers TFEB activation upon starvation, lysosomal Ca^{2+} is likely to play a more active role and provides a signal for TFEB nuclear translocation.

On the other hand, $PI(3,5)P_2$ was reported to regulate mTORC1 signaling in yeast (Jin et al., 2014) and adipocytes (Bridges et al., 2012). However, my results together with reports from other researchers suggest that $PI(3,5)P_2$ may not regulate mTORC1 activity in MCF10A cells, J774.1 macrophages (Krishna et al., 2016b) and Non-Hodgkin lymphoma (B-NHL) cell (Gayle et al., 2017), implying that in some cell types and organisms, $PI(3,5)P_2$ may be uncoupled from mTOR, which is likely the case in my model. However, no final conclusion should be made at this moment considering that the cell types (Epithelial/macrophage/B cells vs. adipocytes) and organisms (Human vs. yeast) employed were different. Moreover, according to Krishna et al's study, regulation of vacuole maturation and nutrient recovery following engulfment in starved yeast cells

by PIKfyve is largely independently of mTORC1 except when cells are dependent on nutrients supplied in the form of engulfed apoptotic corpses (Krishna et al., 2016a), implying the differential effects of $PI(3,5)P_2$ on mTOR under different conditions. More systematic examination considering multiple conditions, cell types and organisms should be taken to elucidate the true relationship between $PI(3,5)P_2$ and mTOR.

2.2 Mechanisms underlying rapid activation of TRPML1 upon starvation.

In chapter II, TRPML1 was shown to contribute the majority of the starvation-induced Ca^{2+} release. However, the underlying mechanism of the immediate activation of TRPML1 (timescale of seconds) in response to acute starvation remains unclear.

$PI(3,5)P_2$ and reactive oxygen species (ROS) are the only known endogenous agonists of TRPML1 (Dong et al., 2010; Zhang et al., 2016). The increase in either of them would induce rapid TRPML1 activation. However, the $PI(3,5)P_2$ level is reduced more than half upon starvation (Li et al., 2013; Zolov et al., 2012), which makes it seems unlikely to be the mediator. However, it is possible that $PI(3,5)P_2$ is recruited to locally to form $PI(3,5)P_2$-rich lipid domains and activate TRPML1. ROS level was reported to be elevated during starvation (Scherz-Shouval et al., 2007). However, starvation-induced TFEB activation was not blocked by N-acetyl-L-cysteine (NAC), which inhibits ROS production (Zhang et al., 2016), implying that a ROS increase may also not be the TRPML1 activation mediator.

Protein phosphorylation by kinases and phosphatases is often a rapid molecular switch in regulating cellular functions (Humphrey et al., 2015) The phosphorylation of TRPML1 by mTOR and protein kinase A (PKA), both of which are shown to be inhibited upon starvation (Barbet et al., 1996; Gomes et al., 2011), have been implicated to negatively regulate TRPML1 activity (Onyenwoke et al., 2015a; Onyenwoke et al., 2015b). It is possible that mTOR or PKA may release the suppression of ML1 upon nutrient deprivation, resulting in an acute Ca^{2+} release.

Moreover, nutrient starvation induces the alkalization of cytoplasmic pH (Korolchuk et al., 2011) and alkalized cytoplasmic pH increases TRPML1 activity (Li et al., 2016). Thus, TRPML1 may be activated by a global cytoplasmic pH increase upon starvation. Interestingly, alkalization of the extracellular pH, which is likely to result in alkalization of the intracellular pH (Fellenz and

Gerweck, 1988), induced strong TEFB nuclear translocation (data not shown). Thus, the pH-regulation of TFEB, even if not TRPML1-related, may be an interesting direction to investigate.

2.3 Sources of starvation-induced Ca^{2+} release.

Pretreatment with GPN, which depletes all lysosomal Ca^{2+}, completely blocked starvation-induced Ca^{2+} release in HEK cells, implying that lysosomal Ca^{2+} may constitute all the Ca^{2+} induced by starvation, however, pretreatment of thapsigargin (TG), which induces ER Ca^{2+} release, also reduced starvation-induced Ca^{2+} release (data not shown), suggesting that the ER may involve in starvation-induced Ca^{2+} release. ER and lysosomes are shown to intimately interact by forming ER-lysosome membrane contact sites (Phillips and Voeltz, 2016), which are dynamic and may mediate translocation of Ca^{2+} from ER into lysosomes (Garrity et al., 2016). It is possible that in response to starvation, the contact sites between ER and lysosomes are increased for optimal cellular adaptation and mediate the necessary Ca^{2+} flux continuously. Consistent with the idea, activation of TRPML1 was shown to trigger global ER Ca^{2+} flux, implicating coupling between lysosomal Ca^{2+} channel TRPML1 and ER Ca^{2+} channels on ER (Kilpatrick et al., 2016). This may also explain why inhibition of the TRPML1 channel, the major lysosomal Ca^{2+} release channel, only partially blocked the starvation-induced Ca^{2+} release. Interestingly, although activation of TRPML1 only plays a permissive role in starvation/PIKfyve inhibition-induced TFEB starvation, TRPML1 may be exclusive in certain conditions. For example, under oxidative stress, the Ca^{2+} required for TFEB activation is totally contributed by TRPML1 (Zhang et al., 2016).

2.4 Downstream sensors of starvation/apilimod-induced Ca^{2+} release

I have shown that $PI(3,5)P_2$ inhibition and starvation induce TFEB translocation via lysosomal Ca^{2+}. However, the downstream players in this pathway remain unclear. Medina et al reported that calcineurin, a Ca^{2+} dependent phosphatase, is one such players. Calcineurin is a heterodimer of calmodulin-binding catalytic subunit and a Ca^{2+} binding regulatory subunit. The catalytic subunit has three isoforms: PPP3CA, PPP3CB, and PPP3CC. Lysosomal Ca^{2+} activates calcineurin, which dephosphorylates TFEB and leads to TFEB activation (Medina et al., 2015). However, based on data in Figure 3.7 and the TFEB translocation experiments performed in PPPC3B (calcineurin catalytic subunit isoform beta) CRISPR KO cells (data not shown), starvation and apilimod-induced TFEB nuclear translocation did not entirely depend on calcineurin,

or at least PPPC3B, considering that FK506/CysA may only partially inhibit calcineurin activity. In a more comprehensive study by Martina et al, TFEB still translocated to the nucleus in more than 50% cells in response to both starvation and ER stress when both known catalytic subunits (PPPC3A and PPPC3B) were knocked down (Martina et al., 2016). Even though the depletion is not complete, it is very likely that additional unidentified factors participate in starvation-/apilimod-induced TFEB activation. Interestingly, CCCP (chlorophenylhydrazone, an oxidative stress inducer)-induced TFEB translocation was largely blocked by co-application of FK506 and CysA (Zhang et al., 2016), implying that the relative contribution of calcineurin on TFEB activation may depend on the type of cellular stress. The additional factors such as other Ca^{2+}-dependent phosphatases and the differential effects of calcineurin on TFEB activation in response to different cellular stresses remain to be further characterized, which will be greatly facilitated by large-scale mass-spectrometry and genome-wide screening.

2.5 Relationship between mTOR, Ca^{2+}, and TRPML1

Beyond the data shown in chapter II-IV, an intriguing question is what is the relationship between TRPML1 and mTOR given that mTOR is activated on lysosomes and Ca^{2+} is implicated as affecting mTOR activity (Gulati et al., 2008; Li et al., 2016b). Multiple groups have probed this question. TRPML1 was suggested to be required for mTOR activation in *Drosophila*, which is essential for *Drosophila*'s viability during pupal stage (Wong et al., 2012). Moreover, using ML1 modulators and a ML1 KO model, the relationship between ML1 and mTOR activity has been examined by Li et al. (Li et al., 2016a). They showed that in mammalian cells, Ca^{2+} release from TRPML1 was required for mTORC1 activation. Deletion of TRPML1 inhibited mTORC1 activity, while activation of TRPML1 further increased basal mTORC1 activity (Li et al., 2016b). However, in a recent study published by my lab, modulation of TRPML1 activity did not affect basal mTORC1 activity (Li et al., 2016c). Moreover, the recruitment of mTOR to the lysosomal membrane remained unaffected (data not shown), suggesting that the regulatory role of TRPML1 on mTORC1 signaling remains to be further examined. The interplay between TRPML1, Ca^{2+}, and mTOR may not be identical in different conditions, cell types, and organisms, requiring more systematic studies to elucidate the observed differential effects.

2.6 Role of other members in MiT/TFE family in lysosomal regulation

Researchers in the lysosome/TFEB field have been massively focused on TFEB, with little work on on other members in MiT/TFE family, such as TFE3 and MITF, which have similar functions and are regulated in a similar manner (Martina et al., 2014; Raben and Puertollano, 2016). This may result in over-claiming of TFEB's effect in many studies since TFE3 and MITF activation may happen simultaneously with TFEB activation. Nezich et al have generated MiT/TFE single CRIPSR KO, double KO and triple KO to probe this issue and found that indeed they have additive effects in regulating transcriptional response. They found that loss of p62 induction, by oligomycin/antimycin A treatment, which strongly induces mitophagy, was only observed in triple TFEB/MITF/TFE3 KO cells, but not in TFEB single KO cells. Besides, mitophagy was moderately defective in TKO cells, but not TFEB KO cells. Similarly, up-regulation of lysosomal genes persists in the TFEB-depleted cell line, implying that TFE3 and TFEB may work in parallel to mediate transcriptional regulation (Martina et al., 2014). Moreover, TFEB and TFE3 are differentially expressed in different cell lines and tissues, for example, ARPE-19 cells have much more TFE3 than TFEB in the cells. Depletion of TFEB in APRE-19 did not prevent starvation-induced lysosomal transcriptional upregulation while depletion of TFE3 did (Martina et al., 2014). Thus, researchers in the field should be more cautious in analyzing TFEB-related effects.

2.7 Future perspectives

Adaptation to environment changes is the theme of evolution. To survive, cells and organisms have evolved a set of adaptation mechanisms. With the emerging role of lysosomes in important cellular signaling pathways and then well-characterized role in degradation, lysosomal adaptation is essential for cellular adaptation in response to nutrient availability and cellular stress. Such adaptation is demonstrated to be orchestrated transcriptionally and is self-regulated via lysosomal Ca^{2+} and $PI(3,5)P_2$. However, accumulating evidence suggests that lysosomal adaptation related signaling pathways diverge in varied conditions, cell types and organisms and are more dynamic. A unique mechanism may be employed for each condition, so this idea requires more careful and systematic examination. With the advance of systems approaches, such as proteomics and metabolomics, identifying novel components in lysosomal regulation on a large scale would

greatly enhance our understanding of lysosomes, which would be challenging but exciting. Understanding lysosomal adaptation at the organism tissue level would be more meaningful. In addition to transcriptional regulation, post-transcriptional and post-translational effects may also play essential roles in lysosomal regulation and would be an interesting direction to explore.

In the past decade, we have gained lots of new understanding of lysosomes . Our traditional view about lysosomes as the degradation center is now greatly expanded to a new era where the lysosomes are also the control center for many cellular signaling events. Dysfunction of lysosomes contributes to numerous diseases including lysosomal storage disorders, neurodegeneration, cardiovascular diseases, cancer and immune diseases. By understanding the basic mechanisms of lysosomal regulation and its application for therapeutic treatments for lysosome-related diseases, we are embracing a bright future where there will be treatments for all lysosome-related disorders.

V-3 Figures

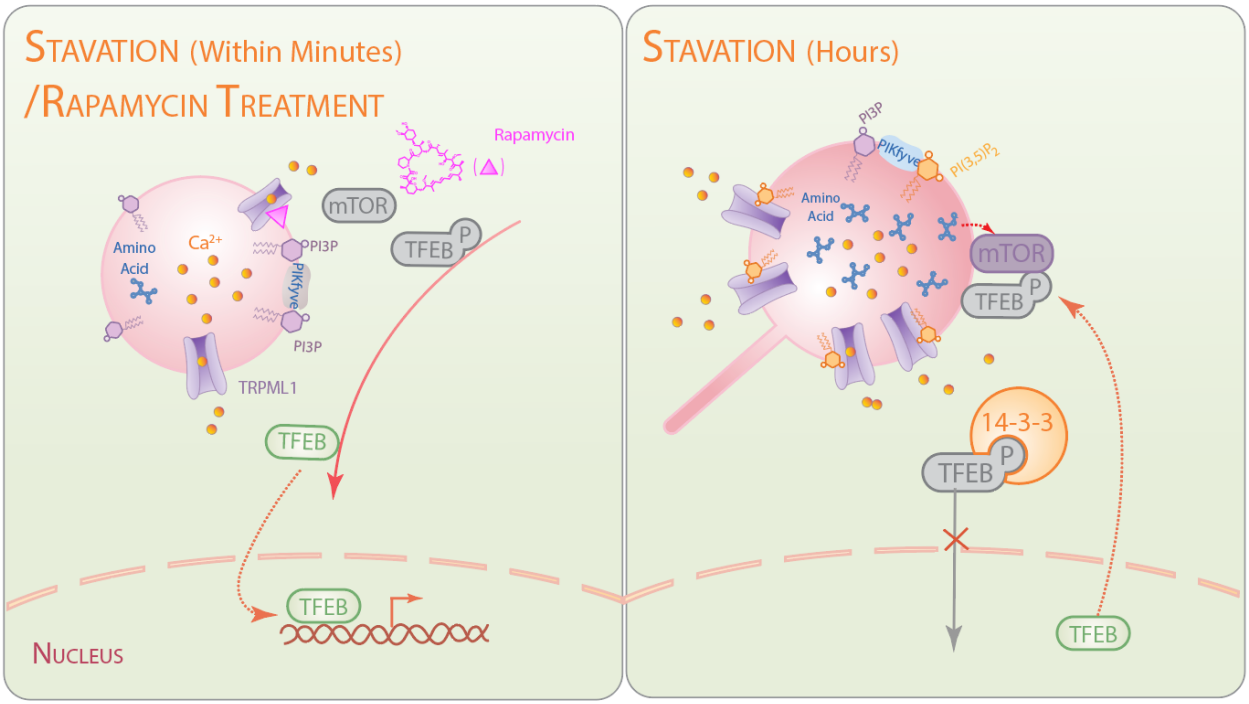

Fig 5.1 Regulation of lysosomal adaptation by lysosomal Ca^{2+}/TRPML1 and $PI(3,5)P_2$/PIKfyve.

Within minutes of withdrawal of nutrients, localized-release of lysosomal Ca^{2+} via TRPML1 and reduction of $PI(3,5)P_2$ trigger a global transcriptional response via activating TFEB and upregulating ML1 expression level. After a few hours of nutrient deprivation, newly synthesized $PI(3,5)P_2$ activates newly expressed TRPML1 to coordinate lysosomal adaptation including increased cellular clearance, enhanced lysosomal proteolytic function, and autophagic-lysosomal reformation, which may reactivate mTOR and inhibit partial TFEB. Furthermore, rapamycin, a clinically widely-used drug and well-known mTOR inhibitor, may activate TRPML1 and promote cellular clearance by activating TFEB via Ca^{2+}.

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