Lysosome Ca\(^{2+}\) Store Refilling Mechanisms

by

Abigail G. Garrity

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Doctoral Committee:

Associate Professor Haoxing Xu, Chair
Professor Richard I. Hume
Professor Edward L. Stuenkel
Professor Joel A. Swanson
Professor Kristen J. Verhey
To my parents, for naming me after the John Prine song “Dear Abby.” Thank you for reminding me that “you are what you are, and you ain’t what you ain’t.”
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# Table of Contents

Dedication ........................................................................................................... ii  
Acknowledgments ............................................................................................. iii  
List of Figures .................................................................................................... vi  
Abstract ........................................................................................................... viii  

## Chapter

I. **INTRODUCTION TO LYSOSOME BIOLOGY** ...................... 1  
   Abstract ......................................................................................................... 1  
   Introduction ................................................................................................... 1  
   References .................................................................................................... 30  

II. THE LYSOSOME V-ATPASE AND pH GRADIENT ARE NOT  
    REQUIRED FOR Ca\(^{2+}\) REFILLING TO THE LYSOSOME .......... 41  
   Abstract ......................................................................................................... 41  
   Introduction .................................................................................................. 41  
   Results .......................................................................................................... 48  
   Discussion .................................................................................................... 54  
   Methods and Materials ............................................................................... 61  
   References .................................................................................................... 81  

III. THE ER Ca\(^{2+}\) STORE IS REQUIRED FOR Ca\(^{2+}\) REFILLING  
    TO THE LYSOSOME .................................................................................. 85  
   Abstract ......................................................................................................... 85  
   Introduction .................................................................................................. 85  
   Results .......................................................................................................... 89  
   Discussion .................................................................................................... 92  
   Methods and Materials ............................................................................... 97  
   References .................................................................................................... 107  

IV. THE IP3-RECEPTOR ON THE ENDOPLASMIC RETICULUM IS  
    REQUIRED FOR REFILLING LYSOSOME CALCIUM STORES ... 112  
   Abstract ......................................................................................................... 112  
   Introduction .................................................................................................. 112  
   Results .......................................................................................................... 116
V. INHIBITION OF IP3-RECEPTORS ON THE ER CAUSES A LYSOSOME STORAGE PHENOTYPE

Abstract
Introduction
Results
Discussion
Methods and Materials
References

VI. CONCLUSIONS AND FUTURE DIRECTIONS

Introduction
References
List of Figures

Figure
1.1 Lysosome Ion Channels and Transporters .............................................................................. 27
1.2 Intracellular Organelles and Trafficking Networks ................................................................. 28

2.1 ML-SA1 application in 0 Ca\(^{2+}\) triggers Ca\(^{2+}\) from an intracellular source and 
    empties lysosomal Ca\(^{2+}\) stores .............................................................................................. 64
2.2 GCaMP3-ML1 is lysosome specific .......................................................................................... 66
2.3 A new assay to assess Ca\(^{2+}\) refilling to lysosomes ............................................................... 68
2.4 Lysosome Ca\(^{2+}\) stores refill in approximately 5 mins ............................................................ 70
2.5 GCaMP3-ML1 remains localized to lysosome membranes after stimulation ...................... 71
2.6 Lysosome Ca\(^{2+}\) refilling is not cell-type specific ..................................................................... 72
2.7 Lysosomes with GCaMP3-ML1 do not differ in pH ............................................................... 73
2.8 Fura-Dextran shows lysosome Ca\(^{2+}\) store refilling ............................................................... 74
2.9 Lysosome membrane permeabilization has off-target effects on the cytosolic Fura-2 Ca\(^{2+}\) 
    indicator and the GCaMP3-ML1 probe .................................................................................. 75
2.10 The endocytic and secretory pathways are not the source of Ca\(^{2+}\) to the lysosome .......... 77
2.11 The proton gradient and V-ATPase are not required for lysosome Ca\(^{2+}\) refilling ............. 79

3.1 ER Ca\(^{2+}\) release is significantly reduced over 5 mins in 0 Ca\(^{2+}\) extracellular solution ................................................................................................................................. 100
3.2 ER SERCA inhibitors block Ca\(^{2+}\) refilling to the lysosome .................................................. 101
3.3 TPEN chelates ER Ca\(^{2+}\) and abolishes lysosome Ca\(^{2+}\) refilling ............................................ 103
3.4 Transfection of IP3R-LBD-ER chronically reduced ER Ca\(^{2+}\) and also affected lysosome 
    Ca\(^{2+}\) refilling .......................................................................................................................... 104
3.5 Lysosomes are in close apposition to ER tubules ................................................................. 105
3.6 Model of ER-Lysosome Interaction ......................................................................................... 106

4.1 HEK cells have ryanodine receptors (RYRs) and IP3-receptors (IP3Rs) ............................... 129
4.2 Specific IP3R antagonist Xestospongin-C prevented Ca\(^{2+}\) refilling to lysosomes ............... 130
4.3 Xesto abolishes Ca\(^{2+}\) refilling to lysosomes measured with luminal Ca\(^{2+}\) indicator Fura-Dextran .......................................................................................................................... 131
4.4 IP3R antagonist 2-APB inhibits lysosomal Ca\(^{2+}\) refilling ...................................................... 132
4.5 PLC inhibitor U73122 blocked Ca\(^{2+}\) refilling to the lysosome ............................................ 133
4.6 Ryanodine receptor blockers did not affect Ca\(^{2+}\) refilling to lysosomes ............................. 134
4.7 DT40 IP3R-TKO cells show typical responses to GPN, normal acidification, 
    and release and refill Ca\(^{2+}\) like WT DT40 cells .................................................................... 135
4.8 Xestospongin-C blocked lysosome Ca\(^{2+}\) store refilling in DT40-WT but not 
    IP3R-TKO cells ...................................................................................................................... 137
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.9</td>
<td>Lysosome Ca(^{2+}) stores are emptied and refilled ongoingly</td>
<td>139</td>
</tr>
<tr>
<td>4.10</td>
<td>A proposed model of Ca(^{2+}) transfer from the ER to lysosomes</td>
<td>141</td>
</tr>
<tr>
<td>5.1</td>
<td>LAMP1 increased significantly after inhibiting ER Ca(^{2+}) and IP3Rs</td>
<td>156</td>
</tr>
<tr>
<td>5.2</td>
<td>LysoTracker staining showed a LSD phenotype in cells treated with IP3R blocker Xesto</td>
<td>157</td>
</tr>
<tr>
<td>5.3</td>
<td>Autofluorescent, lipofuscin-like accumulation in cells treated with IP3R blocker Xesto mimics TRPML1 KO cells</td>
<td>158</td>
</tr>
</tbody>
</table>
ABSTRACT

Lysosomes are acidic intracellular vesicles containing hydrolases that degrade intracellular and extracellular debris delivered through endocytic trafficking and autophagy. Lysosome function requires the establishment of luminal ionic homeostasis for ions including H\(^{+}\) and Ca\(^{2+}\), which are 1,000-5,000 times more concentrated in the lysosome lumen than in the cytosol. Lysosomal H\(^{+}\) homeostasis is required to activate hydrolases and Ca\(^{2+}\) efflux through lysosomal ion channels serves as signals required for precise delivery of hydrolases and cargo and the timely removal of catabolites. Impaired lysosomal Ca\(^{2+}\) homeostasis results in lysosomal dysfunction, lysosomal storage diseases (LSDs), and has been implicated more broadly in neurodegenerative phenotypes. The molecular mechanisms by which lysosomes acquire and refill Ca\(^{2+}\) are unknown. We developed a physiological assay to monitor lysosomal Ca\(^{2+}\) store refilling using specific activators of lysosomal Ca\(^{2+}\) channel TRPML1 to repeatedly induce lysosomal Ca\(^{2+}\) release. In contrast to the prevailing view that lysosomal acidification drives Ca\(^{2+}\) into the lysosome, inhibiting the V-ATPase H\(^{+}\) pump did not prevent Ca\(^{2+}\) refilling. Instead, pharmacological and genetic depletion or chelation of endoplasmic reticulum (ER) Ca\(^{2+}\) prevented lysosomal Ca\(^{2+}\) stores from refilling. More specifically, antagonists of ER IP3 receptors rapidly and completely blocked Ca\(^{2+}\) refilling to lysosomes. Reducing ER Ca\(^{2+}\) or blocking IP3 receptors resulted in a dramatic lysosome storage phenotype. By closely apposing each other, the ER may serve as a direct and primary source of Ca\(^{2+}\) to the lysosome. These findings may clarify seemingly overlapping ER and lysosome Ca\(^{2+}\) stores in some studies and shed light on why ER Ca\(^{2+}\) homeostasis is often involved in LSDs and neurodegenerative diseases.
CHAPTER I
INTRODUCTION TO LYSOSOMAL BIOLOGY

ABSTRACT

Lysosomes are present in every eukaryotic cell with the exception of very few, highly specialized cell-types. Lysosomes function to degrade both intracellular and extracellular materials in order to resupply the cell with the building blocks needed for ongoing growth and repair. To serve their degradative function, lysosomes have a highly acidic lumen and a membrane with high carbohydrate content in order to protect the rest of the cell from the hydrolytic enzymes in the lysosome lumen. Lysosomes have nutrient sensing machinery and in turn regulate endocytic and autophagic pathways to ensure a balance of anabolic and catabolic pathways in the cell. Lysosome Ca\(^{2+}\) content is integral to their degradative capacity and their trafficking. The intraluminal content of all other ions in the lysosome is also essential to many discrete functions as well as to overall ion homeostasis. Lysosomes maintain membrane contact sites with the ER for transfer of materials and many other purposes. Dysfunctions in ER and lysosome Ca\(^{2+}\) are seen in both lysosome storage disorders (LSDs) and neurodegenerative disorders. Therefore the purpose of this work is to examine the possible interactions between ER and lysosome Ca\(^{2+}\) stores.

INTRODUCTION

There are two major degradation mechanisms in the cell, the ubiquitin-proteasome system and lysosomal degradation, which allow for the recycling of cellular components for reuse (Korolchuk et al., 2010). The lysosome is a membrane-bound organelle that breaks down intracellular and extracellular components delivered via two pathways, the endocytic and autophagic pathways. The endocytic pathway carries extracellular materials destined for degradation and the autophagic pathway delivers intracellular components. Lysosomes are able
to fuse with a variety of intracellular vesicles in order to transfer materials for degradation including autophagosomes, late-endosomes, and phagosomes (Li et al., 2013a). Lysosomes also undergo exocytosis by fusing with the plasma membrane to secrete their contents into the extracellular environment (Samie and Xu, 2014).

In addition to breakdown and recycling of materials, lysosomes are highly adaptable to a variety of other intracellular and extracellular signals which allows them to play a role in several other functions. These include plasma membrane repair, cellular homeostasis, energy metabolism, apoptosis, and the immune response (Settembre et al., 2013).

Dysfunction of the lysosome results in a build-up of components targeted for break down in the lysosome and an accumulation of lysosomes, measured by an increased number and/or size of lysosomes. Dysfunction of a specific component of the lysosome results in one of almost 60 known lysosome storage disorders (LSDs) which each have a single gene origin (Boustany, 2013). Lysosome dysfunction is also found in many common neurodegenerative disorders including Alzheimer’s, Huntington’s, and Parkinson’s diseases (Nixon et al., 2008).

**Lysosome Structure and Proteins**

The existence of about 125 different lysosomal resident proteins has been suggested, although only about 25 have been identified and functionally characterized. The lysosome membrane constituents remain poorly characterized due to the low abundance of many proteins and their hydrophobic properties that make purification and functional analysis difficult (Schroder et al., 2010). Studying lysosomal proteomics is also made difficult due to the necessity to separate lysosome-specific organelle constituents from other co-purifying intracellular contaminants, which is more complicated for lysosomes because they serve as the degradation site for many cellular proteins (Schwake et al., 2013). Studying lysosomal hydrolases has proven less difficult, and over 60 different soluble hydrolases have been identified (Settembre et al., 2013).

The lysosome membrane even contains enzymes with catabolic activity, in addition to hydrolytic enzymes within the lysosome lumen (Schroder et al., 2010). The membrane-bound nature of the lysosome protects the rest of the cell from the highly acidic environment of the lysosome, conducinge to its degradative function (Saftig and Klumperman, 2009). Indeed,
lysosome membrane rupture or leakage of lysosomal enzymes into the cytosol is involved in cell death pathways (Guicciardi et al., 2004). Heavily glycosylated integral membrane proteins form a thick, cholesterol-poor glycocalyx on the internal leaflet of the lysosome membrane. This glycocalyx protects the outer part of the limiting membrane and its resident proteins from degradation (Settembre et al., 2013).

Although the study of lysosome membrane proteins is difficult, several membrane proteins have been identified from studies of lysosome storage disorders and their functions have been illuminated. Lysosome membrane proteins are targeted to the lysosome membrane using cytosolic targeting sequences which are typically dilucine- or tyrosine- based (Braulke and Bonifacino, 2009). The most common lysosomal proteins are lysosomal associated membrane proteins (LAMPs), lysosome integral membrane protein-2 (LIMP2), and CD63 (Fig. 1) (Saftig and Klumperman, 2009).

LAMP proteins (LAMP-1 and LAMP-2) account for about 50% of all lysosome membrane proteins. LAMPs are physiologically essential, as depletion of both LAMPs results in an embryonic lethal phenotype in mice between E14 to E16 (Eskelinen et al., 2004). LAMPs have a heavily glycosylated luminal domain and a short cytosolic tail (Fig. 1) (Saftig and Klumperman, 2009). Depletion of either LAMP1 or LAMP2 results in an abnormal accumulation of cholesterol in the lysosome lumen and redistribution of lysosomes to the cell periphery, but has little effect on the proteolytic capacity of lysosomes (Eskelinen et al., 2004; Huynh et al., 2007; Saftig and Klumperman, 2009). LAMP proteins stabilise translocation machinery on lysosome membranes that allows for transport of cytosolic materials into the lysosome for degradation, and likely also help stabilize export machineries as well (Schwake et al., 2013). LAMP proteins are also required for the targeting of many substrates to the lysosome during endosomal and trans-Golgi trafficking. The presence of and glycosylation state of LAMP proteins are also important to lysosome fusion with phagosomes as well as with the plasma membrane and lysosome exocytosis (Schwake et al., 2013).

The lysosome membrane contains transport proteins that facilitate the import of products destined for degradation and the export of catabolites for their reuse in the cell. Few transport proteins on the lysosome membrane have been identified to date, although the vacuolar H⁺-ATPase (V-ATPase) is a well-known transporter of H⁺ ions into the lysosome lumen (Fig. 1).
There are also several identified ion channels on the lysosome membrane for signalling and pH homeostasis. The lysosome membrane is home to the lysosomal nutrient sensing machinery (LYNUS) (Fig. 1), which allows the lysosome to integrate metabolic signals from the cell to allow lysosomal breakdown products to meet intracellular needs (Settembre et al., 2013).

Machinery for trafficking and fusion including SNARE proteins and RABs also reside on the lysosomal membrane. The Ca$^{2+}$ sensor synaptotagmin VII (Syt-VII) and Ca$^{2+}$ release channel TRPML1 are also important for lysosomal exocytosis (Fig. 1) (Samie et al., 2013). Lysosomal membrane glycoprotein CD63, a tetraspanin (Fig. 1), is also important for lysosomal targeting of lysosome membrane proteins like Syt-VII and likely acts as a scaffold protein at the lysosome membrane (Schwake et al., 2013).

**Transport of Lysosomal Components to the Lysosome**

Newly synthesized lysosome proteins from the rough ER are targeted to the lysosome through two pathways. The trans-Golgi network (TGN) to endosome pathway carries receptor targeted substrates, typically hydrolases, directly to the endocytic pathway for subsequent lysosome delivery (Fig. 2A). An indirect pathway also exists that includes transport to the plasma membrane and subsequent endocytic delivery (Fig. 2B).

The most well understood TGN pathway tags newly made proteins with mannose-6-phosphate residues that are recognised and bound by two different mannose-6-phosphate receptors (M6PRs) in the TGN. Clathrin coated vesicles from the TGN containing M6PRs transfer hydrolases from the TGN to endosomes (Fig. 2A), where hydrolases dissociate from M6PRs due to endosomal acidity (Schroder et al., 2010). Because hydrolases could cause cellular damage if active in the cytosol, tagging them with M6P allows them to be inactive through the endosomal pathway and protects the cell from their degradative functions. M6PRs form the basis for lysosomal enzyme replacement therapy in LSDs (Settembre et al., 2013).

M6PRs are notably absent from lysosomes and serve as one way to distinguish lysosomes from endosomes (Schroder et al., 2010). Thus, a commonly accepted operational definition of lysosomes is an acidic organelle in almost all cell types containing mature hydrolases and LAMP proteins that lacks M6PRs (Morgan et al., 2011).
Less is known about how lysosome membrane proteins like LAMPs arrive at the lysosome through more direct, clathrin-independent pathways (Coutinho et al., 2012). However, recent evidence suggests that non-clathrin coated vesicles originating in the Golgi can fuse directly with late-endosomes using SNARE machinery VAMP7 and hVps41 to deliver LAMP proteins (Fig. 2C) (Pols et al., 2013). This more specific targeting of LAMPs may suggest that some lysosomal membrane proteins are delivered at progressing stages of endosome maturation to ensure precision around vesicular specificity and to avoid endosomal sorting processes (Pols et al., 2013).

Lysosome Acidification Mechanisms

The large, multimeric vacuolar H⁺-ATPase (V-ATPase) is the major proton pump in eukaryotic cells (Fig. 1). The V-ATPase is located mainly in the endolysosomal system, but it is also located to a much lower extent on the plasma membrane and Golgi in some specialized cell types (Marshansky and Futai, 2008). The 910 kDa V-ATPase is composed of two large multi-subunit complexes and functions as a rotary proton transport motor. The soluble V₁ sub-complex catalyzes ATP hydrolysis and is made of at least eight subunits. The V₀ sub-complex is embedded in the membrane to translocate protons and is composed of six subunits (Mindell, 2012).

The V-ATPase is important to endocytic and exocytic vesicular trafficking, possibly due to findings suggesting that pH is important to membrane potential and vesicular fusion. pH of organelles in the endocytic pathway exhibit a graded pH (Fig. 2), with lysosomes having a uniquely low pH between 4.0 and 4.7 (Morgan et al., 2011).

Because each organelle maintains a signature pH, regulation of all components of the V-ATPase is a tightly controlled process. Both the V₀ and V₁ domains of the V-ATPase can rapidly associate and dissociate with various organellar membranes and is one way that V-ATPase activity is regulated by the cell (Maxson and Grinstein, 2014). The density of V-ATPase on specific organellar membranes is another way that pH is regulated between compartments (Huotari and Helenius, 2011). Even lipid composition of different membranes has been shown to affect the H⁺ pumping efficiency of the V-ATPase (Maxson and Grinstein, 2014). Subunit isoforms vary between cell and tissue types and combinations of unique isoforms...
distinguish V-ATPase functions on specific membranes (Marshansky and Futai, 2008). For example, targeting of α-subunits of the V₀ domain are compartment specific; the α3-isoform is targeted to the lysosome and sometimes the plasma membrane (Marshansky and Futai, 2008).

**Non-Canonical Functions of the V-ATPase**

The large size and variety of subunits of the V-ATPase are reflected in the many functions it is involved in, including pH homeostasis, coupled transport of small molecules, membrane trafficking, vesicle fusion, and protein degradation. In addition, the subunits of the V-ATPase are also involved directly in vesicular trafficking and play roles far beyond just acidification of compartments. Various subunits of the V-ATPase directly interact with GTPases in an acidification dependent manner. Interactions with a variety of GTPases may function to regulate V-ATPase assembly and function (Maxson and Grinstein, 2014).

The V-ATPase may play a role in fusion and fission by acting as a sensor and transducer of luminal pH (Maxson and Grinstein, 2014). In support of this, various GTPases bind to vesicular membranes in an acidification-dependent manner, suggesting that the V-ATPase plays a role in recruitment of GTPases. Furthermore, acidification may induce changes in V-ATPase conformation to facilitate GTPase docking (Hosokawa et al., 2013). Dissipation of the pH gradient is known to affect various steps in the endosome maturation process, suggesting that the V-ATPase plays a role in recruitment, activation, and/or docking of various other factors (Huotari and Helenius, 2011; Maxson and Grinstein, 2014).

Although the importance of H⁺ gradients in vesicle fusion has been demonstrated, studies have suggested that the V-ATPase also plays a structural role in vesicle fusion (Maxson and Grinstein, 2014). The presence of a V₀ domain has been shown to be required for fusion (Baars et al., 2007; Takeda et al., 2008), and may play a role in the development of a fusion pore (Bayer et al., 2003; Clare et al., 2006). Alternatively, the V₀ domain may promote fusion by association with SNARE proteins (Strasser et al., 2011). The precise mechanism by which the V₀ promotes fusion is still controversial and some believe it is simply the H⁺ gradient that is required for vesicle fusion (Maxson and Grinstein, 2014).

The V-ATPase is also a scaffold protein at the membrane surface. The B and C subunits of the V₁ complex of the V-ATPase have long been known to interact with actin. The V-ATPase
likely regulates cytoplasmic G-actin pools and actin crosslinking and stabilization into filaments. Thus, the V-ATPase is thought to regulate vesicle sorting and transport by linking vesicles to actin filaments, potentially in response to the metabolic state and/or nutrient levels in the cell (Maxson and Grinstein, 2014). The V-ATPase also binds to the Wiskott-Aldrich and scar homolog (WASH) which promotes actin polymerization, providing another mechanism to regulate actin filaments and vesicular attachments (Carnell et al., 2011).

The V-ATPase is also part of the lysosome nutrient sensing (LYNUS) apparatus at the lysosome membrane, serving as a molecular switch between anabolic and catabolic processes at the lysosome (Fig. 1) (Zhang et al., 2014). Mammalian target of rapamycin (mTOR) complex 1 (MTORC1) is a serine/threonine kinase that integrates numerous signals to sense nutrient availability and promote cellular growth when nutrients are available (Shimobayashi and Hall, 2014). AMP-activated protein kinase (AMPK) senses energy stresses in the cell by monitoring levels of ATP and AMP. When bound to AMP, AMPK is activated, which in turn increases cellular catabolic activities (Hardie et al., 2012). The recruitment and activation of both AMPK and MTORC1 requires the lysosomal V-ATPase (Zoncu et al., 2011; Zhang et al., 2014), which may itself sense amino acid availability (Bar-Peled et al., 2012; Xu et al., 2012). It is unclear what role if any luminal acidification has in amino acid sensing (Zoncu et al., 2011; Xu et al., 2012), so the V-ATPase may have a direct role in nutrient sensing. Nutrient status also regulates V-ATPase association with vesicular membranes, at least in yeast (Tabke et al., 2014).

Furthermore, α-subunit of V₀ complex interacts with enzyme 1-phosphofructokinase that catalyses a rate-limiting step in glycolysis (Su et al., 2008).

The Endocytic Pathway to the Lysosome

Endocytosis begins at the plasma membrane, where membrane invagination and subsequent fission takes up nutrients, proteins, lipids, solutes, receptors, macromolecules, cell debris, bacteria and viruses, and many other substances into vesicles (Fig. 2D). These internalized vesicles, nascent endosomes (NE), then undergo a variety of membrane trafficking events that lead back to the plasma membrane through recycling endosomes (RE) (Fig. 2E), or to the lysosome for degradation. The trans-Golgi network also transports newly synthesized proteins, lipids, and hydrolases to late endosomes for integration into the lysosome membrane or degradative lumen to ensure lysosome functioning (Fig. 2A,C) (Huotari and Helenius, 2011).
After internalization, early endosomes recycle back an astonishing 50-180% of the plasma membrane and take in and subsequently release about 30% of total cell volume in extracellular fluid per hour (Steinman et al., 1983). These impressive numbers give a sense of how active and rapid the process of endocytosis is. They also make it clear that the rapidity and constancy of endocytic trafficking provides an easy mechanism for researchers to exploit for internalization of various dyes and indicators used to study membrane trafficking through endocytic pathways. Finally, they support the fact that stringent sorting mechanisms ensure that only a very select amount of internalized substrates remain internalized and are ultimately trafficked to the lysosome. The size of the degradative compartment increases upon stimulation with growth factor receptors suggesting that the pathway to the lysosome is regulated according to metabolic state of the cell (White et al., 2006).

Nascent endosomes (NE) fuse with each other (Fig. 2F) to become early endosomes (EE) (Fig. 2G), where a significant amount of sorting occurs to determine the final destination of endocytosed cargo. A number of pathways converge upon early endosomes to transfer cargo for sorting. The small GTPase Rab5 is a primary regulator of early endosome motility, cargo sorting, and membrane fusion (Rink et al., 2005). Early endosomes have varied size, morphology, and localization in different cell types, although are mostly found in the periphery of the cell. They have both tubular and vacuolar domains which have varied compositions of membrane lipids that create membrane subdomains for different functions including cargo sorting and fission. Vacuolar domains presumably contain all material destined for degradation (Huotari and Helenius, 2011). Early endosomes have small amounts of Ca\(^{2+}\) that is initially lost during progressing acidification (Gerasimenko et al., 1998). The pH of early endosomes ranges from about 6.8 to 5.9 (Maxfield and Yamashiro, 1987).

The switch from Rab5 to Rab7 on the vacuolar membrane of early endosomes marks the conversion to late endosomes (LE) (Fig. 2G) and the progression of cell cargo from the cell periphery towards the cell center (Rink et al., 2005). Late endosomes lack the tubular extensions of early endosomes and become larger in size during their maturation. Late endosomes fuse with other late endosomes to form larger vesicles, receive cargo via kiss-and-run events, and ultimately fuse with lysosomes to transfer cargo for degradation (Fig. 2H). Late endosomes also carry numerous proteins and enzymes that become incorporated into the functional lysosome.
Late endosomes can have a pH that ranges from 6.0 to 5.0 and becomes progressively more acidic as they mature (Maxfield and Yamashiro, 1987). Late endosomes acquire increasing amounts of Cl\(^{-}\) and Ca\(^{2+}\) compared to early endosomes and concentrations of other ions also change during late endosome maturation. Rabs, membrane phosphoinositides, tethering proteins, and ion channels all also change during late endosome maturation (Huotari and Helenius, 2011).

Intraluminal vesicles (ILVs) form within late endosomes (Fig. 2) using ESCRT machinery to ensure receptors are inactive (as opposed to being transported on the surface of endosome membranes). Intraluminal vesicles contain specific, sorted cargo. Thus, ILVs simplify cargo sorting and make delivery to lysosomes efficient and accessible (Huotari and Helenius, 2011). The membrane composition of intraluminal vesicles is far different from that of late endosomes or lysosomes and is conducive to enzyme recruitment (Huotari and Helenius, 2011).

From the perspective of endosome trafficking, the lysosome is the last step in this continuous and highly dynamic pathway. Late endosomes fuse with lysosomes to become endolysosomes (Fig. 2H), where the bulk of cargo degradation occurs. The characteristic low pH of the lysosome facilitates the degradation of complex macromolecules for reuse in biosynthetic pathways. Transporters on the lysosome membrane transport these building blocks out of the lysosome (Xu and Ren, 2015).

The Autophagic Pathway to the Lysosome

Autophagy, or “self-digestion,” is the process by which intracellular components and damaged organelles are delivered to the lysosome via late endosomes and autophagosomes. Basal levels of autophagy function to turnover cytoplasmic components that are obsolete or no longer functioning. Autophagy can also be induced by nutrient starvation to increase the abundance of cellular building blocks for cell growth via mTOR, but can also occur in an mTOR independent manner (Mizushima, 2007).

Autophagy begins through the isolation of cytosolic components in a phagopore (PP), or isolation membrane, which eventually closes to form a double-membrane bound autophagosome (AP) (Fig. 2I) (Mizushima, 2007). Autophagosomes form at ER-mitochondria contact sites (Hamasaki et al., 2013), which may be the source of the newly formed membranes, although this
process is incompletely understood. Little is known about the specificity of materials engulfed by phagopores and subsequent autophagosomes; whether autophagosomes recognize some or all of the contents they engulf remains an unanswered question (Mizushima, 2007).

The outer membrane of autophagosomes fuses with lysosomes to form autolysosomes (Fig. 2J). It has also been suggested that autophagosomes fuse with endosomes to form amphisomes before fusing with lysosomes, possibly to acquire the machinery needed to fuse with lysosomes (Fig. 2K). Then the inner membrane and luminal contents of autophagosomes are degraded by lysosome resident hydrolases (Murrow and Debnath, 2013).

**The Role of Lysosomal Ca\(^{2+}\) in Regulating Endocytosis and Autophagy**

Lysosome Ca\(^{2+}\) channel TRPML1 expression (Fig. 1) and activity has been shown to be upregulated in response to nutrient depletion (Wang et al., 2015b). Ca\(^{2+}\) signalling through TRPML1 activates the phosphatase calcineurin which dephosphorylates TFEB, the transcriptional regulator of autophagy. TFEB phosphorylation promotes its nuclear translocation and activation of target genes for autophagy (Medina et al., 2015). Previous studies have suggested that metabolic status can regulate endocytosis (White et al., 2006), and the recent studies of lysosomal Ca\(^{2+}\) release through TRPML1 suggest that the lysosome regulates autophagy induction as well. These findings suggest that lysosomal nutrient sensing mechanisms have broad regulatory effects on intracellular membrane trafficking pathways, in part through Ca\(^{2+}\) signalling (Medina et al., 2015; Wang et al., 2015b).

**Lysosome Degradation and Recycling**

Exogenous materials destined for breakdown in the lysosome come from various sources in the cell that include endocytosis and pinocytosis, as well as phagocytosis for large particles. Intracellular materials come from macroautophagy, chaperone-mediated autophagy, and microautophagy (Ciechanover, 2005). It is also becoming appreciated that the ubiquitin-proteasome system can also target proteins for degradation in lysosomes through the autophagy pathway (Korolchuk et al., 2010).

There are 60 known lysosomal hydrolases that degrade all kinds of macromolecules of varying sizes into monomeric components. Several activator proteins residing in the lysosome
matrix also exist (Settembre et al., 2013). Furthermore, intramembrane cleaving proteases, like the γ-secretase complex with catalytic subunits presenilin 1 and 2, can process transmembrane substrates (Schwake et al., 2013).

After macromolecules have been digested in the lysosome, soluble monomeric units (amino acids, monosaccharides, nucleotides) are transported back into the cytosol with specific transporters for reuse. A few amino acid and sugar transporters have been identified, but surely there are more to be discovered. A recent candidate for an arginine (and possibly other amino acids) transporter, SLC38A9 (Fig. 1), has been shown to signal to the mTOR pathway, likely to signal amino acid sufficiency and activate MTORC1 (Rebsamen et al., 2015; Wang et al., 2015a).

Insoluble lipids are transported back to the TGN through membrane fusion events (Xu and Ren, 2015). Cholesterol, for example, is not degraded by lysosomal hydrolases and is transported out of lysosomes by cholesterol transporters NPC1 and NPC2 (Fig. 1) (Subramanian and Balch, 2008).

Heavy metal ions resulting from mitophagy or metal storage proteins including Fe^{2+} and Zn^{2+} are transported out of the lysosome, possibly by the principal Ca^{2+} channel, TRPML1 (Fig. 1), because lysosomal heavy metal overload results when TRPML1 is absent (Xu and Ren, 2015).

**Lysosome Ions and Channels**

Ionic balance is tightly regulated throughout all intracellular vesicles. Furthermore, ion channels and concentration gradients are one way that individual vesicles are differentiated along endocytic and autophagic pathways. Notably, after vesicle fusion, concentration gradients in newly formed vesicles can change dramatically, as does the identity of that new compartment. Thus, proper function of lysosomes requires tightly regulated ionic homeostasis to ensure proper delivery of hydrolases and the removal of breakdown products after degradation. Importantly, many ion concentrations depend on the presence and regulation of other ions in the lysosome, so one ion should not be taken out of the context of the balance of all ions (Xu and Ren, 2015). Lysosomal patch-clamping techniques have transformed the understanding of lysosome currents
and channels, and are responsible for most of what is known about lysosome ion channels today (Xu et al., 2007; Dong et al., 2008).

**H⁺**: The acidic environment of the lysosome facilitates degradation by loosening structures of macromolecules, and is optimal for the activity of hydrolases (Pillay et al., 2002). The H⁺ gradient also provides a driving force for catabolite exporters on the lysosome membrane (Ruivo et al., 2009).

The V-ATPase on the lysosome membrane transports 2-4 H⁺ ions per molecule of ATP (Marshansky and Futai, 2008), and the number of H⁺ pumped may be one way the V-ATPase activity is regulated on different membranes (Maxson and Grinstein, 2014). The electrogenic potential of the V-ATPase would cause build-up of positive charge that would prevent further acidification if it was not countered (Demaurex, 2002). Additionally, at chemical equilibrium, the V-ATPase would lower luminal pH to ~2.6 (Grabe and Oster, 2001). The positive membrane potential of the lysosome compared to the cytosol (20 to 40 mV more negative in the cytosol) may contribute to the driving force of proton entry into lysosomes (Morgan et al., 2011). Thus, lysosomal acidification is coupled to other ion concentrations and their transporters to regulate ion homeostasis.

Countercurrents to lysosome acidification have been suggested to be Cl⁻ entry (Graves et al., 2008) or K⁺ and/or Ca^{2+} exit in lysosomes (Steinberg et al., 2010). Cations and anions could both neutralize the charge generated by acidification, but if anions alone were to do the job, considerable osmotic gain would occur which would cause strain on lysosome membranes and possibly membrane lysis. This suggests that the counterions include at least one cation, although a combination of cation and anion involvement seems most plausible (Steinberg et al., 2010). Our studies and those of others show that changes in Ca^{2+} in the lysosome does not change the pH of lysosomes (Christensen et al., 2002), suggesting that Ca^{2+} alone may not be the countercurrent. It is likely that more than one counterion is involved, which may explain why there remains significant controversy.

**Na⁺**: Only two studies to date have addressed Na⁺ concentrations within lysosomes. Na⁺ within isolated lysosomes has been shown to be ~140-150 mM based on estimations of total ion ratios using inductively coupled plasma mass spectrometry (ICP-MS) (Wang et al., 2012). These
results suggest that Na\(^+\) is the major cation in the lysosome. A less direct method using null point titration determinations with a variety of ionophores to dissipate ion concentrations suggested that Na\(^+\) concentrations are lower, at about 20 mM (Steinberg et al., 2010). Notably, Na\(^+\) in the lysosome is higher than cytosolic Na\(^+\) (~12 mM) (Xu and Ren, 2015).

Na\(^+\) channels on lysosome membranes play a role in regulating the membrane potential and excitability of lysosomes. TPC1 and TPC2 channels on lysosome membranes conduct Na\(^+\) from the lysosome lumen to the cytosol (Fig. 1). Their maximal activation requires PI(3,5)P2 (Wang et al., 2012; Cang et al., 2013; Cang et al., 2014). TPC1 currents are activated in response to extracellular amino acid depletion and intracellular ATP removal. TPC1 interacts with MTORC1 to participate in nutrient sensing. TPC1 can also sense voltage and pH changes in the lysosome (Cang et al., 2014).

K\(^+\): Like Na\(^+\), K\(^+\) concentrations are not well studied compared to H\(^+\) and Ca\(^{2+}\) ions in the lysosome due to limited tools. The two studies examining lysosomal K\(^+\) suggest 2-50 mM K\(^+\) in the lysosome, which is lower than cytosolic K\(^+\) (~150 mM) (Steinberg et al., 2010; Wang et al., 2012; Xu and Ren, 2015).

A recent study from our lab (Wang et al, under review) suggests that a new Ca\(^{2+}\) activated K\(^+\) channel is important for lysosome Ca\(^{2+}\) store refilling. While little is known about this new K\(^+\) channel, these findings suggest that despite its lower concentrations in the lysosome, K\(^+\) is important to ion homeostasis. Lysosome membrane potential is also known to be affected by a yet to be identified K\(^+\) channel using patch-clamp techniques on enlarged lysosomes (Cang et al., 2014; Xu and Ren, 2015).

Fe\(^{2+}\) and Zn\(^{2+}\): Metal bound proteins degraded in the lysosome release Cu\(^{2+}\), Fe\(^{3+}\), Fe\(^{2+}\), and Zn\(^{2+}\) into the lysosome lumen, which is the major store for these ions in the cell. These ions are important to lysosome function but their functional role has yet to be fully realized. Zn\(^{2+}\) has been shown to be required for the activity of hydrolases, but high concentrations of Zn\(^{2+}\) have negative consequences, which includes inhibiting cathepsins (Xu and Ren, 2015). Furthermore, depletion of Zn\(^{2+}\) blocks endosome fusion (Aballay et al., 1995). Lysosomal Fe\(^{2+}\) is required for the Fenton reactions, ROS production, and the survival of lysosome resident pathogens (Xu and Ren, 2015).
TRPML1 is a nonselective cation channel that conducts Fe$^{2+}$ and Zn$^{2+}$ out of the lysosome, as well Ca$^{2+}$, Na$^{+}$ and K$^{+}$, although it does not conduct H$^{+}$ (Xu et al., 2007; Dong et al., 2008). Zn$^{2+}$ transporters ZIP3 and ZIP8, part of the SLC39 family, are localized to the lysosome and serve to export Zn$^{2+}$ out of the lysosome (Jeong and Eide, 2013).

Cl$^{-}$: Like H$^{+}$, Cl$^{-}$ concentrations increase along the endocytic pathway. Lysosomes contain about 80 mM Cl$^{-}$ compared to about 5-40 mM in the cytosol (Stauber and Jentsch, 2013). Chloride transporter CLC-7 is present on lysosome membranes (Fig. 1) and transports 2Cl$^{-}$ into the lysosome for each H$^{+}$ out. CLC-7 appears to be important for trafficking and/or fusion/fission events to and from the lysosome. Consequently, its loss results in severe pathology in mice and humans including accumulation of autophagic structures, osteopetrosis, and a lysosome-storage like phenotype (Weinert et al., 2010). Studies suggest that CLC-7 is not the only Cl$^{-}$ conductance in lysosomes, the molecular identities of other transporters and channels are not yet known (Stauber and Jentsch, 2013).

Although there is disagreement in the literature regarding the role of Cl$^{-}$ in lysosome acidification (Steinberg et al., 2010), Cl$^{-}$ may have additional roles in trafficking particularly in regulating ion homeostasis through co-transport as well as regulating osmolarity to assist in vesicular volume and shape (Stauber and Jentsch, 2013). Cl$^{-}$ efflux may play a role in Ca$^{2+}$ release from the lysosome (Luzio et al., 2007), possibly for counterion transport which has been shown for other organelles like the ER (Scott and Gruenberg, 2011).

**Lysosome Ca$^{2+}$ Release Channels and Ca$^{2+}$ Signaling**

Lysosomes are a significant Ca$^{2+}$ store in the cell, with measurements suggesting about 0.4 to 0.6 mM total Ca$^{2+}$ within the lysosome (Christensen et al., 2002; Lloyd-Evans et al., 2008). Indeed, the concentration of Ca$^{2+}$ in lysosomes is higher than all other intracellular vesicles other than the ER (Fig. 2) (Morgan et al., 2011). Using the fast Ca$^{2+}$ chelator BAPTA, Ca$^{2+}$ release from lysosomes has been shown to be required for late endosome-lysosome fusion in cell-free assays (Pryor et al., 2000). Lysosomal exocytosis (Samie et al., 2013), phagocytosis, membrane repair, signal transduction (Reddy et al., 2001; Lewis, 2007; Steen et al., 2007), and possibly lysosome reformation (Pryor et al., 2000).
The only known lysosomal Ca\(^{2+}\) channel is TRPML1 (Fig. 1) (Wang et al., 2014). Whereas human mutations of trpml1 cause type IV Mucolipidosis (ML-IV) (Sun et al., 2000), pathogenic inhibition of TRPML1 leads to several other LSDs (Chen et al., 1998; Shen et al., 2012). PI(3,5)P\(_2\) is an endogenous regulator of TRPML1 activity, and also plays a role in the recruitment of cytoplasmic effector proteins to regulate lysosome trafficking (Wang et al., 2014; Xu and Ren, 2015). Levels of PI(3,5)P2 have been shown to increase transiently before lysosomal exocytosis and lysosome fusion (Li et al., 2013b; Samie et al., 2013). Plasma membrane localized PI(4,5)P2 inhibits TRPML1, suggesting that TRPML1 may be inactive after lysosomal exocytosis when it is localized transiently to the plasma membrane (Zhang et al., 2012).

Ca\(^{2+}\) sensors help to ensure specificity of Ca\(^{2+}\) signalling from the lysosome. In yeast, calmodulin signals the completion of vesicle docking and triggers terminal steps in vesicle fusion (Peters and Mayer, 1998). In mammalian cells, the only identified Ca\(^{2+}\) sensor specific to the lysosome is the penta-EF-hand protein ALG-2. ALG-2 binds to the NH-terminal cytosolic tail of TRPML1 and likely regulates Ca\(^{2+}\) release (Vergarajauregui et al., 2009). Ca\(^{2+}\) sensors such as C2 domain–containing synaptotagmin VII (Syt-VII) are required for lysosomal exocytosis. Syt-VII is found on the lysosome membrane (Fig. 1) and throughout the cell (Samie et al., 2013; Samie and Xu, 2014). There are likely many other Ca\(^{2+}\) sensors involved in vesicle fusion and fission that have yet to be identified.

**Ca\(^{2+}\) Buffering**

Ca\(^{2+}\) buffers contain a highly acidic domain that binds Ca\(^{2+}\) with a low affinity but high capacity (Gelebart et al., 2005). 50-90% of ER Ca\(^{2+}\) is reversibly bound by Ca\(^{2+}\) buffers such as calnexin, calreticulin, and calsequestrin (Michalak et al., 2002). Little is known about Ca\(^{2+}\) buffers in acidic stores including lysosomes and how they regulate luminal Ca\(^{2+}\) stores as well as Ca\(^{2+}\) release and refilling (Morgan et al., 2011; Dickson et al., 2012). It has been suggested that some Ca\(^{2+}\) chelation occurs in acidic vesicles from abundant phosphate and sulphate groups in the lumen which are strong acids and can complex even at low pH (Morgan et al., 2011; Dickson et al., 2012). Polyanionic matrices like phosphate and other proteins and small organic acids (e.g. oxalic acid) within acidic vesicles also play a role (Nguyen et al., 1998; Morgan et al., 2011; Dickson et al., 2012). Notably, H\(^{+}\) competition at low pH reduces the affinity of Ca\(^{2+}\) buffers,
and as pH increases to become more neutral, the affinity of Ca\textsuperscript{2+} buffers for Ca\textsuperscript{2+} increases dramatically (Morgan et al., 2011; Dickson et al., 2012).

The relationship between free and total Ca\textsuperscript{2+} is better understood in mitochondria when compared to lysosomes (Chalmers and Nicholls, 2003). Uptake of Ca\textsuperscript{2+} buffering phosphate occurs when mitochondria take up Ca\textsuperscript{2+}, for which the \( P_i \) transporter has been identified (Iacobazzi et al., 2005). There is controversy over whether mitochondria serve as Ca\textsuperscript{2+} buffers more generally in the cell. They are indeed able to take up significant amounts of Ca\textsuperscript{2+} when exposed to non-physiological high Ca\textsuperscript{2+}, but whether this occurs under normal conditions has been doubted (Williams et al., 2013). Mitochondria do use Ca\textsuperscript{2+} signalling transiently for a wide variety of purposes.

**Refilling of Intracellular Ca\textsuperscript{2+} Stores**

How intracellular Ca\textsuperscript{2+} stores are acquired and refill has become a topic of considerable interest in recent years. Ca\textsuperscript{2+} store maintenance is best understood for the ER. Upon depletion of Ca\textsuperscript{2+} stores in the ER, ER proteins rearrange to facilitate Ca\textsuperscript{2+} entry. ER protein STIM1 is located diffusely throughout the ER membrane at rest, and has an EF-hand sequence on its luminal side which allows it to detect Ca\textsuperscript{2+} levels. When Ca\textsuperscript{2+} levels decrease, the EF hand of STIM1 unbinds Ca\textsuperscript{2+} which allows it to oligomerize. STIM1 oligomers then move into discrete puncta at ER-plasma membrane contact sites (Lewis, 2007). The exact mechanism by which STIM1 moves is not well understood but may be through passive diffusion or through an active, microtubule dependent mechanism. Orai1 is located in the plasma membrane, and upon store depletion it relocates directly apposing STIM1 at contact sites. STIM1-Orai1 interactions result in Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} current (CRAC) current after ER stores have been at least 25% depleted. The CRAC-activating domain of STIM1 interacts with Orai1 channels through electrostatic interactions to open the channel. 8 STIM1 are required to maximally open the Orai1 channel. SERCA pumps on the ER membrane then pump Ca\textsuperscript{2+} into the ER at these high Ca\textsuperscript{2+} microdomains. Ca\textsuperscript{2+} dependent inactivation of CRAC occurs within tens of milliseconds. After store refilling, both Orai1 and STIM1 redistribute throughout the ER and plasma membranes respectively (Lewis, 2011).
While ER Ca\(^{2+}\) store refilling is well understood, how lysosomes acquire and refill their Ca\(^{2+}\) is less well understood. It has been suggested that the pH gradient is important to drive Ca\(^{2+}\) into the lysosome (Christensen et al., 2002). However pH affects Ca\(^{2+}\) buffering in acidic stores significantly (Dickson et al., 2012), suggesting that alternative mechanisms of Ca\(^{2+}\) refilling may be at play. Ca\(^{2+}\) refilling of lysosomes will be discussed in more detail in Chapter 2.

**Lysosome Storage Disorders**

Impaired lysosome function is found in nearly 60 lysosome storage diseases (LSDs) whose underlying cause is a single gene mutation. Although each LSD is rare, combined they are found in about 1:5000 births. LSDs are typically caused by mutations in genes encoding catabolic enzymes, hydrolase activators, membrane proteins, or transporters (Boustany, 2013). Using a classical but slightly out-dated categorization of these diseases by accumulated material, there are glycogen storage diseases, glycoproteinoses, (sphingo)lipidoses, mucopolysaccaridoses, and neuronal ceroid lipofuscinoses (NCLs) (Schroder et al., 2010). NCLs, also known as Batten Disease, is a family of eight genetically distinct neurodegenerative disorders that result in the accumulation of a variety of lipofuscin-like materials that exhibit autofluorescent properties (Jalanko and Braulke, 2009).

LSDs share several commonalities despite having unique genetic causes. Accumulation of un-degraded materials that become toxic to the cell, impaired lipid trafficking, increased inflammation, disturbed ER Ca\(^{2+}\) homeostasis, and enhanced cellular stress responses like the unfolded protein response all characterize LSDs (Boustany, 2013).

Given the varied genes that cause LSDs, a variety of therapies have been effective in different diseases. Enzyme replacement therapy, pharmacological chaperones, substrate reduction therapy, and ER Ca\(^{2+}\) channel blockers are currently being used to varying degrees of success in several LSDs (Boustany, 2013). These therapies, stem cell therapies, and other hypothesis driven therapies including ion channel targeting are currently being examined in animal models and several new treatments for LSDs will hopefully emerge (Boustany, 2013; Xu and Ren, 2015).
About two thirds of people with LSDs have a significant neurological component that ranges in severity and manifestation (Parenti et al., 2014). Brain inflammation, alteration of intracellular trafficking, and autophagy impairment that are secondary to lysosome dysfunction in LSDs are also found in common adult neurodegenerative diseases including Alzheimer’s, Parkinson’s, and Huntington’s diseases which all have protein accumulation in common (Appelqvist et al., 2013).

**Lysosomes in Neurodegenerative Diseases**

Improper turnover of proteins and degradation is found in many common, multifactorial diseases like Alzheimer’s Disease, atherosclerosis, cancer, and Parkinson’s Disease (Levine and Kroemer, 2008; Nixon et al., 2008; Kirkegaard and Jaattela, 2009; Martinet and De Meyer, 2009; Schroder et al., 2010; Nixon, 2013). This is due at least in part to deregulated autophagic flux, as fusion of lysosomes with autophagosomes and the degradative activity of auto-lysosomes are the final steps in autophagy. Even inactivation of key autophagy related genes Atg-5 or Atg-7 in mice results in pathological neurodegeneration (Hara et al., 2006; Komatsu et al., 2006).

Neurons are especially sensitive to lysosome and autophagy defects because they are: (1) limited in their regenerative potential; (2) post-mitotic, and therefore unable to dilute stored materials during cell division; (3) heavily reliant on autophagy because they lack compensatory metabolic pathways; (4) dependent on intracellular trafficking to move materials out of the cell body and into axons and dendrites (Bellettato and Scarpa, 2010; Appelqvist et al., 2013).

Restoring lysosome function and autophagy in mouse models of Alzheimer’s disease has proven to be very beneficial and may hold hope for therapeutic targets (Pickford et al., 2008; Caccamo et al., 2010; Butler et al., 2011; Yang et al., 2011; Bahr et al., 2012; Steele et al., 2013a; Steele et al., 2013b).

**Altered Ca$^{2+}$ Homeostasis in LSDs and Neurodegenerative Diseases**

Aberrant Ca$^{2+}$ homeostasis and signalling has been implicated as a central cause of Alzheimer’s disease related dysfunction, particularly because it occurs long before other pathologic hallmarks of the disease (Pierrot et al., 2004; Stutzmann, 2007; Thibault et al., 2007; Supnet and Bezprozvanny, 2010; Chakroborty et al., 2012). ER Ca$^{2+}$ homeostasis has also been implicated
in LSDs and suggests an important potential target for current research. Furthermore, the histopathological hallmarks of LSDs and AD, like accumulated substrates such as amyloid, can result in further Ca$^{2+}$ deregulation, enhancing the disease progression in a feed-forward manner (Stutzmann, 2007; Demuro et al., 2010).

Altered ER Ca$^{2+}$ release through RYRs has been shown to play a role in the pathogenesis of AD (Kelliher et al., 1999; Stutzmann et al., 2006; Chakroverty et al., 2009; Goussakov et al., 2010; Goussakov et al., 2011; Oules et al., 2012; Peng et al., 2012). Supporting this, altered RYR expression in adults with mild cognitive impairment and AD has been demonstrated (Bruno et al., 2012). Additionally, IP3Rs have also been implicated in Alzheimer’s pathology (Stutzmann et al., 2004; Cheung et al., 2008; Cheung et al., 2010; Muller et al., 2011).

ER Ca$^{2+}$ is altered in a variety of ways in LSDs. For example, Gaucher disease is characterized by increased Ca$^{2+}$ release into the cytosol through ryanodine receptors (RYRs). GM1- and GM2-gangliosidoses have aberrant Ca$^{2+}$ uptake into the ER due to inhibition of the Sarcoplasmic/Endoplasmic Ca$^{2+}$ ATP-ase (SERCA) transporter. Reduced expression of the SERCA is also found in Neimann Pick Type A (NPA) (Boustany, 2013). Pompe disease has altered Ca$^{2+}$ homeostasis that is alleviated using RYR blockers (Lim et al., 2015). RYR blockers diltiazem and verapamil, which are used for treatment of hypertension, have been shown to restore enzyme function in patient fibroblasts from alpha-mannosidosis, Gaucher disease, and MPS IIIa (Mu et al., 2008; Platt and Lachmann, 2009).

Precise mechanisms of ER Ca$^{2+}$ regulation and dysregulation are not yet well understood in LSDs and neurodegenerative disorders, but it is clear that abnormal release and altered levels of ER Ca$^{2+}$ have negative consequences on lysosomes. Furthermore, accumulated substrates in the ER and lysosomes further alter Ca$^{2+}$ signalling and homeostasis. Thus, further investigation into the relationship between ER and lysosome Ca$^{2+}$ may help shed light on this phenomenon.

**Membrane Contact Sites: The ER as a Central Connector**

Membrane contact sites are ongoing links between a variety of membranes, typically between the ER and other organelles or the plasma membrane. Accumulated evidence suggests that the ER forms membrane contact sites with the plasma membrane (Giordano et al., 2013; Malmersjo and Meyer, 2013), mitochondria (Rizzuto et al., 1993; Szabadkai et al., 2006; de Brito and Scorrano,
ER-membrane contact sites facilitate the rapid back-and-forth exchange of materials for synthesis without requiring specific vesicles for transport, and the rapid and discrete exchange of ions. Membrane contact sites contain regulatory complexes and facilitate intraorganellar communication about nutrient state and bioenergetics. Membrane contact sites are typically 5–50 nm in diameter, are enriched locally with specific lipids and proteins, and are linked ongoingly or transiently by tethers that facilitate contact and exchange (Prinz, 2014).

A primary function of the rough ER is synthesis of membrane and secretory proteins (Dallner et al., 1963). However, the function of smooth ER, which is interspersed between areas of rough ER, has been more difficult to elucidate (Lynes and Simmen, 2011). In recent years, it has been proposed that a primary function of smooth ER is to store Ca\(^{2+}\) and to regulate membrane contact sites, exchanging proteins, ions, and lipids between the ER and other organelles (Lynes and Simmen, 2011; English and Voeltz, 2013).

Contact sites between the ER and plasma membrane have been shown to facilitate lipid synthesis, phosphatidylinositol metabolism, sterol exchange, and Ca\(^{2+}\) exchange particularly during store operated Ca\(^{2+}\) entry (SOCE) (English and Voeltz, 2013; Giordano et al., 2013; Stefan et al., 2013). ER-plasma membrane contact sites are so stable that it is necessary to eliminate six different proteins to even reduce the extensive connections between the two membranes (Manford et al., 2012).

ER-Golgi contact sites regulate the transfer of secreted proteins and sterols (Peretti et al., 2008; Mesmin et al., 2013; Prinz, 2014).

ER-endosome contact sites increase during endosome maturation (Friedman et al., 2013) and have been shown to play a role in receptor trafficking (Eden et al., 2010), cholesterol exchange (Rocha et al., 2009; Du et al., 2011; Du et al., 2012; Alpy et al., 2013; van der Kant et al., 2013; van der Kant and Neefjes, 2014) and most recently the timing and position of endosome fission (Rowland et al., 2014).
Perhaps the most studied membrane contact sites are between the ER and mitochondria. By understanding the specifics of these contact sites, the form and function ER-lysosome contact sites may be better understood. Because of this, ER-mitochondria interactions will be described in greater detail below.

**ER-Mitochondria Contact Sites: The Most Studied Membrane Contact Sites**

Contact sites between the ER and mitochondria are the best characterized inter-organelar contact sites. Microdomains of Ca\(^{2+}\) between the IP3-releasable Ca\(^{2+}\) stores of the ER and mitochondria have been suggested for many years (Rizzuto et al., 1993), and as much as 20% of mitochondrial surface membranes are in contact with the ER (Rizzuto et al., 1998). Mitochondria are also proposed to shape ER Ca\(^{2+}\) signals and buffer ER released Ca\(^{2+}\) (Rizzuto et al., 2004).

GRP75 is a proteinaceous tether linking voltage-dependent anion channel (VDAC) channels of the outer mitochondrial membrane with IP3Rs on the ER membrane. The GRP75-IP3R contact site has been shown to regulate Ca\(^{2+}\) transfer between the ER and mitochondria, although varying the length of this tether does not affect Ca\(^{2+}\) signalling, suggesting that another protein plays that role (Szabadkai et al., 2006). More recently, constitutive IP3R Ca\(^{2+}\) release has been shown to regulate bioenergetics of mitochondria and serve as a signal to suppress macroautophagy in times of nutrient abundance (Cardenas et al., 2010). Indeed, in times of ER stress, mitochondria-ER contact increases (Bravo et al., 2011). The ER has also been shown to regulate mitochondrial division sites (Friedman et al., 2011), which likely includes Ca\(^{2+}\) signalling as a final trigger.

Imaging Ca\(^{2+}\) dynamics at interorganelle contact sites between the ER and mitochondria has been done using drug-inducible, synthetic interorganellar linkers localized to the outer mitochondrial membrane and the mitochondrial-associated membrane of the ER (Csordas et al., 2010). This elegant study provided the first direct evidence of a high Ca\(^{2+}\) microdomain exceeding 10 µM at ER-mitochondria junctions, and suggested that the distance between these two organelles varies for different purposes and regulates Ca\(^{2+}\) transfer (Csordas et al., 2010).
ER-Mitochondria Contact Sites: A Model for ER-Lysosome Contact Sites?

Our understanding of ER-mitochondrial contact sites may provide hints to contact sites between lysosomes and the ER. While there are many obvious differences between mitochondria and lysosomes, there are particular similarities that when examined, may shed light on lysosomal coordination with the ER. As such, those similarities and relevant mitochondrial functions will be reviewed here briefly.

Mitochondria are known as the powerhouse of the cell, but also participate in the coordination and regulation of a variety of intracellular processes, particularly those requiring ATP. Like lysosomes, mitochondria maintain a pH gradient, which is driven by the electron transport chain coupled to the production of ATP in mitochondria (Szabadkai and Duchen, 2008). As such, mitochondrial function, like lysosome function, is tightly coupled to the energy/nutrient state of the cell.

The ER requires important resources from both lysosomes and mitochondria, providing one mechanism by which they may modulate ER function. Numerous examples exist of mitochondria modulating ER function. An obvious example is the requirement of ATP from mitochondria for function of the SERCA pumps (Szabadkai and Duchen, 2008). Likewise, lysosomes recycle all macromolecules in the cell. As the making center of the cell, the ER requires a supply of building blocks from lysosomes.

Mitochondrial-nuclear communication occurs to signal mitochondrial functional state and coordinate cellular bioenergetics. Although not well-understood in eukaryotic cells, it is known to involve Ca\(^{2+}\) (Szabadkai and Duchen, 2008). Nuclear signalling to and from lysosomes has been the focus of many recent studies and has been shown involve transcription factor EB (TFEB) (Sardiello et al., 2009; Settembre et al., 2011; Roczniak-Ferguson et al., 2012; Settembre et al., 2012).

The involvement of Ca\(^{2+}\) in lysosome-to-nucleus signalling has just been recognized to involve the lysosomal Ca\(^{2+}\) channel TRPML1 (Medina et al., 2015). The importance of Ca\(^{2+}\) in mitochondrial functioning is illustrated by the fact that the major rate limiting steps in the citric acid cycle are Ca\(^{2+}\) dependent (McCormack et al., 1990). Indeed, a major role for Ca\(^{2+}\) uptake in mitochondria is to regulate mitochondrial metabolism (Szabadkai and Duchen, 2008). This is
supported by the fact that an increase in mitochondrial Ca\textsuperscript{2+} from the ER stimulated by histamine increases ATP production (Jouaville et al., 1999). Thus, Ca\textsuperscript{2+} is required for normal function of lysosomes and mitochondria, both of which store and signal with Ca\textsuperscript{2+} ongoingly.

**Ca\textsuperscript{2+} Exchange at ER-Mitochondria Contact Sites: A Model for Ca\textsuperscript{2+} Exchange at Membrane Contact Sites**

Mitochondria take in Ca\textsuperscript{2+} through a voltage dependent anion channel (VDAC) on the outer mitochondrial membrane (OMM), fuelled by a membrane potential about 200 mV more negative than the cytosol (-200mV). VDAC was originally known for its role in channelling metabolites (Rizzuto et al., 2004). Knockdown of VDAC does not change Ca\textsuperscript{2+} handling in the mitochondria as would be expected, suggesting that there may be other pore-forming channels that also participate in Ca\textsuperscript{2+} uptake, but these have not yet been identified (Szabadkai and Duchen, 2008). Ca\textsuperscript{2+} travels to the inner mitochondrial membrane (IMM) through the mitochondrial calcium uniporter (MCU), which may be gated by Ca\textsuperscript{2+} (Kirichok et al., 2004). The molecular identity and functional specifics of the MCU remained largely elusive until recently (Baughman et al., 2011; De Stefani et al., 2011). Further, findings exist to support an additional rapid uptake mode of Ca\textsuperscript{2+} into the IMM (Szabadkai and Duchen, 2008).

Mechanisms of Ca\textsuperscript{2+} extrusion from the mitochondria are not well understood, and may be coupled to Na\textsuperscript{+} and/or H\textsuperscript{+} efflux, or Ca\textsuperscript{2+} may possibly exit directly through the VDAC (Szabadkai and Duchen, 2008). Confusion over Ca\textsuperscript{2+} efflux likely exists because it is coupled to at least one other ion, whose concentration and the overall membrane potential also play a role in regulating Ca\textsuperscript{2+} exit from mitochondria.

The importance of ER-mitochondrial contact sites was first appreciated when it was discovered that mitochondria could take up Ca\textsuperscript{2+} released from the ER at microdomains between the two vesicles (Rizzuto et al., 1993). The rate of Ca\textsuperscript{2+} entry and magnitude of Ca\textsuperscript{2+} taken into mitochondria corresponded with local microdomains of significantly higher Ca\textsuperscript{2+} when compared to that of the cytosolic increase due to IP3R stimulation (Rizzuto et al., 1993). Because of these findings, the previously observed close apposition of mitochondria to the ER in electron micrographs was appreciated (Satoh et al., 1990; Takei et al., 1992).
About 12-20% of the outer mitochondrial membrane (OMM) is estimated to be directly apposing both the smooth and rough ER; these sites are often called “membrane contact sites.” The distance between the ER and OMM is about 10-50 nm (Csordas et al., 2006), although the length varies (Pacher et al., 2000). Many contact sites exist between the ER membrane and each mitochondria, suggesting multiple junctions for different purposes. Junctions between the ER and mitochondria display “quasi-synaptic” signal transmission, particularly for Ca$^{2+}$ (Csordas et al., 1999). Said another way, these close interactions allow for rapid, efficient, and specific exchange materials and coordination of signals.

Ca$^{2+}$ uptake into mitochondria occurs quickly after IP3 induced Ca$^{2+}$ release from the ER at specific microdomains, or “hotspots” (Rizzuto et al., 1993; Rizzuto et al., 1998; Montero et al., 2000; Szabadkai et al., 2004; Gerencser and Adam-Vizi, 2005). These Ca$^{2+}$ microdomains have even been shown to be resistant to BAPTA in cardiomyocytes (Dell'agnello et al., 2007). This quick uptake of Ca$^{2+}$ by mitochondria ensures that Ca$^{2+}$ induced inactivation of IP3Rs does not occur and allows for more Ca$^{2+}$ to be transmitted (Hajnoczky et al., 1999). Knockdown of IP3 receptors does not abolish contact between the ER and mitochondria (Csordas et al., 2006), nor does disruption of microtubules or intermediate filaments (Soltys and Gupta, 1992). Even completely altering ER morphology with a mutation to decrease ER tubulation does not abolish ER-mitochondrial contact (Friedman et al., 2011).

ER-to-mitochondria tethers are known to be proteinaceous, as they are abolished after proteolysis of isolated ER-mitochondrial preparations. Ca$^{2+}$ within the mitochondria of these preparations is reduced if these tethers are abolished (Csordas et al., 2006). Intriguingly, the distance between the ER and mitochondria seems to be regulated by Ca$^{2+}$ (Csordas et al., 2006). Moreover, specific tether GRP-75 links the ligand binding domain of IP3Rs to VDAC1 on mitochondrial membranes (Szabadkai et al., 2006). Multiple different tethers exist, some of which have been identified (Szabadkai et al., 2006; de Brito and Scorrano, 2008; De Vos et al., 2012), to support a variety of processes at ER-mitochondrial junctions, and abolishing just one does not prevent tethering of these two organelles.

Ca$^{2+}$ signalling between the ER and mitochondria aids in coordinating cellular energy needs, sensed by the ER, and couples them to their production in mitochondria (Hayashi et al., 2009). Increasing IP3 and Ca$^{2+}$ in mitochondria increases ATP production (Jouaville et al., 2009).
1999). Ca\(^{2+}\) levels in the mitochondria also promote enzyme activity and protein transport (Hayashi et al., 2009). Ca\(^{2+}\) is required for the transport of PtdSer synthase from the ER MAM to mitochondria (Kuge et al., 2001). The mitochondrial associated membrane (MAM) of the ER is enriched with enzymes for lipid and glucose metabolism and biosynthesis (Hayashi et al., 2009). Because many lipid synthesizing enzymes are Ca\(^{2+}\) dependent and lipid metabolism depends on ATP, it is likely that ER-mitochondria Ca\(^{2+}\) signalling regulates lipid synthesis at multiple steps in the process (Hayashi et al., 2009). Indeed, these complexes between the ER and mitochondria have been shown to be required for synthesis of cytochrome C oxidase in the mitochondria as well as phospho- and glycosphingolipids (Voelker, 2005).

Ca\(^{2+}\) has also been shown to be involved in mitochondrial division (Breckenridge et al., 2003; Cribbs and Strack, 2007). More recently, ER tubules have been shown to mark sites of mitochondrial division (Friedman et al., 2011), presumably at least in part for Ca\(^{2+}\) transfer. Mitochondrial positioning is also regulated by Ca\(^{2+}\), with two major GTPases containing EF-hand Ca\(^{2+}\) binding domains (Fransson et al., 2006).

Mitochondria can in turn regulate the amount of Ca\(^{2+}\) released from the ER (Jouaville et al., 1995). Mitochondria release cytochrome c in response to activation of apoptotic pathways which binds to IP3 receptors increasing Ca\(^{2+}\) flux from the ER and increasing apoptotic signalling. In response to apoptotic inducing agents, the gap between the ER and mitochondria gets smaller (Csordas et al., 2006), because exchange of Ca\(^{2+}\) to the mitochondria from the IP3Rs on the ER is required for apoptosis (Wozniak et al., 2006).

In summary, membrane contact sites between the ER and mitochondria require Ca\(^{2+}\) and other tethers, coordinate multiple functions of both the ER and mitochondria, and one function is to provide Ca\(^{2+}\) to mitochondria. Considering these junctions between ER and mitochondria may provide clues to our current and future studies to understand ER-lysosome interactions and Ca\(^{2+}\) signalling.

**Research Objectives**

As discussed above, lysosome Ca\(^{2+}\) stores are essential to proper lysosome function and to trafficking in both the endosomal and autophagic pathways. However, little is known about how the lysosome acquires and maintains its Ca\(^{2+}\) store, as few studies have closely examined this
question. We first sought to test the hypothesis that pH is required for lysosome Ca\textsuperscript{2+} store refilling and show that it is not required for Ca\textsuperscript{2+} store refilling or maintenance (Chapter II). Then, encouraged by converging lines of evidence that the ER and lysosomes interact, we hypothesized that the ER Ca\textsuperscript{2+} store may directly refill lysosome Ca\textsuperscript{2+} stores. We show that ER Ca\textsuperscript{2+} stores are required for lysosomes to maintain their Ca\textsuperscript{2+} stores (Chapter III). In Chapter IV we examine which ER Ca\textsuperscript{2+} channel is important to refill lysosome Ca\textsuperscript{2+} stores. Finally, we hypothesized that altered ER Ca\textsuperscript{2+} transfer to lysosomes would impair lysosome function and showed that by blocking ER Ca\textsuperscript{2+} release, a lysosome storage phenotype results (Chapter V). This research will facilitate a better understanding of the current literature on lysosome biology and provides a foundation for many future questions (Chapter VI).
Lysosomes are the second largest Ca\(^{2+}\) store in the cell, containing 0.4 – 0.6 mM Ca\(^{2+}\). Lysosomes are also the most acidic organelles in the cell, with pH ranging from as low as 4.0 to about 4.9. Lysosome membrane proteins LAMP1 and LAMP2 make up 50% of lysosome membrane proteins. CD63 and LIMP2 are also abundant lysosome membrane proteins, providing structural support to other transporters. Syt-7 is a membrane trafficking protein that facilitates Ca\(^{2+}\) dependent fusion of membranes. Syt-7 is involved in Ca\(^{2+}\) dependent lysosome exocytosis, and is stabilized by CD63. TRPML1 is a non-selective cation channel on the lysosome membrane that conducts Ca\(^{2+}\) as well as Fe\(^{2+}\) and Zn\(^{2+}\) into the cell cytosol. A putative Ca\(^{2+}\) pump likely resides on the lysosome membrane to create the high Ca\(^{2+}\) gradient found in the lysosome. Cl\(^{-}\) transporter CLC-7 transfers 2 Cl\(^{-}\) into the lysosome lumen for each H\(^{+}\) out. The V-ATPase on the lysosome uses ATP to pump H\(^{+}\) into the lysosome. TPC1 and TPC2 are Na\(^{+}\) channels on the lysosome that release Na\(^{+}\) into the cytosol. TPC1 interacts with MTORC1 and may play a role in nutrient sensing. The lysosome nutrient sensing (LYNUS) apparatus is composed of the V-ATPase and several regulator proteins that associate and dissociate with the V-ATPase in a nutrient dependent manner to activate MTORC1. MTORC1 activation triggers anabolic processes in the cell. Conversely, AMPK associates with the V-ATPase when bound to AMP, which triggers catabolic processes in the cell. NPC1 in the lysosome membrane and NPC2 in the lysosome lumen are essential for cholesterol transport out of the lysosome.

**Figure 1.1 Lysosome Ion Channels and Transporters.** Lysosomes are the second largest Ca\(^{2+}\) store in the cell, containing 0.4 – 0.6 mM Ca\(^{2+}\). Lysosomes are also the most acidic organelles in the cell, with pH ranging from as low as 4.0 to about 4.9. Lysosome membrane proteins LAMP1 and LAMP2 make up 50% of lysosome membrane proteins. CD63 and LIMP2 are also abundant lysosome membrane proteins, providing structural support to other transporters. Syt-7 is a membrane trafficking protein that facilitates Ca\(^{2+}\) dependent fusion of membranes. Syt-7 is involved in Ca\(^{2+}\) dependent lysosome exocytosis, and is stabilized by CD63. TRPML1 is a non-selective cation channel on the lysosome membrane that conducts Ca\(^{2+}\) as well as Fe\(^{2+}\) and Zn\(^{2+}\) into the cell cytosol. A putative Ca\(^{2+}\) pump likely resides on the lysosome membrane to create the high Ca\(^{2+}\) gradient found in the lysosome. Cl\(^{-}\) transporter CLC-7 transfers 2 Cl\(^{-}\) into the lysosome lumen for each H\(^{+}\) out. The V-ATPase on the lysosome uses ATP to pump H\(^{+}\) into the lysosome. TPC1 and TPC2 are Na\(^{+}\) channels on the lysosome that release Na\(^{+}\) into the cytosol. TPC1 interacts with MTORC1 and may play a role in nutrient sensing. The lysosome nutrient sensing (LYNUS) apparatus is composed of the V-ATPase and several regulator proteins that associate and dissociate with the V-ATPase in a nutrient dependent manner to activate MTORC1. MTORC1 activation triggers anabolic processes in the cell. Conversely, AMPK associates with the V-ATPase when bound to AMP, which triggers catabolic processes in the cell. NPC1 in the lysosome membrane and NPC2 in the lysosome lumen are essential for cholesterol transport out of the lysosome.
Figure 1.2 Intracellular Organelles and Trafficking Networks. This schematic view of the cell illustrates primary intracellular vesicles as well as known Ca\textsuperscript{2+} concentrations and pH (in red) when available. The largest contiguous organelle in the cell, the endoplasmic reticulum (ER) is partially illustrated here in blue forming membrane contact sites with all organelles in the cell and the plasma membrane. (A) Clathrin-coated vesicles from the Golgi transport M6PR receptors carrying lysosomal hydrolases to late endosomes. (B) Vesicles budded from the Golgi also traffic to the plasma membrane to deliver receptor bound materials destined for the lysosome. These will be endocytosed and trafficked to the lysosome. (C) Non-clathrin coated vesicles originating in the Golgi can also fuse with late endosomes. (D) Endocytosis begins with membrane invagination and budding from the plasma membrane to form nascent endosomes (NE). (E) Recycling endosomes transport materials back to the plasma membrane that are not destined for degradation. (F) Nascent endosomes fuse to form early endosomes (EE). (G) Early endosomes mature into late endosomes through several GTPase switching steps, the final of which (Rab 5 to 7) marks the conversion to late endosomes (LE). (H) Late endosomes fuse with lysosomes so that cargo carried in intraluminal vesicles (ILVs) can be degraded by the lysosome. Late endosomes also deliver lysosome membrane proteins and hydrolases to the lysosome from the Golgi. (I) Phagopores enclose intracellular components for degradation and upon membrane closure form autophagosomes (AP). (J) Autophagosomes fuse with lysosomes for degradation
of intracellular materials. (K) Alternatively, autophagosomes fuse with late endosomes which subsequently fuse with lysosomes.
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CHAPTER II
THE LYOSOME V-ATPASE AND pH GRADIENT ARE NOT REQUIRED FOR Ca^{2+} REFILLING TO THE LYOSOME

ABSTRACT

Ion homeostasis in the lysosome lumen regulates a variety of lysosomal processes integral to lysosome function. Previous studies have suggested that the proton gradient in the lysosome, or the V-ATPase on the lysosome membrane, drives Ca^{2+} into the lysosome, and this is the prevailing view of the field. Using a new assay of Ca^{2+} release and refilling, we show that the pH gradient and V-ATPase are not required for lysosome Ca^{2+} store refilling. Instead, altering the pH of the lysosome may disrupt Ca^{2+} buffering in the lysosome and decrease the free, releasable Ca^{2+} in the lysosome lumen. The lysosome membrane-disruptor GPN results in a pH induced change in the intracellular Ca^{2+} indicator Fura-2, which may have contributed to the misunderstanding of the relationship between pH and Ca^{2+} stores in lysosomes. In addition to showing that pH is not integral for Ca^{2+} store regulation in lysosomes, we believe this work highlights the problems with previous methods of studying lysosome Ca^{2+}, specifically that disrupting membrane proteins and ion homeostasis of the lysosome is problematic when determining physiologic Ca^{2+} stores in lysosomes. We highlight the need for BAPTA-AM as a control in all intracellular Ca^{2+} assays.

INTRODUCTION

Normal function of the lysosome requires on-going maintenance of luminal ion homeostasis for ions like H^{+} and Ca^{2+}, which are 1,000-5,000 times more abundant in the lysosome lumen than the cytosol (Xu and Ren 2015). The lysosome also contains Cl^{-}, Cu^{+}, Fe^{2+}, K^{+}, Na^{+}, Zn^{2+}, and other ions (Luzio, Bright et al. 2007; Scott and Gruenberg 2011; Xu and Ren 2015), the precise spatio-temporal balance of which is likely to affect lysosome function in yet to be realized ways. Together, these ions generate a membrane potential that has been measured to range from 20 to
40 mV more negative than the cytosol (-20 to -40mV) (Xu and Ren 2015). Membrane potential is also likely to vary depending on the lysosome functional state. It seems probable that membrane potential and specific intraluminal ion concentrations directly regulate lysosome ion channels and transporters and thus lysosome function as well.

The lysosome lumen is highly acidic (pH ~4.5), driven by a vacuolar-type H⁺-ATPase (V-ATPase) on the lysosome membrane (Mindell 2012). The low pH of the lysosome plays a role in sorting processes and activates hydrolytic enzymes for the breakdown of macromolecules (Yoshimori, Yamamoto et al. 1991; Luzio, Bright et al. 2007). Improper acidification of lysosomes and/or a reduction in their Ca²⁺ stores has been found in an array of lysosome storage disorders and neurodegenerative diseases (Maxfield 2014). Additionally, the V-ATPase on the lysosome is part of the RAGulator complex for nutrient sensing at the lysosome membrane (Zoncu, Bar-Peled et al. 2011), suggesting that the role of the V-ATPase on the lysosome is far more complicated than previously thought.

Lysosome Ca²⁺ Store Regulation

Although direct measurement of luminal Ca²⁺ concentration is difficult, it has been shown to be about 0.4–0.6 mM in lysosomes (Christensen, Myers et al. 2002; Lloyd-Evans, Morgan et al. 2008). However, the source of Ca²⁺ to the lysosome has been misunderstood to date. Unlike the major Ca²⁺ store in the cell, the endoplasmic reticulum (ER), emptying lysosome Ca²⁺ stores does not induce store operated Ca²⁺ entry (SOCE) (Haller, Volkl et al. 1996). Most Ca²⁺ taken up through endocytosis is quickly lost during the course of endosomal acidification (Gerasimenko, Tepikin et al. 1998; Sherwood, Prior et al. 2007).

Ca²⁺ Refilling to the Lysosome: The pH Hypothesis

It is commonly thought that the Ca²⁺ gradient in the lysosome is established by an unidentified Ca²⁺/H⁺ exchanger or a Ca²⁺ transporter that depends on the H⁺ gradient (Morgan, Platt et al. 2011). However, much of the research supporting this view has been performed in highly specialized cell types (Lopez, Camello-Almaraz et al. 2005) or in subcellular preparations (Klemper 1985; Schulz, Thevenod et al. 1989; Docampo, Scott et al. 1995). Studies in yeast and plant vacuoles also demonstrate a Ca²⁺/H⁺ exchanger (Pittman 2011), but these findings are difficult to extrapolate to mammalian lysosomes.
Initial studies in intact mammalian cells examined secretory granules (SG) in polarized pancreatic acinar cells and suggested the V-ATPase was responsible for refilling of SG Ca\(^{2+}\) stores (Camello, Pariente et al. 2000). These conclusions were based on their findings that a cytosolic Ca\(^{2+}\) gradient initiated by ER SOCE dissipates after application of the ionophore nigericin and the V-ATPase blocker Concanamycin-A (Con-A). Importantly, they include no direct measurement of luminal Ca\(^{2+}\) or SG specific Ca\(^{2+}\) release (Camello, Pariente et al. 2000), making their conclusions largely speculative. Secretory granules are significantly less acidic than lysosomes (pH 5.5-6.0) (Tompkins, Nullmeyer et al. 2002), are insensitive to lysosome specific reagents (Gerasimenko, Sherwood et al. 2006), and have very different functions in the cell (Petersen 2015; Xu and Ren 2015) making it difficult to extrapolate these findings directly to lysosomes. Moreover, SG Ca\(^{2+}\) stores have been shown to be releasable though IP3R agonists (Gerasimenko, Gerasimenko et al. 1996) like the ER, which is not true of lysosomal Ca\(^{2+}\) stores.

Using a luminal Ca\(^{2+}\) indicator, Christensen et al. showed that by inhibiting the lysosomal V-ATPase with Baf-A, free Ca\(^{2+}\) levels in the lysosome lumen decreased slowly from 0.6 mM to 285 nM, with a corresponding increase in lysosome pH (Christensen, Myers et al. 2002). Then, using NH\(_4\)Cl, Christensen et al. also showed that cytosolic Ca\(^{2+}\) increased rapidly by 40 nM after increasing the pH of lysosomes (Christensen, Myers et al. 2002).

Because these findings are consistent with studies in yeast showing that the Ca\(^{2+}/H^+\) exchangers establish the vacuolar Ca\(^{2+}\) gradient (Pittman 2011), this “pH hypothesis” has been widely accepted (Christensen, Myers et al. 2002; Cribbs and Strack 2007; Lloyd-Evans, Morgan et al. 2008; CalcRAFT, Ruas et al. 2009; Morgan, Platt et al. 2011; Shen, Wang et al. 2012). However, large changes of luminal pH may indirectly affect many other lysosomal processes, including luminal Ca\(^{2+}\) buffering (Grabe and Oster 2001), lysosomal trafficking, or the activity of other transporters and exchangers. Thus, how lysosomal Ca\(^{2+}\) stores are refilled under more physiological conditions remains to be tested.

### Ca\(^{2+}\) Refilling to Lysosomes: The Ca\(^{2+}\) Buffer Hypothesis

More recently, Dickson et al. similarly found that when NH\(_4\)Cl was used to increase the pH of SGs and the ER, levels of free, luminal Ca\(^{2+}\) in both SGs and the ER decreased rapidly with a concomitant cytosolic Ca\(^{2+}\) increase of 40 nM. Importantly, this change was immediately
reversible upon removal of NH$_4$Cl and was observed in both SGs and the ER (Dickson, Duman et al. 2012). This finding is important because even though the pH of the ER is 6.9, just slightly lower than the cytosolic pH, it demonstrates that any changes in pH can have major consequences on luminal Ca$^{2+}$. Using CCCP to collapse SG pH, they found a similar rapid, reversible change in Ca$^{2+}$ levels that tracks with pH changes (Dickson, Duman et al. 2012). Based on their findings, Dickson et al proposed the “Ca$^{2+}$ buffer hypothesis”, which is supported by the fact that Ca$^{2+}$ buffers bind Ca$^{2+}$ much more effectively after being deprotonated (when pH increases). After inducing a non-physiologic, high pH the amount of bound-buffered Ca$^{2+}$ in the lumen of both the SGs and ER increases rapidly. This decreases free Ca$^{2+}$ within organellar compartments (Dickson, Duman et al. 2012). The concomitant small increase in Ca$^{2+}$ in the cytosol may actually be due to pH changes that affect cytosolic Ca$^{2+}$ indicators (Rudolf, Mongillo et al. 2003), because when both ER and SG Ca$^{2+}$ is released through IP3-receptors or ryanodine receptors, the Ca$^{2+}$ response is much higher. These findings support the notion that Ca$^{2+}$ binding proteins are sensitive to pH and affect the level of free Ca$^{2+}$ in acidic vesicles. However, they do not shed light onto the source of Ca$^{2+}$ to the lysosome.

**Tools to Study Lysosomes in Living Cells**

Lysosomes generally have a perinuclear localization around the microtubule organizing center. Lysosomes are difficult organelles to study due to their highly acidic lumen which can impact luminal indicators, particularly Ca$^{2+}$ indicators. However, taking advantage of the lysosome’s terminal location in the endocytic pathway and lysosome membrane proteins has enabled many methods to probe lysosome function.

**Utilizing Endocytic Pathways to Examine Lysosomes**

As the terminal compartment of the endocytic and phagocytic pathways, probes incubated in the cell culture medium will reach and accumulate within lysosomes after several hours. Fluorescent-dye dextran conjugates are the most useful in studies of lysosomes because they are relatively resistant to degradation by lysosomes. Different dextran conjugates can be used to measure pH and Ca$^{2+}$ within the lysosome. There are limitations to this method in certain cell types that have low rates of fluid phase uptake, but this does not impede studies in most cell types (Pryor 2012).
Membrane-resident receptors that are endocytosed such as the EGF receptor are another way of utilizing the endocytic pathway to study lysosomes. Tools such as fluorescent BODIPY-Lactosylceramide can also be incorporated into the plasma membrane and its trafficking to and from the lysosome can be studied (Pryor 2012).

Membrane Permeable Dyes to Study Lysosomes

Membrane permeant dyes can also be used to study lysosomes. LysoTracker probes consist of a fluorophore attached to a weak base that is only partially protonated at neutral pH. LysoTracker dyes are readily membrane permeant at neutral cytosolic pH and are highly selective for lysosomes, particularly when compared to other dyes neutral red or acridine orange dyes (Pryor 2012). Their pH sensitivity allows them to be used to measure the impact of a particular cell treatment on pH maintenance (Chazotte 2011). LysoTracker dyes can also be used to measure the size of lysosome compartments, which are enlarged in LSD phenotypes, and are a tool used to screen efficacy of treatments of LSDs (Xu, Liu et al. 2014). LysoTracker probes can be visualized at a variety of different wavelengths and require very short incubation times (15-30 mins) and low loading concentrations ~50 nM in order to effectively label lysosomes (Chazotte 2011).

Fluorescently Tagged Proteins

Transfecting cells with fluorescently tagged proteins membrane proteins is a convenient and less costly way to study lysosomes. Ideally, fluorescent tags reside on the cytosolic side of the lysosome membrane to avoid the need to use protease inhibitors to prevent the degradation of luminally-located tags by lysosome hydrolases. Furthermore, fluorescent tags should be inserted in a manner that avoids the interference with lysosome targeting sequences. Because LAMP proteins make up 50% of lysosome membrane proteins and have a cytosolic tail that can be tagged (Schwake, Schroder et al. 2013), they are commonly tagged to GFP or mCherry to provide a quick and easy way to visualize lysosomes (Pryor 2012). A similar method can be used to tag lysosomal ion channels which also reside on the membrane like TRPML1.

Stable Cell Lines for Expression of Lysosomal Proteins
Generating cell lines stably expressing lysosome membrane proteins with fluorescent tags provides greater control over expression levels. Stable cell lines also avoid saturating membranes with excessive exogenous protein and can avoid off-target effects of overexpression. This method also prevents any side-effects resulting from reagents used for transient transfection (Pryor 2012).

**Tools to Manipulate Lysosome pH**

Because pH is easy to manipulate given the currently available antagonists and other tools, the effects of pH on lysosome function are well studied. The V-ATPase can be inhibited by various macrolide antibiotics. The highly specific Bafilomycin-A (Baf-A) and Concanamycin-A (Con-A) are two such macrolides that have made substantial contributions to the study of acidic compartments (Marshansky and Futai 2008). Both Baf-A and Con-A bind to and inhibit the rotation of the $V_0$ domain in order to prevent $H^+$ translocation. Bafilomycin is impressively specific to the V-ATPase over other ATPases in the cell (Bowman, Siebers et al. 1988).

**Tools to Examine Lysosome Ca$^{2+}$**

Like pH, tools to study intracellular Ca$^{2+}$ are abundant, although many of them are still not ideal for examining the highly acidic lumen of the lysosome. Many Ca$^{2+}$ indicators are sensitive to pH, which should be considered in all analyses (Rudolf, Mongillo et al. 2003). The most commonly used tools, GPN and NAADP, have major limitations that prevented us from using them in our current study. Luminal Ca$^{2+}$ indicators have been used previously, but also have limitations. Finally, lysosome membrane targeted Ca$^{2+}$ indicators are increasing in popularity because they are not affected by low luminal pH.

NAADP is a Ca$^{2+}$ mobilizing messenger that has been suggested to be lysosome specific (Churchill, Okada et al. 2002; Calcra, Ruas et al. 2009), although this finding is controversial (Gerasimenko, Maruyama et al. 2003; Gerasimenko, Sherwood et al. 2006; Steen, Kirchberger et al. 2007; Wang, Zhang et al. 2012). Furthermore, NAADP is difficult to use because it is membrane impermeable, and not useful for a real-time refilling assay requiring repeated application because of its strong desensitization (Morgan, Platt et al. 2011).
The most common and longest-standing method of examining lysosomal Ca\textsuperscript{2+} is to disrupt lysosomal membranes using glycyld-L-phenylalanine 2-naphthylamide (GPN) (Jadot, Colmant et al. 1984; Berg, Stromhaug et al. 1994; Haller, Dietl et al. 1996; Haller, Volkl et al. 1996; Steinberg, Huynh et al. 2010; Morgan, Platt et al. 2011). GPN is a membrane-permeable di-peptide that readily diffuses into lysosomes. GPN is cleaved by the lysosome-specific exopeptidase Cathepsin C, which causes the accumulation of osmotically active products in the lysosome lumen, drawing water into the lysosome (Jadot, Colmant et al. 1984; Berg, Stromhaug et al. 1994; Steinberg, Huynh et al. 2010). This results in a transient permeabilization of the lysosome membrane (Berg, Stromhaug et al. 1994). The membrane permeability caused by GPN results in the leakage of small solutes into the cytosol including ions, changes in the pH (Fig 2.8B), changes in Ca\textsuperscript{2+} buffering within lysosomes, and in certain experimental conditions, membrane damage (Berg, Stromhaug et al. 1994; Kilpatrick, Eden et al. 2013). Short treatments with GPN are thought to be reversible after removal of GPN from the bath solution. Lysosome membranes reseal within minutes, demonstrated by their ability to take up acidotropic dyes quickly after GPN removal (Steinberg, Huynh et al. 2010).

Fura-Dextran (Christensen, Myers et al. 2002) and low-affinity Rhod-Dextran (Lloyd-Evans, Morgan et al. 2008) have been used previously in the only studies to directly examine lysosomal Ca\textsuperscript{2+} content. However, the K\textsubscript{d} of many dextrans is affected by the low pH of the lysosome (Christensen, Myers et al. 2002) and accuracy can only be achieved if the variable falls within one degree of magnitude on either side of the probe’s K\textsubscript{d} (Christensen, Myers et al. 2002; Pryor 2012). Notably, many dextrans are no longer produced because this method of analysis is being forgone for newer Ca\textsuperscript{2+} and pH indicators (based on my conversations with Invitrogen). For example, the Ca\textsuperscript{2+} sensitive, low-affinity Rhod-Dextran (Lloyd-Evans, Morgan et al. 2008) is no longer available, making direct comparisons to previous data increasingly difficult.

**Genetically Encoded Ca\textsuperscript{2+} Indicators**

In place of many luminal indicators and their associated difficulties, genetically encoded Ca\textsuperscript{2+} indicators are increasingly popular for studies of lysosome Ca\textsuperscript{2+} release unimpeded by lysosome pH. Our lab developed a lysosomally targeted, genetically encoded Ca\textsuperscript{2+} probe by fusing GCaMP3 to the N-terminus of TRPML1 (Fig. 2.1A) (Shen, Wang et al. 2012). GCaMP3-ML1 is localized on the cytosolic side of the lysosome membrane, thereby allowing Ca\textsuperscript{2+} release from
the lysosome to be measured without being affected by the highly acidic lysosome lumen. We have since established a stable cell line to allow for precise targeting and expression of our GCaMP3-ML1 probe in order to study lysosome Ca\textsuperscript{2+} release.

Utilizing lysosomal associated membrane protein-1 (LAMP1), a fluorescence resonance energy transfer (FRET)-based Ca\textsuperscript{2+} sensor was also developed to measure lysosome Ca\textsuperscript{2+} release, LAMP1-YCaM (McCue, Wardyn et al. 2013). LAMP1-YCaM has shown similar findings to our GCaMP3-ML1 that will be discussed further in Chapter 3.

**Focus of the Present Study**

The mechanisms of Ca\textsuperscript{2+} store refilling to the ER have been elucidated in recent years (Lewis 2011). However, studies examining the source of Ca\textsuperscript{2+} refilling to the lysosome are minimal and often speculative, as described above. The suggestion that the proton gradient in the lysosome is responsible for maintaining and/or refilling lysosome Ca\textsuperscript{2+} stores may have mislead many findings, and must be confirmed with newer assays. This study first sought to determine whether the pH gradient in the lysosome was required for Ca\textsuperscript{2+} refilling to the lysosome to resolve a yet unproven theory in the field.

**RESULTS**

**Genetically encoded Ca\textsuperscript{2+} sensor fused to lysosomal TRPML1 to detect intracellular Ca\textsuperscript{2+} release**

To measure lysosomal Ca\textsuperscript{2+} release without directly disrupting intraluminal ion homeostasis, we tagged the lysosomal Ca\textsuperscript{2+} channel TRPML1 with the genetically encoded Ca\textsuperscript{2+} indicator GCaMP3 (Fig. 2.1A). We generated HEK293 cell lines stably-expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells) in order to avoid off-target effects of over-expression and transfection. We used ML-SA1, a membrane-permeable synthetic agonist of the lysosome Ca\textsuperscript{2+} channel TRPML1 (Shen, Wang et al. 2012) to release Ca\textsuperscript{2+} stores (Fig 2.1). Bath application of ML-SA1 (30s) in a zero (< 10 nM) Ca\textsuperscript{2+} external solution produced robust lysosomal Ca\textsuperscript{2+} release measured by GCaMP3 fluorescence (ΔF/F\textsubscript{0} > 0.5; Fig. 2.1B).

Throughout all experiments, lysosomal Ca\textsuperscript{2+} release was recorded in a 0 Ca\textsuperscript{2+} extracellular solution, marked in blue across the top of each graph, to ensure responses were
from an intracellular source (Fig. 2.1B). Importantly, the fast, intracellular Ca\(^{2+}\) chelator BAPTA also blocked the response to ML-SA1 (Fig. 2.1C). This confirms that the GCaMP3-ML1 response to ML-SA1 in a 0 Ca\(^{2+}\) solution was specifically measuring intracellular Ca\(^{2+}\) release.

**ML-SA1 application for 30s depleted lysosome Ca\(^{2+}\) stores**

Notably, repeat applications of ML-SA1 in 0 Ca\(^{2+}\) directly after the first response evoked a much smaller or no Ca\(^{2+}\) response, suggesting that Ca\(^{2+}\) stores were largely released after first application (Fig. 2.1B). Upon second application, a significantly smaller response was sometimes evoked, which may be due to unbuffering of previously buffered Ca\(^{2+}\) stores in the lysosome, or to immediate refilling of Ca\(^{2+}\) stores. The response to ML-SA1 declined after 30s even during prolonged application (1m 30s) (Fig 2.1D), further supporting the notion that stores were emptied after ML-SA1 application.

To rule out channel desensitization, we transfected HEK cells with the plasma-membrane targeted TRPML1 mutant (TRPML1-4A). TRPML1 conducts Ca\(^{2+}\) into the cytosol regardless of what membrane it resides on, so we used the cytosolic Ca\(^{2+}\) indicator Fura-2 to measure Ca\(^{2+}\) entry through TRPML1-4A from the extracellular solution into the cytosol. Unlike TRPML1 in 0 Ca\(^{2+}\), TRPML1-4A showed repeatable Ca\(^{2+}\) responses of similar magnitude (Fig. 2.1E). This demonstrates that in unlimited Ca\(^{2+}\) conditions, the Ca\(^{2+}\) response to ML-SA1 by TRPML1 is of the same magnitude. Taken together, these findings indicate that ML-SA1 applied to HEK-GCaMP3-ML1 cells releases Ca\(^{2+}\) from an intracellular source that is largely depleted after a 30s application.

**Lysosome specificity of GCaMP3-ML1**

To confirm lysosome specificity, we assessed the location of the GCaMP3-ML1 probe. The GCaMP3-ML1 probe co-localized well with lysosomal associated membrane protein-1 (LAMP1) (Fig. 2.2A), but not with markers for the ER (Fig. 2.2B), mitochondria (Fig. 2.2C), or early endosomes (Fig. 2.2D). Pre-treatment with the lysosome-disrupting reagent GPN (Berg, Stromhaug et al. 1994) also abolished the naïve response (Fig. 2.2E), further supporting the specificity of the response. GPN is only broken down by Cathepsin-C in lysosomes, which results in osmotic lysis of lysosome membranes. Importantly, as membranes begin to reform,
responses to ML-SA1 returned gradually (Fig. 2.2E). Ca\(^{2+}\) release was also completely blocked by the TRPML-specific, synthetic antagonists ML-SI1 or ML-SI3 (Samie, Wang et al. 2013) (Fig. 2.2F). Collectively, these findings support the conclusion that responses to ML-SA1 in HEK-GCaMP3-ML1 cells are lysosome specific.

**A physiological assay to monitor lysosomal Ca\(^{2+}\) refilling**

Under physiological conditions, lysosome Ca\(^{2+}\) stores are utilized for normal signalling processes in the cell and require ongoing refilling to maintain the store. To determine the source of Ca\(^{2+}\) to the lysosome, we developed a new assay that allowed us to directly activate TRPML1, a Ca\(^{2+}\) channel on the lysosome, to deplete the original, “naïve” Ca\(^{2+}\) store. This allowed us to apply pharmacological agonists and antagonists directly during refilling to determine the source of Ca\(^{2+}\). This refilling assay is shown in Fig. 2.3A and a detailed timeline is provided in Fig. 2.3B. Responses were very consistent, and the second and third ML-SA1 responses are often slightly higher than the first, naïve response (ΔF/F\(_0\) ~ 1.4; Fig. 2.3C). Unless otherwise specified, each figure represents an average of 30-40 cells; Fig. 2.3D shows an un-averaged trace illustrating each individual cell’s response to ML-SA1. Importantly, repeatable responses to ML-SA1 were also observed in the presence of La\(^{3+}\) (Fig. 2.3E), a membrane-impermeable Ca\(^{2+}\) channel blocker (Dong, Cheng et al. 2008). For the remainder of experiments, however, we applied ML-SA1 in a 0 Ca\(^{2+}\) solution.

**Lysosome Ca\(^{2+}\) stores refill completely in 5 mins**

In order to precisely modulate store refilling, we needed to fully understand the time-course of refilling. Refilling was largely abolished without a washout in 2mM Tyrode’s, likely due to a scarcity of releasable ER Ca\(^{2+}\) that will be discussed in further detail in Chapter 3 (Fig. 2.4A). Increasing the time interval of Tyrode’s washout between consecutive applications of ML-SA1 allowed for greater refilling to occur, observable by the magnitude of the response to ML-SA1. After 1 minute in Tyrode’s, refilling began but was not complete (Fig. 2.4B). There was little difference between 5 min of refilling and 8 min of refilling (Fig. 2.4C), and we concluded that it takes approximately 5 min to fully refill lysosome Ca\(^{2+}\) stores (Fig. 2.4D). We chose 5 min of refilling time for the rest of our experiments, which allowed sufficient time to apply agonists and antagonists in Tyrode’s solution to assess the source of Ca\(^{2+}\) refilling.
The GCaMP3-ML1 probe measures Ca\textsuperscript{2+} from the same lysosomes throughout experiments

To rule out the possibility that the probe trafficked to other compartments after first stimulation, we conducted live-cell imaging experiments to show that the GCaMP3-ML1 probe remained in the lysosome after ML-SA1 treatment (Fig. 2.5A). Importantly, the probe still responded to ionomycin (Fig. 2.5B), a Ca\textsuperscript{2+} ionophore, and the number of lysosomes did not change after ML-SA1 stimulation (Fig. 2.5A). These findings also support the fact that our measurements were not from the generation of new lysosomes, but from refilling of the original, “naïve” Ca\textsuperscript{2+} store in lysosomes. Taken together, these results ensure that lysosomal Ca\textsuperscript{2+} stores can be emptied and refilled repeatedly and consistently in a time-dependent manner.

**Ca\textsuperscript{2+} refilling to lysosomes is not cell type specific**

Similar Ca\textsuperscript{2+} refilling of lysosomes was also observed in GCaMP3-ML1-transfected Cos-7 cells (Fig. 2.6A), human fibroblasts (Fig. 2.6B), and DT40 chicken B cells (Fig. 2.6C). These findings and additional results from our lab in primary mouse macrophages and mouse myoblasts suggest that Ca\textsuperscript{2+} refilling is not cell type specific.

Taken together, the aforementioned findings support the use of our lysosome-specific GCaMP3-ML1 probe and the use of ML-SA1 as a way to release Ca\textsuperscript{2+} stores from lysosomes. These tools together allow for precise and physiological way to assay the refilling mechanisms of lysosomes.

**The pH of GCaMP3-ML1 containing lysosomes is not different**

The expression of GCaMP3-ML1 on lysosome membranes does not significantly change the pH of lysosomes, as LysoTracker co-localizes strongly with GCaMP3-ML1 containing lysosomes (Fig 2.7A).

**An intraluminal Ca\textsuperscript{2+} indicator, Fura-Dextran, confirms that lysosome Ca\textsuperscript{2+} stores can be emptied by ML-SA1 and refill in a time-dependent manner**

To confirm that lysosomal Ca\textsuperscript{2+} stores can be emptied and refilled using an alternative method, we measured lysosomal Ca\textsuperscript{2+} with the Ca\textsuperscript{2+} indicator Fura-Dextran. When Fura-Dextran is pulsed into cells and then chased in indicator-free medium, it is endocytosed and then
trafficked to lysosomes (Christensen, Myers et al. 2002), evidenced by its presence inside LAMP1-positive vesicles (Fig. 2.8A). Application of the lysosome specific membrane permeabilizer GPN resulted in a small but observable decrease in the luminal Ca\(^{2+}\) indicator, further confirming lysosome specificity (Fig. 2.8B). Furthermore, ML-SA1 application in a 0 Ca\(^{2+}\) solution to HEK cells transfected with TRPML1-mCherry also decreased the luminal Ca\(^{2+}\) indicator signal (Fig. 2.8C). These findings confirm that GPN and ML-SA1 act on the same LAMP1-positive Ca\(^{2+}\) source intracellularly, and that lysosome stores can be emptied and refilled consistently. The luminal signal increased after first application of both ML-SA1 and GPN, possibly as a result of store refilling. The second application of both ML-SA1 and GPN did induce a slightly smaller response, possibly due to the increase in Fura-Dextran signal without a change in the amount of Ca\(^{2+}\) released. Because these experiments were used to confirm the ability to release and refill Ca\(^{2+}\) stores to lysosomes as a control for our GCaMP3-ML1 assay, these possibilities were not explored further.

**GPN is a lysosome-specific membrane-permeabilizer resulting in leakage of intraluminal contents into the cytosol and pH changes**

GPN is a membrane permeable substrate broken down solely by Cathepsin-C in the lysosome. After breakdown, it accumulates in the lysosome, which causes osmotic rupture of the lysosome membrane, resulting in the leakage of solutes from the lysosome lumen into the cytosol (Fig. 2.9A,B). GPN abolishes LysoTracker staining after short-term treatment (1 min) (Fig. 2.9B). Pre-treatment with GPN abolished the initial, “naïve” response to ML-SA1, confirming the GCaMP3-ML1 probe’s lysosome specificity (Fig. 2.4B). After pre-treatment, subsequent ML-SA1 responses returned gradually during lysosomal-membrane resealing (Fig. 2.4B). Additionally, using the Ca\(^{2+}\) indicator Fura-2 in untransfected HEK293T cells, GPN can be used to induce repeated responses, suggestive of Ca\(^{2+}\) refilling (Fig. 2.9C). Responses do begin to dwindle, possibly as a result of membrane damage (Berg, Stromhaug et al. 1994; Kilpatrick, Eden et al. 2013).

GPN is a useful tool to confirm lysosome specificity of intracellular assays, but it has limitations that may have confounded previous findings in the literature. We found that GPN induced a signal in Fura-2 that persisted in untransfected HEK293T cells pre-treated with membrane permeable BAPTA-AM in order to chelate intracellular Ca\(^{2+}\) (Fig. 2.9D). When
compared to ATP and GPN responses without BAPTA (Fig 2.9E), it is clear that the response to ATP is completely abolished, whereas a portion, albeit small, of the GPN response persists (Fig. 2.9D compared to 2.9E). These findings suggest that the membrane permeabilization induced by GPN results in a decreased cytosolic pH due to the leakage of the acidic contents of the lysosome lumen, which results in an increase in the signal of the Ca\(^{2+}\) indicator Fura-2. The portion of the GPN response resulting from a pH change may have been misinterpreted in the literature as entirely due to Ca\(^{2+}\) release. It is more likely that the GPN effect represents a combination of Ca\(^{2+}\) and pH effects on the Fura-2 signal.

In HEK-GCaMP3-ML1 cells pre-treated with membrane-permeable BAPTA-AM, GPN induced a non-specific increase of GCaMP3 fluorescence that persisted in the presence of the Ca\(^{2+}\) chelator BAPTA (Fig. 2.9F). GFP-based Ca\(^{2+}\) indicators are known to be particularly sensitive to pH (Rudolf, Mongillo et al. 2003) and this limited the use of GPN in GCaMP3-based assays. These findings suggest that using GPN to confirm the presence of lysosomal Ca\(^{2+}\) may be misleading, and therefore we used ML-SA1 induced Ca\(^{2+}\) release as a more precise measure of lysosome Ca\(^{2+}\) stores and release.

**The secretory and endocytic pathways are not required for lysosome Ca\(^{2+}\) refilling**

Inhibition of endocytosis using dynasore (Fig. 2.10A,E) and organelle mobility using cytoskeleton inhibitors such as nocodazole (Fig. 2.10B,E) and trichostatin A (Fig. 2.10C,E) did not block Ca\(^{2+}\) refilling to the lysosome. Furthermore, Golgi disruption using Brefeldin-A (Lippincott-Schwartz, Yuan et al. 1989; Dolman, Gerasimenko et al. 2005) also had no effect on refilling (Fig. 2.10D,E).

**K\(^{+}\) homeostasis is important for lysosome Ca\(^{2+}\) refilling**

Interestingly, impairing K\(^{+}\) homeostasis with the ionophore nigericin (Markin, Sokolov et al. 1975; Feinstein, Henderson et al. 1977; Fruth and Arrizabalaga 2007) abolished the response to ML-SA1 and lysosomal Ca\(^{2+}\) refilling (Fig. 2.11A). Hence, the secretory and endocytic pathways are not directly involved in Ca\(^{2+}\) refilling, although cation homeostasis is important to lysosome Ca\(^{2+}\) store maintenance.

**The pH gradient and V-ATPase are not required for lysosome Ca\(^{2+}\) refilling**
Previous findings suggest that the pH gradient in the lysosome may be important to Ca\(^{2+}\) refilling. However, few studies have thoroughly investigated this possibility. Bafilomycin-A (Baf-A) and Concanamycin-A (Con-A) are specific inhibitors of the V-ATPase and increase the pH of the lysosome (Bowman, Siebers et al. 1988; Yoshimori, Yamamoto et al. 1991; Drose, Bindseil et al. 1993; Futai, Oka et al. 2000). V-ATPase inhibitors have been shown to increase the pH of lysosomes above 6.5, which is the upper limit of pH indicators for acidic vesicles (Steinberg, Huynh et al. 2010). These effects were confirmed in our hands. Both Baf-A and Con-A abolished LysoTracker staining within minutes after application (Fig. 2.11B).

Surprisingly, acute application of Baf-A did not affect the subsequent response to ML-SA1, and had little effect on refilling (Fig. 2.11C). Pre-treatment with Baf-A for 1, 3 (Fig. 2.11D,E), or 16 hrs also did not significantly affect lysosome Ca\(^{2+}\) store refilling. Similarly, pre-treatment with Con-A also had no effect on Ca\(^{2+}\) refilling of lysosomes, measured either by ML-SA1 in HEK-GCaMP3-ML1 cells (Fig. 2.11F,G) or by GPN in HEK293T cells (Fig. 2.11H). These findings suggest that contradictory to previous conclusions, the pH gradient and V-ATPase are not required to refill lysosome Ca\(^{2+}\) stores. Interestingly, disrupting both H\(^{+}\) and K\(^{+}\) ion homeostasis did affect refilling. These data raise the possibility that an unidentified transporter or exchanger dependent on K\(^{+}\) plays a part in supplying Ca\(^{2+}\) to lysosomes.

**DISCUSSION**

**A new, more physiological lysosome Ca\(^{2+}\) refilling assay**

By fusing a genetically-encoded Ca\(^{2+}\) sensor directly to a lysosomal Ca\(^{2+}\) channel, we have developed a robust and sensitive method to directly measure lysosomal Ca\(^{2+}\) release independent of intraluminal pH. Our assay allows for time-lapse examination of the depletion and refilling processes of lysosome Ca\(^{2+}\) stores. It also allows for acute (<5 min) application of various pharmacological reagents, which has many advantages over prolonged treatments used in previous studies.

Our refilling assay demonstrates that Ca\(^{2+}\) stores in the lysosome refill maximally in approximately 5 minutes, and they can be released and refilled repeatedly, a phenomenon that is not cell type specific. Our GCaMP3-ML1 probe shows good co-localization with late endosome/lysosome marker LAMP1, but does not co-localize with markers of the ER,
mitochondria, and early endosomes. The initial response to ML-SA1 is abolished after pre-treatment with the lysosome disrupting agent, GPN, and as membrane reformation occurs, subsequent responses to ML-SA1 increase. All Ca\(^{2+}\) release measurements were performed in a 0 Ca\(^{2+}\) extracellular solution to ensure the response was from an intracellular Ca\(^{2+}\) source. Supporting this, the intracellular Ca\(^{2+}\) chelator BAPTA abolishes the response to ML-SA1. The membrane-impermeable TRPML blocker La\(^{3+}\) in Tyrode’s solution reproduces the effects of ML-SA1 application in 0 Ca\(^{2+}\), ruling out extracellular Ca\(^{2+}\) influx. Collectively, these data support the lysosome specificity of our GCaMP3-ML1 & ML-SA1 assay.

Lysosome Ca\(^{2+}\) stores were maximally released after 30s application of ML-SA1. In support of this, repeat applications of ML-SA1 on the plasma-membrane targeted channel mutant TRPML1-4A showed that the magnitude of Ca\(^{2+}\) response did not change when Ca\(^{2+}\) is unlimited (in 2 mM extracellular solution). When compared to ML-SA1 applied to GCaMP3-ML1 in a 0 Ca\(^{2+}\) solution, which shows a depleted response after 30s even during prolonged application, these findings support the suggestion that intracellular stores are depleted. Because there was a small response to ML-SA1 immediately after the first response, stores may not be completely depleted. It seems likely that after the release of Ca\(^{2+}\) from the lysosome lumen, there is an unbuffering of lysosome Ca\(^{2+}\) which allows for more Ca\(^{2+}\) to be released. Alternatively, refilling could have begun immediately after release. While these are difficult to test, lysosome Ca\(^{2+}\) stores were not considered refilled unless they were within 75% of the naïve response. This ensured that even if stores were not completely depleted after the first application, we were still able to detect when stores were refilled.

GCaMP3-ML1 was not trafficked elsewhere, nor were new lysosomes formed, which was observed in live-cell imaging experiments using ML-SA1 and then ionomycin, a Ca\(^{2+}\) ionophore. Importantly, during repeated applications of ML-SA1, no significant changes were observed in the size, location, or number of lysosomes. Thus, we are measuring Ca\(^{2+}\) release from the same lysosomes during the course of our experiments.

**An alternative assay using Fura-Dextran confirms lysosome Ca\(^{2+}\) store refilling**

In an additional assay, we used the Ca\(^{2+}\) indicator Fura-Dextran in lysosomes as a further confirmation that lysosomal stores can be emptied and refilled repeatedly. Fura-Dextran did load
only LAMP1 positive vesicles, but only a few LAMP1 positive vesicles were filled despite high concentrations of dye loaded. Indeed, the $K_d$ of Fura-Dextran is not ideal for the highly acidic lysosome lumen (Christensen, Myers et al. 2002). The signal from Fura-Dextran was also small, which might make it difficult to observe small differences in refilling. However, it provided a lysosome-specific control to our GCaMP3-ML1 assay. These findings support our findings that lysosome Ca$^{2+}$ stores are emptied completely and refilled, and there is no change in the lysosomal source of Ca$^{2+}$ being measured.

**Endocytic and secretory pathways are not required for lysosome Ca$^{2+}$ store refilling**

Our findings support the conclusion that the secretory and endocytic pathways are not required for lysosome Ca$^{2+}$ refilling. While secretory vesicles do fuse with lysosomes to deliver hydrolases and other integral lysosome proteins (Huotari and Helenius 2011), we showed that abolishing the Golgi had no effect on lysosome Ca$^{2+}$ stores. Endocytosis and microtubule inhibitors also did not affect lysosome Ca$^{2+}$ stores. Previous findings show that Ca$^{2+}$ is lost after endocytosis during the initial acidification of endosomes (Gerasimenko, Tepikin et al. 1998). Luminal ion concentrations vary between different endocytic vesicles to support their individual functions, and are likely to be changing ongoingly (Scott and Gruenberg 2011). Ion concentrations including H$^+$, Cl$^-$, and Ca$^{2+}$ differ with vesicle identity and are all higher in lysosomes than early endocytic vesicles. Thus, ion homeostasis is dynamically regulated and changes throughout the endocytic pathway, supporting our findings that ion concentrations are not maintained from endocytosis until lysosome maturation.

**GPN causes lysosome membrane permeabilization and a pH artefact and is not ideal to measure lysosome Ca$^{2+}$ stores**

GPN proved to be an advantageous control to ensure lysosome specificity, but not ideal for accurately measuring lysosome Ca$^{2+}$ specifically. When GPN is used with the cytosolic Ca$^{2+}$ indicator Fura-2, it results in a signal that is not completely due to Ca$^{2+}$ release because it was not abolished by BAPTA loading. Instead, GPN produces a signal in Fura-2 that is likely a combination of Ca$^{2+}$ and pH. Depending on the length of application and permeabilizing agent, lysosome membrane permeabilization can cause dramatic cytosolic pH changes, acidifying the cytosol to a pH of about 6.0-6.6 (Appelqvist, Johansson et al. 2012). It is possible that the signal
resulting from pH alone has obscured previous findings in the literature, especially those utilizing ER Ca\(^{2+}\) inhibitors (Lloyd-Evans, Morgan et al. 2008), which will be discussed further in Chapter 3. GPN should still be used as a control to ensure for lysosome specificity. If cytosolic Ca\(^{2+}\) indicator Fura-2 must be used with GPN, a more detailed analysis should be performed to subtract the pH signal and isolate Ca\(^{2+}\).

GPN disrupts homeostasis of all lysosomal ions by causing osmotic lysis of lysosome membranes, which certainly affects multiple on-going processes in the lysosome. In fact, permeabilization of lysosomes with the lysosomotropic reagent MSDH initiates a cell-death pathway (Li, Yuan et al. 2000). Lysosomal enzymes have been shown to be involved in programmed cell death (Guicciardi, Leist et al. 2004; Zuzarte-Luis, Montero et al. 2007; Kreuzaler, Staniszewska et al. 2011) and even partially permeabilizing lysosome membranes can cause apoptosis and necrosis (Turk, Stoka et al. 2002). Microinjection of lysosome specific Cathepsins into cells can also induce apoptosis (Roberg, Kagedal et al. 2002; Bivik, Larsson et al. 2006; Schestkowa, Geisel et al. 2007). While our GPN application was short (<1 min) and most lysosomal enzymes require acidic environments below pH 5.7 to be active (Appelqvist, Johansson et al. 2012), it is possible that GPN has off-target effects that effect cell health. Therefore, the consequences of permeabilizing lysosome membranes are incompletely understood and are likely more consequential than previously understood. We believe that using a more physiological and direct method of Ca\(^{2+}\) release from lysosomes, especially one that is not affected by lysosome pH, will allow a clearer picture of Ca\(^{2+}\) release and refilling of the lysosome Ca\(^{2+}\) stores.

**BAPTA-AM should be used in all intracellular Ca\(^{2+}\) assays**

BAPTA-AM did not completely block the signal caused by GPN in Fura-2. It also did not block the odd signal caused by GPN on the GCaMP3-ML1 probe. This quickly indicated to us that GPN causes signals not due to Ca\(^{2+}\) on Ca\(^{2+}\) indicators. However, BAPTA-AM controls are rarely performed in the literature. These data support the necessity of using BAPTA-AM as a control in all lysosome Ca\(^{2+}\) release assays.

**Lysosome Ca\(^{2+}\) refilling assay reveals pH does not affect lysosome Ca\(^{2+}\) stores**
Using our powerful refilling assay, we found that in contrast to previous conclusions, dissipating the proton gradient in the lysosome has little or no impact on naïve Ca\(^{2+}\) stores or their refilling. Secondary changes in lysosomal Ca\(^{2+}\) buffering and changes to other ion concentrations as a result of pH changes are likely to be the cause of incorrect interpretation of previous findings in the literature. In addition, the observed sensitivity of lysosomal Ca\(^{2+}\) stores to various pharmacological treatments using cytosolic Ca\(^{2+}\) indicators may have contained a contaminating pH component that can be easily misinterpreted.

**The Ca\(^{2+}\) buffer hypothesis explains effects of pH on Ca\(^{2+}\) stores**

A compelling study demonstrated that in secretory granules, increasing luminal pH changed the Ca\(^{2+}\) buffering capacity of SG lumen and therefore free \([\text{Ca}^{2+}]_{\text{SG}}\), and caused a minimal (~ 40 nM) increase in cytosolic Ca\(^{2+}\) (Dickson, Duman et al. 2012). Their precise measurement of luminal and cytosolic Ca\(^{2+}\) allowed them to determine that after pH changes, very little Ca\(^{2+}\) is lost from vesicle lumen. Instead, Ca\(^{2+}\) remains within vesicles and is bound by intraluminal Ca\(^{2+}\) buffers, thereby decreasing free, releasable Ca\(^{2+}\). Their findings very closely replicate those by Christiensen et al. demonstrating that while free Ca\(^{2+}\) decreases in the lysosome, the amount released into the cytosol is minimal (Christensen, Myers et al. 2002). This “buffer hypothesis” can explain the reported pH-dependence of lysosomal Ca\(^{2+}\) in some studies (Camello, Pariente et al. 2000; Christensen, Myers et al. 2002; Lopez, Camello-Almaraz et al. 2005; Morgan, Platt et al. 2011).

**Studying ions while inhibiting important lysosome functions is not ideal**

It is important to consider that the V-ATPase has other functions beyond just lysosomal pH that are just beginning to be understood. For example, the V-ATPase and its activity are required for amino acid sensing at the lysosome membrane (Zoncu, Bar-Peled et al. 2011). These findings raise the possibility that inhibition of the V-ATPase has additional repercussions beyond just pH that may have confounded previous findings in the literature. Studies inhibiting the function of any integral membrane protein on the lysosome to study lysosome ion gradients are not ideal.

The lysosome contains substantial amounts of Ca\(^{2+}\) and H\(^{+}\), but also Cl\(^{-}\), Cu\(^{+}\), Fe\(^{2+}\), K\(^{+}\), Na\(^{+}\), and Zn\(^{2+}\) (Wang, Zhang et al. 2012; Xu and Ren 2015). Although H\(^{+}\) and Ca\(^{2+}\) are commonly studied due to the availability of tools to measure these ions, all ions in the lysosome
are important to lysosome function. It seems likely that disrupting one ion in the lysosome lumen would disrupt the homeostasis of all ions in the lysosome. This is supported by the multitude of already identified channels and pumps in the lysosome membrane that are dependent on the presence of other ions. For example, it is known that acidification of the lysosome lumen must be coupled to the flux of other ions to prevent a run up of membrane potential that inhibits the V-ATPase (Steinberg, Huynh et al. 2010). Thus, even acidification requires a “counterion flux.” Ionic concentrations also vary as endosomes traffic and mature (Scott and Gruenberg 2011). Furthermore, even small imbalances in ionic charge can result in dramatic changes in membrane potential (Ishida, Nayak et al. 2013). There are numerous studies demonstrating that concentrations of luminal ions affect discrete cellular processes. For example, luminal chloride concentrations higher than 82mM inhibit a Ca\(^{2+}\) channel on the endosome (Saito, Hanson et al. 2007).

Consequently, any studies that directly inhibit the ion flux of a particular vesicle cannot conclusively determine the concentration of any other ion in that particular vesicle. It seems likely that many studies that attribute findings to the disruption of the pH gradient may not result from simply the change in luminal H\(^+\), but may be secondary to the change in other ion concentrations due to a widespread change in the electrochemical gradient, or a disruption of a particular ion transporter or exchanger (Scott and Gruenberg 2011). It has proven difficult, and it may be impossible, to uncouple the effects of each ion on the proper function of the lysosome due to their interdependence. This at least requires that several assays on any particular organelle be utilized to ensure they all converge on the same result.

**Potential mechanisms for Ca\(^{2+}\) refilling to lysosomes**

Our findings are in direct contrast with the common belief that lysosome Ca\(^{2+}\) stores depend on the proton gradient and/or the V-ATPase (Fig. 2.10). Our findings confirm largely ignored findings from isolated lysosomes that the Ca\(^{2+}\) uptake mechanism is different than the V-ATPase that acidifies the lysosome lumen (Ezaki, Himeno et al. 1992).

While the literature has focused on the role of H\(^+\) in maintaining lysosome Ca\(^{2+}\) stores, it has not considered the actual source of Ca\(^{2+}\). A high affinity uptake system that could operate at the extremely low levels of cytosolic Ca\(^{2+}\) (100 nM) would be required on the lysosome if an
intracellular source of Ca\(^{2+}\) was not utilised. Indeed, endoplasmic reticulum (ER) Ca\(^{2+}\) stores are maintained by the high affinity Sarcoplasmic/Endoplasmic reticulum Ca\(^{2+}\)-ATP-ase (SERCA) pumps, but these do not reside on lysosomes (Lewis 2007). Given the close proximity of the ER to endosomes and lysosomes (Friedman, Dibenedetto et al. 2013), it is interesting to consider that both Ca\(^{2+}\) stores would use high affinity Ca\(^{2+}\) pumping mechanisms, because this would put them in direct competition with each other.

Our findings do not rule out the possibility that the pump is ATP dependent (Klemper 1985), but they do support the hypotheses first proposed in isolated lysosome studies that Ca\(^{2+}\) may come from an intracellular source (Lemons and Thoene 1991; Ezaki, Himeno et al. 1992; Adachi, Arai et al. 1996). A low affinity uptake mechanism was suggested many years ago from a study of isolated lysosomes (Lemons and Thoene 1991), but would require a source of Ca\(^{2+}\) that could provide much higher concentrations of Ca\(^{2+}\) than cytosolic Ca\(^{2+}\). Further investigation into the molecular identity of Ca\(^{2+}\) pumps on the lysosome is warranted (Patel and Docampo 2010), but based on our results, H\(^{+}\) ions are not required for Ca\(^{2+}\) transport into the lysosome.

**A role for K\(^{+}\) in Ca\(^{2+}\) homeostasis in the lysosome?**

The finding that nigericin, a K\(^{+}\)/H\(^{+}\) antiporter, but not V-ATPase inhibitors Baf-A and Con-A that abolish the H\(^{+}\) gradient in the lysosome, inhibit lysosomal refilling is suggestive of a role for K\(^{+}\) in store maintenance or refilling. K\(^{+}\) fluxes are thought to provide a counterion flux to Ca\(^{2+}\) release in the ER (Kuum, Veksler et al. 2014). Importantly, recent and yet unpublished findings from our lab suggest the presence of a new K\(^{+}\) channel on the lysosome membrane that, when inhibited, prevents Ca\(^{2+}\) refilling to the lysosome. Thus, a K\(^{+}\) gradient in the lysosome is required for Ca\(^{2+}\) store refilling of lysosomes, potentially through its regulation of lysosome membrane potential (Wang et al, *under review in Science*).

**Limitations of the present studies**

The study of lysosome Ca\(^{2+}\) stores has been limited due to the low pH of the lysosome lumen, which limits the use of many Ca\(^{2+}\) indicators. The use of our GCaMP3-ML1 probe, located on the cytosolic side of the lysosome membrane, allowed us to overcome this limitation. The
experiments using Fura-Dextran were not calibrated and corrected for the low pH of the lysosome lumen, and therefore we did not use them to directly measure the luminal Ca\(^{2+}\) content of the lysosome.

Our studies involve the overexpression of a lysosome Ca\(^{2+}\) channel, which is an important limitation. Ca\(^{2+}\) release from lysosomes is likely of a smaller magnitude when TRPML1 is not overexpressed. Thus, overexpression of TRPML1 likely impacts the timecourse of lysosome Ca\(^{2+}\) store emptying as well as store refilling.

Whether lysosome Ca\(^{2+}\) stores are completely emptied in 30s of ML-SA1 application is difficult to elucidate. While there is no further Ca\(^{2+}\) release after 30s and the Ca\(^{2+}\) signal on GCaMP3-ML1 diminishes even during longer applications of ML-SA1, it is possible that there is still Ca\(^{2+}\) in the lysosome because there was a small response during the second application of ML-SA1 immediately following the first. Some Ca\(^{2+}\) refilling may have occurred within this time. However, the most likely explanation is that after Ca\(^{2+}\) release from the lysosome, releasable Ca\(^{2+}\) stores increase due to an unbuffering of Ca\(^{2+}\) within the lysosome lumen.

The primary Ca\(^{2+}\) store is the ER

Given that lysosomal H\(^+\) ions specifically were not required for Ca\(^{2+}\) refilling to lysosomes, we next sought to determine the source of Ca\(^{2+}\) to the lysosome. Because the ER is the largest intracellular Ca\(^{2+}\) store in the cell, we hypothesized that it may be the source of Ca\(^{2+}\) to the lysosome.

METHODS AND MATERIALS

Molecular biology. Genetically-encoded Ca\(^{2+}\) indicator GCaMP3 was fused directly to the N-terminus of ML1 (GCaMP3-ML1) (Shen, Wang et al. 2012). The pECFP-ER plasmid was obtained from CLONTECH. LAMP1-mCherry was made by fusing mCherry with the C-terminus of LAMP1. All constructs were confirmed by DNA sequencing.

Mammalian Cell Culture. HEK293 cells stably expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells) were generated using the Flip-In T-Rex 293 cell line (Invitrogen). Human fibroblasts
were obtained from the Coriell Institute for Medical Research (NJ, USA). All cells were cultured in a 37°C incubator with 5% CO₂. HEK293T cells, HEK-GCaMP3-ML1 cells, Cos-7 cells, and human fibroblasts were cultured in DMEM F12 (Invitrogen) supplemented with 10% (vol/vol) FBS or Tet-free FBS.

Human fibroblasts were transiently transfected using the Invitrogen Neon electroporation kit (1200V, 1 pulse, 30 s.) with 100µg GCaMP3-ML1. HEK293T cells, HEK-GCaMP3-ML1 cells, and Cos-7 cells were transfected using Lipofectamine 2000 (Invitrogen). All cells were used for experiments 24-48 hrs after transfection.

**Confocal imaging.** Live imaging of cells was performed on a heated and humidified stage using a Spinning Disc Confocal Imaging System. The system includes an Olympus IX81 inverted microscope, a 100X OiI objective NA 1.49 (Olympus, UAPON100XOTIRF), a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor). MetaMorph Advanced Imaging acquisition software v.7.7.8.0 (Molecular Devices) was used to acquire and analyze all images.

LysoTracker (50 nM; Invitrogen) was dissolved in culture medium and loaded into cells for 30 min before imaging as described previously (Chazotte 2011). MitoTracker (25nM; Invitrogen) was dissolved in the culture medium and loaded into cells for 15 min before imaging. Coverslips were washed 3 times with Tyrode’s and imaged in Tyrode’s.

**GCaMP3-ML1 Ca²⁺ imaging.** GCaMP3-ML1 expression was induced in Tet-On HEK-GCaMP3-ML1 cells with 0.01µg/mL doxycycline 20-24h prior to experiments. GCaMP3-ML1 fluorescence was monitored at an excitation wavelength of 470 nm (F470) using a EasyRatio Pro system (PTI). Cells were bathed in Tyrode’s solution containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Glucose, and 20 mM Hepes (pH 7.4). Lysosomal Ca²⁺ release was measured in a zero Ca²⁺ solution containing 145 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 10 mM glucose, 1 mM EGTA, and 20 mM HEPES (pH 7.4). Ca²⁺ concentration in the nominally free Ca²⁺ solution is estimated to be 1–10 μM. With 1 mM EGTA, the free Ca²⁺ concentration is estimated to be < 10 nM based on the Maxchelator software (http://maxchelator.stanford.edu/). Experiments were carried out 0.5 to 6 hrs after plating.

**Fura-2 Ca²⁺ imaging.** Cells were loaded with Fura-2 (3 µM) and Plurionic-F127 (3 µM) in the culture medium at 37°C for 45-60 min, and then culture medium was replaced for 30 min to
allow sufficient de-esterification of intracellular AM esters. Fluorescence was recorded using the EasyRatio Pro system (PTI) at two different wavelengths (340 and 380 nm) and the ratio (F_{340}/F_{380}) was used to calculate changes in intracellular [Ca^{2+}]. All experiments were carried out 1.5 to 6 hrs after plating.

**Reagents.** All reagents were dissolved and stored in DMSO or water and then diluted in Tyrode’s and 0 Ca^{2+} solutions for experiments. Con-A and Doxycycline were from Sigma; GPN was from Santa Cruz; LysoTracker, MitoTracker, Fura-2 and Plurionic F-127 were from Invitrogen; Baf-A was from L.C. Laboratories; ML-SA1 was from Chembridge.

**Data analysis.** Data are presented as mean ± SEM. All statistical analyses were conducted using GraphPad Prism. Paired t-tests were used to compare the average of three or more experiments between treated and untreated conditions. A value of P <0.05 was considered statistically significant.
Figure 2.1 ML-SAl application in 0 Ca\(^{2+}\) triggers Ca\(^{2+}\) from an intracellular source and empties lysosomal Ca\(^{2+}\) stores. (A) GCaMP3 fused to the N-terminus of TRPML1. When TRPML1 agonist ML-SAl is applied, Ca\(^{2+}\) release from the lysosome is measured directly. (B) Second application of ML-SAl directly after first application shows a dramatically reduced Ca\(^{2+}\) response. Trace represents the average of 30-40 HEK GCaMP3-ML1 cells from one coverslip. (C) After 30 min pre-treatment with 20 µM BAPTA-AM, individual responses of HEK-GCaMP3-ML1 cells to ML-SAl are abolished, demonstrating that the signal is due to Ca\(^{2+}\). (D)
Prolonged application of ML-SA1 demonstrates the Ca^{2+} signal depletes and no more Ca^{2+} is available to be released. (E) HEK cells transfected with TRPML1-4A (mutation in lysosome targeting sequence) results in TRPML1 expression on the plasma membrane. In 2mM Ca^{2+} external solution, ML-SA1 can be applied repeatedly in cells with TRPML1-4A and induce similar Ca^{2+} responses.
Figure 2.2 GCaMP3-ML1 is lysosome specific. (A) Cos7 cells transfected with Lysosome Associated Membrane Protein 1 (LAMP1) and GCaMP3-ML1. (B) Cos7 cells transfected with CFP-ER and GCaMP3-ML1. (C) Cos7 cells transfected with GCaMP3-ML1 and loaded with MitoTracker (25 nM) for 15 min. (D) Cos7 cells transfected with Early Endosome Antigen 1 (EEA1) and GCaMP3-ML1. (E) ML-SA1 application to HEK-GCaMP3-ML1 cells after pretreatment with lysosome specific substrate GPN in the bath solution to induce osmotic swelling of lysosome membranes. (F) TRPML1 inhibitor ML-SI3 co-applied with ML-SA1 during the
second ML-SA1 application. Scale bar for panels A-D represents 15 µm. Panels E, and F represent the average of 30-40 HEK GCaMP3-ML1 cells from one coverslip.
Figure 2.3 A new assay to assess Ca\(^{2+}\) refilling to lysosomes. (A) In HEK293 cells stably expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells), bath application of the TRPML1 channel agonist ML-SA1 (20 µM) in a 0 Ca\(^{2+}\) (<10 nM) external solution induced an increase in GCaMP3 fluorescence (F\(_{470}\)). After 5 minutes of washout, repeated applications of ML-SA1...
induced responses that were similar to the first one. Data represents the average of 30-40 cells on one coverslip. (B) An illustrated timecourse of the first two applications of ML-SA1 highlighting the “naïve” vs refilled response and the Tyrode’s washout period as refilling. (C) The average Ca$^{2+}$ responses of three ML-SA1 applications at intervals of 5 min (n=26 coverslips). Data represent mean ± SEM. (D) Raw traces of ML-SA1 induced GCaMP3 Ca$^{2+}$ responses of individual HEK-GCaMP3-ML1 cells on one coverslip. (E) GCaMP3 induced ML-SA1 responses after pre-treatment and on-going application of membrane impermeable TRPML1 blocker La$^{3+}$ (100 µM). Data represents the average of 30-40 cells on one coverslip.
Figure 2.4 Lysosome Ca\(^{2+}\) stores refill in approximately 5 mins. (A) Third ML-SA1 application in 0 Ca\(^{2+}\) external solution reduces the response. (B) After 1 minute of Ca\(^{2+}\) refilling to the lysosome, application of ML-SA1 resulted in a second response that was smaller than the first. (C) There was little difference between responses to ML-SA1 after 5 and 8 minutes of refilling. (D) Time dependence of lysosomal Ca\(^{2+}\) refilling. Panels A-D represent the average of 30-40 HEK-GCaMP3-ML1 cells from one coverslip.
Figure 2.5 GCaMP3-ML1 remains localized to lysosome membranes after stimulation. (A) Live cell imaging of HEK-GCaMP3-ML1 cells at basal level and after application of ML-SA1 (20 uM) and Ionomycin (10 µM). Scale bar represents 15 µm. (B) Average of 30-40 cells from one coverslip of Ca$^{2+}$ imaging of HEK-GCaMP3-ML1 cells after first application of ML-SA1 and then subsequent application of Ionomycin.
Figure 2.6 Lysosome Ca\textsuperscript{2+} refilling is not cell-type specific. (A) Cos7 cells transfected with GCaMP3-ML1. Repeated applications of ML-SA1 (20 µM) after refilling demonstrates Ca\textsuperscript{2+} refilling in these cells. (B) Human fibroblasts electroporated with GCaMP3-ML1. Repeated applications of ML-SA1 (20 µM) after refilling demonstrates Ca\textsuperscript{2+} refilling. (C) Repeated applications of ML-SA1 (20 µM) after refilling demonstrates Ca\textsuperscript{2+} refilling in DT40 chicken B cells electroporated with GCaMP3-ML1. All panels represent the average of 15-20 cells from one coverslip.
Figure 2.7 Lysosomes with GCaMP3-ML1 do not differ in pH. (A) Cos7 cells transfected with GCaMP3-ML1 show strong colocalization with LysoTracker, suggesting their acidity is not changed by the presence of the GCaMP3-ML1 probe.
Figure 2.8 Fura-Dextran shows lysosome Ca\textsuperscript{2+} store refilling. (A) After 12h pulse of Fura-Dextran in cell culture medium followed by a 4-6h chase, Fura-Dextran localized within LAMP1 positive vesicles. Scale bar represents 15 µm. (B) Repeated applications of GPN induced a reduced signal from Fura-Dextran, indicating a decrease in Ca\textsuperscript{2+} within the lysosome lumen. (C) Repeated applications of ML-SA1 (50 µM) in 0 Ca\textsuperscript{2+} decreased the Fura-Dextran within the lysosome lumen. Panels B and C represent the average of 30-40 cells from one coverslip representing n=3 experiments for GPN and ML-SA1.
Figure 2.9 Lysosome membrane permeabilization has off-target effects on the cytosolic Fura-2 Ca$^{2+}$ indicator and the GCaMP3-ML1 probe. (A) GPN induces osmotic permeabilization of lysosome membranes which results in a leakage of luminal ions and possibly other contents into the cytosol. (B) GPN abolishes LysoTracker staining in HEK293T cells, indicating it increases the pH of the lysosome lumen. ML-SA1 does not change LysoTracker staining. Scale bar represents 15 µm. (C) GPN can be applied repeatedly in HEK293T cells.
(D) Pre-treatment for 2h with 20 µM BAPTA-AM abolishes the response to ATP which releases Ca\(^{2+}\) from the ER through IP3-receptors, but not GPN in HEK293T cells. (E) Typical responses to ATP and GPN in HEK293T cells. (F) Pre-treatment and co-application of 50uM BAPTA-AM does not abolish the GPN induced signal on GCaMP3 in HEK-GCaMP3-ML1 cells, suggesting that pH is causing an artefact on the GCaMP3-ML1 probe due to membrane permeabilization by GPN. Panels C and F represent the average of 30-40 cells from one coverslip.
Figure 2.10 The endocytic and secretory pathways are not the source of Ca\(^{2+}\) to the lysosome. (A) Co-application of 50 μM Dynasore, the dynamin GTPase inhibitor, during repeated applications of ML-SA1 does not block Ca\(^{2+}\) refilling to the lysosome. (B) Pretreatment and co-application of microtubule inhibitor (5 μM) nocodazole does not prevent lysosomal Ca\(^{2+}\) refilling. (C) Co-application of microtubule inhibitor Trichostatin-A (125 nM)
after pre-treatment for 30 min. does not abolish Ca\textsuperscript{2+} refilling of lysosome stores. (D) Golgi inhibitor Brefeldin-A does not block Ca\textsuperscript{2+} refilling after pre-treatment and co-application. All panels represent the average of 30-40 HEK-GCaMP3-ML1 cells from one coverslip. (E) Quantification of 1\textsuperscript{st}, 2\textsuperscript{nd}, and 3\textsuperscript{rd} ML-SAI responses after control (Ctrl) (n=3), dynasore (Dyna) (n=2), brefeldin-A (BFA) (n=2), nocodazole (Noc) (n=4), and trichostatin-A (TSA) (n=3). For all pharmacological treatments, there were no significant differences between each response after treatments compared to controls.
Figure 2.11 The proton gradient and V-ATPase are not required for lysosome Ca$^{2+}$ refilling. (A) K$^+$ ionophore nigericin (10 µM, 5 min.) blocks refilling to lysosomes, which quickly returns after removal of nigericin. (B) V-ATPase inhibitors Bafilomycin-A (Baf-A) and Concanamycin (Con-A) quickly (<1 min.) abolished LysoTracker staining in HEK293T cells. (C) Acute application of Baf-A (5 µM) for 5 min during refilling did not abolish Ca$^{2+}$ refilling to
lysosomes. (D) Prolonged application of Baf-A (5 µM, 3h and in bath solutions) did not block Ca\textsuperscript{2+} refilling to lysosomes. (E) Quantification of 1\textsuperscript{st} (p value= 0.11), 2\textsuperscript{nd} (p= 0.01), and 3\textsuperscript{rd} (p= 0.004) ML-SA1 responses upon Baf-A treatment (n=8) compared to control traces (n=6). (F) Prolonged treatment (1h) with Con-A did not prevent lysosomes from refilling their Ca\textsuperscript{2+} stores. (G) Quantification of 1\textsuperscript{st} (p= 0.90), 2\textsuperscript{nd} (p= 0.33), and 3\textsuperscript{rd} (p= 0.66) ML-SA1 responses with Con-A pre-treatment (n=3). (H) Con-A did not reveal differences in Ca\textsuperscript{2+} refilling responses to repeated applications of GPN in untransfected HEK293T cells. Panels A, C, D, F, and H are the average of 30-40 cells from one representative coverslip/experiment. The data in panels E and G represent mean ± SEM from at least three independent experiments. All experiments were performed in HEK-GCaMP3-ML1 cells.
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CHAPTER III

THE ER Ca\textsuperscript{2+} STORE IS REQUIRED FOR Ca\textsuperscript{2+} REFILLING TO THE LYSOSOME

ABSTRACT
After concluding that the lysosomal V-ATPase and H\textsuperscript{+} gradient and are not responsible for refilling Ca\textsuperscript{2+} stores in lysosomes, we sought to determine the source of Ca\textsuperscript{2+} to the lysosome. The ER is the largest intracellular source of Ca\textsuperscript{2+} and was a likely candidate. Using pharmacological and genetic depletion as well as chelation of ER Ca\textsuperscript{2+} stores, we show that lysosome Ca\textsuperscript{2+} refilling requires ER Ca\textsuperscript{2+}. Additionally, in low extracellular Ca\textsuperscript{2+} environments, the releasable Ca\textsuperscript{2+} from the ER is diminished, and lysosome stores do not refill, corroborating our pharmacologic and genetic findings. We also show that the ER and lysosomes are in close contact during live cell imaging experiments and using TEM, which supports previous findings in the literature. Membrane contact sites between the ER and lysosomes, possibly mediated by proteinaceous tethers, are likely responsible for local microdomains of Ca\textsuperscript{2+} that refill lysosomal stores.

INTRODUCTION

In Chapter 2 our data strongly indicated that that lysosome Ca\textsuperscript{2+} stores do not require the H\textsuperscript{+} gradient or the V-ATPase using three different assays to converge on the same findings. The endoplasmic reticulum (ER) provides Ca\textsuperscript{2+} to mitochondria (Csordas et al., 1999), which suggests that it may also provide Ca\textsuperscript{2+} to the lysosome. Because of this, we first examined the potential for the ER to be the primary source of Ca\textsuperscript{2+} to the lysosome.
Previous Studies Support the ER as a Possible Source of Ca\textsuperscript{2+} to the Lysosome

An interaction between ER and lysosome Ca\textsuperscript{2+} stores was suggested many years ago (Haller et al., 1996a), but these results were overlooked due to a lack of specific tools to thoroughly probe individual Ca\textsuperscript{2+} stores. In a study of secretory granules (SGs) and their interaction with the ER, a model of ER Ca\textsuperscript{2+} tunnelling was suggested, where store operated Ca\textsuperscript{2+} entry (SOCE) after depletion of ER stores resulted in Ca\textsuperscript{2+} entry into the ER which was then “tunelled” through the cytosol (measured as a cytosolic Ca\textsuperscript{2+} gradient) to SGs (Mogami et al., 1997). Studies in SGs should not be extrapolated to lysosomes, but the idea that Ca\textsuperscript{2+} from the ER, the largest Ca\textsuperscript{2+} store in the cell, is transmitted to smaller intracellular Ca\textsuperscript{2+} stores is supported by the high amount of Ca\textsuperscript{2+} that other intracellular stores contain (0.4 – 0.6 mM for Ca\textsuperscript{2+} in the lysosome, for example).

Instead of “tunnels” of high Ca\textsuperscript{2+} in the cytosol, microdomains of high Ca\textsuperscript{2+} within small nanojunctions between vesicles is more likely (Lam and Galione, 2013), particularly because cytosolic Ca\textsuperscript{2+} is spatio-temporally regulated and functions as a final trigger in many signalling processes (Berridge et al., 2000). These would require contact sites between organelles, which in recent years have come to be appreciated (Helle et al., 2013). Indeed, mitochondria have been shown to receive Ca\textsuperscript{2+} from SOCE after its presence in the ER through close contact sites (Demaurex et al., 2009).

NAADP May Target ER Ca\textsuperscript{2+} Stores, Not Lysosome Ca\textsuperscript{2+} Stores

Ca\textsuperscript{2+} signalling between lysosomes and the ER has been suggested by studies examining nicotinic acid-adenine dinucleotide phosphate (NAADP) mediated Ca\textsuperscript{2+} signalling (Churchill et al., 2002; Kinnear et al., 2004; Calcraft et al., 2009; Fameli et al., 2014; Ronco et al., 2015). However the source of NAADP triggered Ca\textsuperscript{2+} release is highly controversial and has been shown in numerous studies to include the ER (Mojzisova et al., 2001; Hohenegger et al., 2002; Gerasimenko et al., 2003; Langhorst et al., 2004; Dammermann and Guse, 2005; Gerasimenko et al., 2006; Steen et al., 2007; Dammermann et al., 2009; Walseth et al., 2012; Wang et al., 2012; Ronco et al., 2015). The discrepancy over the source of NAADP induced Ca\textsuperscript{2+} release has led to significant confusion in the field (Guse, 2012; Lin-Moshier et al., 2012).
Interestingly, many studies utilizing NAADP are examining “acidic vesicles” which are often not well defined, and are difficult to distinguish from other organelles in cells like sea urchin eggs. Often, these studies are in fact examining secretory granules and not lysosomes (Gerasimenko et al., 2006), which do have similar Ca\textsuperscript{2+} release and refilling mechanisms to the ER (Dickson et al., 2012). Secretory granules are significantly less acidic than lysosomes (pH 6.3-5.8) and have very different functions (Tompkins et al., 2002), so these findings should not be extrapolated to lysosomes. Unfortunately, the literature has often not made the distinction between acidic organelles, which has contributed to the misunderstanding of lysosome Ca\textsuperscript{2+} stores.

**Possible Bidirectional Ca\textsuperscript{2+} Signalling Between the ER and Lysosomes**

Bidirectional Ca\textsuperscript{2+} signalling between the ER and lysosomes has been suggested in a study that used Baf-A and GPN to modulate lysosomal Ca\textsuperscript{2+} stores (Morgan et al., 2013). Baf-A and GPN are problematic tools to differentiate the effects of pH from Ca\textsuperscript{2+} (see Chapter 2). GPN causes damage to lysosomes and possibly other harmful consequences to the cell (Li et al., 2000; Guicciardi et al., 2004). Agents used to alter pH also change Ca\textsuperscript{2+} buffering within lysosomes, thereby decreasing the free, releasable Ca\textsuperscript{2+} within the lysosome (Dickson et al., 2012). Thus, bidirectional signalling between the ER and lysosomes is possible, but this should be examined with more specific reagents.

Lysosomes have also been suggested to sequester Ca\textsuperscript{2+} from the ER and modulate IP3-evoked signals (Lopez-Sanjurjo et al., 2013), which seems likely but again the findings were confounded by the lack of tools to appropriately distinguish lysosome pH from Ca\textsuperscript{2+}. Lysosome Ca\textsuperscript{2+} release (after GPN application) has also been implicated in stimulating ER Ca\textsuperscript{2+} release (Kilpatrick et al., 2013), which again seems possible but should be examined with more specific pharmacological tools.

**Limitations of Previous Studies Examining Lysosome Ca\textsuperscript{2+} Stores**

The prevailing view of lysosome Ca\textsuperscript{2+} store refilling is that the H\textsuperscript{+} gradient drives a putative Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger that refills Ca\textsuperscript{2+} to the lysosome. In addition to the aforementioned studies, this conclusion was reached from a few studies performed in subcellular fractions that differ in important ways from intact cells (Thomas et al., 2000). Many studies have used highly
specialized cell types including sea urchin eggs (Churchill et al., 2002; Morgan et al., 2013) and platelets (Lopez et al., 2005), which may have specialized mechanisms for Ca\(^{2+}\) store maintenance and release.

Importantly, no consideration was given to additional ions in the lysosome that may depend on H\(^+\) gradients but may be more directly responsible for Ca\(^{2+}\) transport. For example, a secondary pump utilizing an electrochemical gradient to transport Ca\(^{2+}\) into the lysosome may utilize an ion that itself depends more directly on H\(^+\) concentrations. This is primarily due to the limitations of available tools, which are more abundant for Ca\(^{2+}\) and H\(^+\) than for other ions. Gross disruption of lysosome ion homeostasis or membrane integrity are not ideal to probe lysosome function under physiologic conditions, however they are the only ones available until recently.

Most studies implicating H\(^+\) in maintaining the lysosome Ca\(^{2+}\) gradient included no direct measurement of lysosomal Ca\(^{2+}\). As discussed above, cytosolic Ca\(^{2+}\) measurements are often used in place of a more direct method of lysosome Ca\(^{2+}\) stores or release, but these can be confounded by pH release from lysosomes, resulting from V-ATPase inhibitors and GPN.

**Lysosome Contact with the ER**

Endosome maturation leads to increasing contact with ER (Friedman et al., 2013), which may be responsible for providing Ca\(^{2+}\) to endosomes during trafficking. Electron microscopy has also shown close contact between the ER and lysosomes (Kilpatrick et al., 2013). Ca\(^{2+}\) exchange between these nanojunctions has not yet been demonstrated due to the limitations of microscopy, but nanojunctions between the ER and endosomes have been suggested to facilitate cholesterol exchange (Rocha et al., 2009; Du et al., 2011; Du et al., 2012) and EGFR trafficking (Eden et al., 2010).

**Focus of the Present Study**

Nevertheless, studies are converging on an important Ca\(^{2+}\)-related link between the ER and lysosomes that should be examined in more detail with more specific tools. Thus, these studies sought to test the hypothesis that the ER was involved in refilling lysosome Ca\(^{2+}\) stores utilizing a more specific assay of lysosome Ca\(^{2+}\) refilling.
RESULTS

As discussed in Chapter 2, blocking Ca\(^{2+}\) entry into the cell using the generic cation channel blocker La\(^{3+}\) did not prevent Ca\(^{2+}\) refilling to lysosomes (Fig. 2.2A). These findings corroborate previous findings suggesting that Ca\(^{2+}\) in the lysosome is from an intracellular source rather than from store operated Ca\(^{2+}\) entry (SOCE) (Haller et al., 1996a). Lysosomal Ca\(^{2+}\) refilling was drastically reduced upon removal of extracellular Ca\(^{2+}\) during refilling, however (Fig. 2.4A). ER stores are passively depleted and do not release Ca\(^{2+}\) in 0 Ca\(^{2+}\) extracellular environment (Pacaud et al., 1994; Wu et al., 2006), which suggests that the ER could be the intracellular source of Ca\(^{2+}\) to the lysosome.

**ER Ca\(^{2+}\) Stores are Passively Depleted in 0 Ca\(^{2+}\) Solution.**

To confirm that releasable Ca\(^{2+}\) by the ER was reduced in 0 Ca\(^{2+}\) extracellular solution, we applied ATP to HEK293T cells. ATP stimulates endogenous P2Y receptors on the HEK cell surface (Schachter et al., 1997) which results in the formation of intracellular inositol triphosphate (IP3) (Burgess et al., 1984) and triggers release of ER Ca\(^{2+}\) stores (Schulz et al., 1989). ATP causes a significant Ca\(^{2+}\) release into the cytosol through IP3 receptors on the ER membrane (Fig 3.1A) but almost no Ca\(^{2+}\) release in 0 Ca\(^{2+}\) extracellular solution (Fig 3.1A, B). Given that lysosome Ca\(^{2+}\) stores show dramatically reduced refilling when extracellular Ca\(^{2+}\) is removed (Fig. 2.4A) and because of the demonstrated role of extracellular Ca\(^{2+}\) in ER store refilling (Lewis, 2007; Berridge, 2012), we sought to test the hypothesis that the ER is required for lysosome Ca\(^{2+}\) store refilling.

**Inhibiting SERCA Pumps on the ER Prevents Lysosome Ca\(^{2+}\) Refilling.**

Sarcoplasmic/Endoplasmic Calcium-ATPase (SERCA) pumps on the ER sequester Ca\(^{2+}\) from the cytosol due to their affinity for Ca\(^{2+}\) (K\(_m\) ~0.5-2 µM) (Lytton et al., 1992). Because of this, SERCA inhibitors can be used to release Ca\(^{2+}\) from ER Ca\(^{2+}\) stores into the cytosol down the concentration gradient. We first used thapsigargin (TG), a specific inhibitor of the ER SERCA pump that induces SOCE but does not affect cellular IP3 levels (Thastrup et al., 1990). TG rapidly and completely abolished Ca\(^{2+}\) refilling to lysosomes (Fig. 3.2A and C). A rapid block of Ca\(^{2+}\) refilling was also observed for another SERCA pump inhibitor Cyclopiazonic Acid (CPA) (Fig. 3.2B and C). The depletion of ER Ca\(^{2+}\) down its concentration gradient results in
Ca²⁺ leakage into the cytosol. Because lysosomes and the ER are in close contact, this Ca²⁺ leak is observable on our GCaMP3-ML1 probe because it is located on the lysosome lumen. Contact sites between the lysosome and ER are estimated to be between 10-50 nm. The exit of Ca²⁺ on the ER, which is also observed using Fura-2, can be detected on our GCaMP3-ML1 probe. This phenomenon has been observed on another lysosomally targeted, cytosolically located Ca²⁺ indicator (McCue et al., 2013).

CPA also reduced the response to GPN but did not completely abolish it, suggesting that it reduced Ca²⁺ stores but did not change the pH of the lysosome (see Chapter 2 for a discussion of GPN and pH in Fura-2) (Fig. 3.2D).

Notably, TG did not affect the initial, naïve ML-SA1 response (Fig. 3.2E, compare first response to second response marked with an arrow; after SERCA inhibition mean=1.08±0.07 vs. control mean=1.39±0.09, p=0.2024) or lysosomal pH (Fig. 3.2F). These results suggest that SERCA inhibitors had no direct effect on the naïve Ca²⁺ store in lysosomes, but specifically affected lysosomal Ca²⁺ refilling.

**Chelating ER Ca²⁺ Blocks Lysosome Ca²⁺ Refilling.**

We next turned towards ways to modulate ER Ca²⁺ stores less invasively. [Ca²⁺]ER, but not cytosolic Ca²⁺, can be specifically chelated by N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane-permeable metal chelator with a low affinity for Ca²⁺. TPEN has been shown to specifically chelate ER Ca²⁺ in previous studies (Hofer et al., 1998; Caroppo et al., 2003; Morgan et al., 2012). To ensure TPEN did indeed chelate ER Ca²⁺, a short application of TPEN was shown to quick block ER Ca²⁺ release stimulated by the endogenous P2Y receptor agonist ATP (Fig 3.3A compared to Fig 3.3B). Importantly, TPEN did not chelate the naïve lysosomal Ca²⁺ store, observed with GPN in Fura-2 (Fig 3.3C compared to Fig 3.3B). The magnitude of the GPN. Thus, TPEN is an ideal tool to examine Ca²⁺ refilling to lysosomes because it had no direct effect on the naïve Ca²⁺ store in lysosomes, but specifically chelated ER Ca²⁺.

Acute application of TPEN during refilling completely blocked lysosomal Ca²⁺ refilling (Fig. 3.3D,E). When TPEN was applied for 20 mins in the bath solution, however, the responses to ATP and GPN were both abolished, suggesting that disrupting ER Ca²⁺ stores and Ca²⁺ release...
long-term also disrupts ongoing lysosome Ca\(^{2+}\) store maintenance (Fig 3.3F). These findings suggest that disrupting ER Ca\(^{2+}\) without affecting Ca\(^{2+}\) release into the cytosol prevents Ca\(^{2+}\) refill to lysosomes.

**Chronic Ca\(^{2+}\) Store Reduction of the ER Prevents Lysosome Ca\(^{2+}\) Refilling.**

The ER Ca\(^{2+}\) store can also be genetically and chronically reduced without raising intracellular Ca\(^{2+}\) levels by transfecting cells with the IP3R ligand-binding domain with an ER targeting sequence (IP3R-LBD-ER) (Varnai et al., 2005). As shown previously, IP3R-LBD-ER expression decreased IP3R Ca\(^{2+}\) release stimulated by the P2Y receptor agonist ATP (Fig. 3.4A compared to 3.3B). Importantly, basal cytosolic levels of Ca\(^{2+}\) were not different after IP3R-LBD-ER transfection (Fig. 3.4A), which was also found in the original study (Varnai et al., 2005). Interestingly, IP3R-LBD-ER also significantly reduced the GPN induced lysosomal Ca\(^{2+}\) release in HEK293T cells, which may indicate a complete depletion of lysosomal Ca\(^{2+}\) if the pH artefact in Fura-2 is taken into account (Fig 3.4A compared to 3.3B). In HEK-GCaMP3-ML1 cells transfected with IP3R-LBD-ER, lysosomal Ca\(^{2+}\) release was significantly reduced when compared to untransfected cells on the same coverslip (Fig. 3.4B). These findings show that by genetically reducing ER Ca\(^{2+}\) stores ongoinly, lysosomal Ca\(^{2+}\) stores are also reduced. The ER is still able to release Ca\(^{2+}\) and Ca\(^{2+}\) stores refill ongoinly, but are significantly smaller (Varnai et al., 2005). Here, a non-pharmacological method of reducing ER Ca\(^{2+}\) converges on findings using pharmacological manipulations of ER Ca\(^{2+}\) to support the notion that lysosome Ca\(^{2+}\) stores are maintained and refilled by ER Ca\(^{2+}\) stores.

**The ER and Lysosomes are in Close Contact which may Facilitate Ca\(^{2+}\) Exchange.**

Collectively, the aforementioned findings suggest that the ER, the major Ca\(^{2+}\) store in the cell, is essential for refilling and the ongoing maintenance of lysosomal Ca\(^{2+}\) stores. Interestingly, the Ca\(^{2+}\) released from SERCA inhibition on the ER was detectable on our GCaMP3-ML1 probe (Fig. 3.2), likely due to membrane contact sites between the ER and lysosomes (Penny et al., 2014). Ca\(^{2+}\) release from the ER was also observed on LAMP1-YCaM, another genetically encoded, lysosomally targeted chameleon Ca\(^{2+}\) sensor (McCue et al., 2013).

Using time-lapse confocal imaging, we found that the majority of lysosomes, marked by LAMP1-mCherry, move and traffic together with ER tubules, labelled with CFP-ER, throughout
the cell (Fig. 3.5A,B). White boxes highlight areas within the same cell where the ER appears to move and reform to maintain contact with the lysosome (Fig. 3.5A,B), and even generate new tubules to continue contact with lysosomes (Fig. 3.5A see difference between 0:02 and 0:03).

We also used TEM to probe potential ER-lysosome contact sites. ER tubules (outlined in red) are found directly apposing lysosomes (outlined in blue) (Fig 3.5C). This has been shown previously with BSA loaded lysosomes (Kilpatrick et al., 2013). In Fig. 3.5C the distance between the ER and lysosome membrane is 23.4 nM. Thus, the ER could be the direct source of Ca\(^{2+}\) to lysosomes through nanojunctions to facilitate Ca\(^{2+}\) exchange.

DISCUSSION

ER is the Source of Ca\(^{2+}\) to the Lysosome

Under conditions of low extracellular Ca\(^{2+}\), the amount of Ca\(^{2+}\) released from the ER is significantly reduced, which we showed to significantly decrease lysosomal Ca\(^{2+}\) refilling in Chapter 2. Then, using SERCA inhibitors we depleted the ER Ca\(^{2+}\) gradient and showed that lysosome Ca\(^{2+}\) store refilling was abolished. Using pharmacological chelation of ER Ca\(^{2+}\) with TPEN, we showed that Ca\(^{2+}\) refilling to lysosomes is again blocked when releasable Ca\(^{2+}\) from the ER is abolished. Importantly, manipulations of ER Ca\(^{2+}\) do not affect the naïve lysosome Ca\(^{2+}\) store, but do impact Ca\(^{2+}\) store refilling to lysosomes. We also significantly diminished ER Ca\(^{2+}\) ongoingly using genetic manipulation of IP3-receptors with an IP3R-LBD-ER to deplete releasable Ca\(^{2+}\) (Varnai et al., 2005). Ca\(^{2+}\) refilling to lysosomes was significantly diminished using this genetic approach in HEK-GCaMP3-ML1 cells. After this long-term manipulation of ER Ca\(^{2+}\), naïve Ca\(^{2+}\) stores in lysosomes released using GPN are also significantly decreased. Thus, inhibiting ER Ca\(^{2+}\) store maintenance, chelating ER Ca\(^{2+}\), and genetically reducing ER Ca\(^{2+}\) stores all demonstrate that ER Ca\(^{2+}\) is important for lysosome Ca\(^{2+}\) store refilling (see summary Fig. 3.6).

Our results not only provide an explanation for the reported sensitivity of the Ca\(^{2+}\) stores of acidic organelles to long-term treatment with ER disrupting agents (Haller et al., 1996a; Menteyne et al., 2006; Patel and Docampo, 2010), but are also consistent with the observations that lysosomes may buffer cytosolic Ca\(^{2+}\) released from the ER (Lopez-Sanjurjo et al., 2013). Our findings suggest a new role for ER-lysosome contact sites that have already been found to
regulate cholesterol exchange (Rocha et al., 2009; Du et al., 2011; Toulmay and Prinz, 2011; Du et al., 2012) and receptor trafficking (Eden et al., 2010).

Membrane Contact Sites May Facilitate Ca\textsuperscript{2+} Exchange between the ER and Lysosomes

Our time lapse imaging and TEM findings corroborate previous findings that membrane contact sites exist between the ER and lysosomes (Kilpatrick et al., 2013). Nanojunctions of 50 nm or less are ideal for Ca\textsuperscript{2+} exchange (Fameli et al., 2014), making these sites between the ER and lysosomes ideal to serve as “nano-domains” of Ca\textsuperscript{2+} exchange. ER-endosome membrane contact, although currently still difficult to study, is proposed to facilitate cholesterol transport from endosomes to the ER (Rocha et al., 2009; Du et al., 2011; Du et al., 2012; van der Kant and Neefjes, 2014). Given the established role of lysosomal Ca\textsuperscript{2+} release in cholesterol transport (Shen et al., 2012), lysosomal Ca\textsuperscript{2+} may have a direct role in regulating this ER-lysosome interaction.

The ability to observe the TG induced Ca\textsuperscript{2+} signal on our GCaMP3-ML1 probe further supports the close contact of these two organelles. A very different LAMP1-targeted Ca\textsuperscript{2+} indicator was able to observe a similar phenomenon (McCue et al., 2013). In mitochondria, the estimated distance of 10-25 nm has been suggested to be far enough to isolate mitochondria from a slow leak of Ca\textsuperscript{2+} from the ER (Csordas et al., 2006). Given the widespread distribution of SERCA pumps on the ER, it seems likely that Ca\textsuperscript{2+} leak from ER SERCA pumps could be observed on our overexpressed GCaMP3-ML1 probe.

Specific tethers that link IP3R Ca\textsuperscript{2+} release channels on the ER to the voltage dependent anion channel (VDAC) on the outer mitochondrial membrane may function to ensure Ca\textsuperscript{2+} transfer specificity (Szabadkai et al., 2006). Similarly, more specific Ca\textsuperscript{2+} transfer to lysosomes likely occurs in specialized regions between the ER and lysosomes, which would not be observable on our GCaMP3-ML1 probe. Interestingly, during ER stress, Ca\textsuperscript{2+} overload from SERCA leaks increases ER-mitochondrial contact sites and triggers the mitochondrial apoptotic pathway (Chami et al., 2008). It is possible that Ca\textsuperscript{2+} overload during ER stress also functions at ER-lysosome contact sites to trigger lysosomal apoptotic pathways as well.
Functional Interactions between ER and Lysosome Ca\textsuperscript{2+} stores.

A functional interaction between ER and lysosome Ca\textsuperscript{2+} stores was previously suggested (Haller et al., 1996b; Haller et al., 1996a), but these results have been largely ignored, presumably due to the lack of specific tools required for definitive interpretation. Several recent studies have suggested that the ER and lysosome Ca\textsuperscript{2+} stores interact (Kilpatrick et al., 2013; Lopez-Sanjurjo et al., 2013; Morgan et al., 2013; Lopez Sanjurjo et al., 2014). However, these studies used problematic methods for probing lysosome Ca\textsuperscript{2+} or apply more broadly to an acidic Ca\textsuperscript{2+} store that is likely not the lysosome, making their interpretation difficult. A closer look at all of these papers supports an interaction between the ER and acidic Ca\textsuperscript{2+} stores, but raises the issue that these are all indirect observations of luminal Ca\textsuperscript{2+} release measured using cytosolic Ca\textsuperscript{2+} indicators and thus very speculative.

In some cell types, lysosome permeabilization with GPN has been shown to trigger ER Ca\textsuperscript{2+} release (Kilpatrick et al., 2013), and in other cell types ER Ca\textsuperscript{2+} release has been shown to subsequently trigger Ca\textsuperscript{2+} release from acidic vesicles (Morgan et al., 2013). Lysosomes have been suggested to absorb Ca\textsuperscript{2+} release from IP3-evoked Ca\textsuperscript{2+} signals on the ER, measured before and after Baf-A treatment (Lopez-Sanjurjo et al., 2013). A rapid recycling between IP3-sensitive stores and lysosomes has also been suggested, evidenced by a difference in cytosolically measured Carbachol (CCh) responses before and after Baf-A treatment (Lopez Sanjurjo et al., 2014). Collectively, these observations can seem in direct opposition to each other, but could also simply suggest that the two Ca\textsuperscript{2+} stores are in close proximity and engage in signalling with each other. Even studies closely examining the effects of NAADP on ER and lysosome Ca\textsuperscript{2+} stores suggest this possibility (Ronco et al., 2015). Indeed, many intracellular Ca\textsuperscript{2+} stores have been implicated in Ca\textsuperscript{2+} oscillations in the cell. These studies do not provide a physiological basis for Ca\textsuperscript{2+} signalling because they are all based on observations following lysosome disrupting agents. Our findings provide one function of ER-lysosome Ca\textsuperscript{2+} exchange that can be observed in physiological conditions in intact cells.

The ER as the Source of Ca\textsuperscript{2+} to the Lysosome May Explain the NAADP Controversy

The overlap of ER and lysosome stores, which is at the center of the NAADP controversy, is likely fuelled at least in part by the fact that TG does not affect the naïve Ca\textsuperscript{2+} store but instead
the refilled store ([Fig 3.2E](#) and see Fig. 3 in (Patel and Docampo, 2010). TPEN was shown to block lysosome stores after 20 mins, and these findings support the hypothesis that lysosome stores are being released ongoingly, and refilling must be continuous. This possibility will be examined in greater detail in Chapter 4, and may partly explain why the literature often sees overlap between pharmacological agents that affect the ER Ca\(^{2+}\) store after short-term application, but affect both stores after long-term application.

A nuanced analysis of store overlap for NAADP and other Ca\(^{2+}\) mobilizing agents using varied time courses has not yet been performed. NAADP may be one such case of this overlap confusion, especially since it has been shown to be insensitive to the lysosome specific GPN (Gerasimenko et al., 2006) and to require ryanodine receptors for Ca\(^{2+}\) release (Gerasimenko et al., 2003; Dammermann and Guse, 2005). Another possibility is that contact sites between lysosomes and RYRs on the ER are the site of NAADP Ca\(^{2+}\) signalling, which has been suggested (Kinnear et al., 2008). This contact site may serve a different functional purpose than sites to refill Ca\(^{2+}\) that involve IP3Rs. It is also possible to speculate that NAADP functions to couple lysosomes to the ER or to trigger ER Ca\(^{2+}\) transfer to lysosomes (Ronco et al., 2015). Store specific analyses and better resolution will allow for a better understanding of how NAADP acts to mobilize Ca\(^{2+}\) stores.

**Previously Assumed to be ER Specific Reagents also Affect Lysosome Ca\(^{2+}\) Stores**

In addition, our work reveals that, depending on the treatment conditions (acute *versus* prolonged treatment), many assumed-to-be ER-specific reagents may indirectly affect lysosome Ca\(^{2+}\) stores after long-term treatment, and this may impact the interpretations of a large body of literature on Ca\(^{2+}\) signalling. For example, previous studies have attempted to improve LSD symptoms by increasing lysosomal Ca\(^{2+}\) using ER SERCA inhibitors such as Curcumin (Lloyd-Evans et al., 2008). In my hands, I found curcumin to increase cytosolic Ca\(^{2+}\) measured with Fura-2 as expected, and also to completely abolish lysosomal Ca\(^{2+}\), measured with GPN in Fura-2 as well as in GCaMP3-ML1 cells (data not shown). These effects may be due to curcumin’s inhibitory effects on SERCA pumps (Bilmen et al., 2001), or its inhibition of IP3Rs (Dyer et al., 2002). Other studies have noted that SERCA inhibitors antagonize the GPN signal, but have not expounded upon this finding (Haller et al., 1996b; Sivaramakrishnan et al., 2012). Notably,
however, any alteration of ER Ca\(^{2+}\) may affect lysosomal Ca\(^{2+}\) after a short time, and should be considered carefully.

**ER-to-Lysosome Ca\(^{2+}\) Transport May be altered in Diseased States.**

Dysfunction of lysosomal Ca\(^{2+}\) stores and Ca\(^{2+}\) release has been implicated in the pathogenesis of acute pancreatitis (Gerasimenko et al., 2009), Alzheimer’s Disease (Coen et al., 2012), and lysosome storage disorders (Shen et al., 2012). The unexpected role of the ER in maintaining Ca\(^{2+}\) stores in lysosomes may help resolve the long-standing mystery of how impaired ER Ca\(^{2+}\) homeostasis is commonly seen in lysosomal storage diseases (LSDs) (Cribbs and Strack, 2007; Coen et al., 2012), and manipulating ER Ca\(^{2+}\) reduces lysosome storage (Lloyd-Evans et al., 2008; Mu et al., 2008; Ong et al., 2010).

**Limitations**

Pharmacological studies always have limitations that should be considered, as many pharmacological reagents can have multiple targets and effects within the cell. As such, multiple lines of pharmacological manipulations should converge upon the same findings to rule out off target effects. Although these studies did utilize multiple mechanisms of pharmacological inhibition, additional effects of these treatments should be considered.

SERCA pumps are localized to the ER and also reside on the Golgi, which interacts with lysosomes through trafficking in the trans-Golgi network (TGN) (Wuytack et al., 2002). Abolishing the Golgi with Brefeldin-A (Chapter 2) did not affect Ca\(^{2+}\) store refilling to lysosomes, suggesting that the effects of inhibition of SERCA pumps on lysosome Ca\(^{2+}\) stores is due to their effects on ER Ca\(^{2+}\) stores.

TPEN has been shown previously to chelate ER Ca\(^{2+}\), but it is also known to chelate Zn\(^{2+}\) which is a co-factor for many enzymes and a modulator of a variety of ion channels. TPEN abolished Ca\(^{2+}\) release from IP3Rs but not Ca\(^{2+}\) release from lysosomes measured with GPN, supporting its use as an ER specific chelator. However, an additional control could be performed to show that it does not abolish the naïve response to ML-SA1 in HEK-GCaMP3-ML1 cells to further support the notion that it does not chelate lysosomal Ca\(^{2+}\). TPEN may also be
contributing to abolishing Ca\textsuperscript{2+} refilling if Zn\textsuperscript{2+} is required for this process, and should be kept in mind.

**Future Directions**

Knowing that pharmacological agents that affected IP3-releasable pools blocked Ca\textsuperscript{2+} refilling to lysosomes (Authi et al., 1993; Aulestia et al., 2011), we next sought to determine which ER Ca\textsuperscript{2+} channel was responsible for Ca\textsuperscript{2+} refilling to lysosomes in Chapter IV.

**METHODS AND MATERIALS**

**Molecular biology.** Genetically-encoded Ca\textsuperscript{2+} indicator GCaMP3 was fused directly to the N-terminus of ML1 (GCaMP3-ML1) as described previously (Shen et al., 2012). The IP3R-LBD-ER construct (Varnai et al., 2005) was a kind gift from Dr. Thomas Balla (National Institute of Child Health and Human Development, NIH). The pECFP-ER plasmid was obtained from CLONTECH. LAMP1-mCherry was made by fusing mCherry with the C terminus of LAMP1.

**Mammalian Cell Culture.** All cells were cultured in a 37°C incubator with 5% CO\textsubscript{2}. HEK293T cells, Tet-On HEK293 cells stably expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells), and Cos-7 cells were cultured in DMEM F12 (Invitrogen) supplemented with 10% (vol/vol) FBS or Tet-free FBS.

   HEK293T cells, HEK-GCaMP3-ML1 cells, and Cos-7 cells were transfected using Lipofectamine 2000 (Invitrogen). All cells were used for experiments 24-48 hrs after transfection.

**Confocal imaging.** Live imaging of cells was performed on a heated and humidified stage using a Spinning Disc Confocal Imaging System. The system includes an Olympus IX81 inverted microscope, a 100X Oil objective NA 1.49 (Olympus, UAPON100XOTIRF), a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor). MetaMorph Advanced Imaging acquisition software v.7.7.8.0 (Molecular Devices) was used to acquire and analyze all images.
Lysotracker (50nM) was loaded into cells for 30 minutes in cell culture medium (Chazotte, 2011). After LysoTracker loading, coverslips were washed 3 times with Tyrode’s and imaged in Tyrode’s.

**TEM.** Confluent Cos7 cell cultures in 10 mm dishes were prepared for TEM by washing twice in Sorensen’s buffer and then fixing for 30 min in 2.5% glutaraldehyde in 0.1M Sorensen’s buffer. Post-fixation was performed for 15 min with 1% osmium tetroxide in 0.1M Sorensen’s buffer. After rinsing, cells were stained in 7% uranyl acetate dissolved in ddH2O for 15 min. After rinsing in ddH2O, cells were dehydrated in increasing concentrations of ethanol and infiltrated with Epon. After infiltration, cells were pelleted and polymerized for 24h at 60ºC. After pelleting and application on TEM grids, cells were post-stained with 7% Uranyl acetate.

A Philips CM-100 TEM microscope was used for TEM and image acquisition was performed with a AmtV600 camera.

**GCaMP3-ML1 Ca²⁺ imaging.** GCaMP3-ML1 expression was induced in Tet-On HEK-GCaMP3-ML1 cells 20-24h prior to experiments using 0.01µg/mL doxycycline. GCaMP3-ML1 fluorescence was monitored at an excitation wavelength of 470 nm (F₄₇₀) using a EasyRatio Pro system (PTI). Cells were bathed in Tyrode’s solution containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Glucose, and 20 mM Hepes (pH 7.4). Lysosomal Ca²⁺ release was measured in a zero Ca²⁺ solution containing 145 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 10 mM glucose, 1 mM EGTA, and 20 mM HEPES (pH 7.4). Ca²⁺ concentration in the nominally free Ca²⁺ solution is estimated to be 1–10 μM. With 1 mM EGTA, the free Ca²⁺ concentration is estimated to be < 10 nM based on the Maxchelator software (http://maxchelator.stanford.edu/). Experiments were carried out 0.5 to 6 hrs after plating.

**Fura-2 Ca²⁺ imaging.** Cells were loaded with Fura-2 (3 μM) and Plurionic-F127 (3 μM) in the culture medium at 37°C for 60 min. Florence was recorded using the EasyRatio Pro system (PTI) at two different wavelengths (340 and 380 nm) and the ratio (F₃₄₀/F₃₈₀) was used to calculate changes in intracellular [Ca²⁺]. All experiments were carried out 1.5 to 6 hrs after plating.
**Reagents.** All reagents were dissolved and stored in DMSO or water and then diluted in Tyrode’s and 0 Ca\(^{2+}\) solutions for experiments. ATP, CPA, Doxycycline, TG, TPEN were from Sigma; GPN was from Santa Cruz; ML-SA1 was from Chembridge.

**Data analysis.** Data are presented as mean ± SEM. All statistical analyses were conducted using GraphPad Prism. Paired t-tests were used to compare the average of three or more experiments between treated and untreated conditions. A value of P <0.05 was considered statistically significant.
Figure 3.1  ER Ca$^{2+}$ release is significantly reduced over 5 mins in 0 Ca$^{2+}$ extracellular solution.  (A) The response to ATP in HEK293T cells loaded with Fura-2 was significantly diminished after perfusing cells with 0 external Ca$^{2+}$ for 5 min.  (B) 0 Ca$^{2+}$ extracellular solution for 5 mins reduces ATP stimulated IP3R release from the ER Ca$^{2+}$ store. All panels represent the average of 30-40 HEK cells.
Figure 3.2 ER SERCA inhibitors block \( \text{Ca}^{2+} \) refilling to the lysosome. (A) Acute treatment with the ER SERCA inhibitor Thapsigargin (TG) (2 \( \mu \text{M} \)) abolishes refilling to the lysosome (arrow marks application of ML-SA1). (B) Cyclopiazonic Acid (CPA) is a SERCA inhibitor that when applied acutely (100 \( \mu \text{M} \)) abolishes \( \text{Ca}^{2+} \) refilling to lysosomes. (C) TG (\( p=0.008 \)) and CPA (\( p=0.0069 \)) both significantly reduced \( \text{Ca}^{2+} \) refilling to lysosomes. Data is presented as
mean ± SEM. (D) Short application of CPA to HEK293T cells affected the amplitude of GPN response, suggesting that the response is only due to pH. (E) Application of TG did not affect the naïve, initial response to ML-SA1, but did abolish the refilled response. The leak of Ca^{2+} from ER Ca^{2+} stores into the cytosol is observable on the cytosolically located GCaMP3-ML1 probe, likely due to close contact between the ER and lysosomes. Control naïve response 1.39±0.09 (n=3); Naïve response after TG 1.08±0.07 (n=3); p=0.2024) (F) TG did not abolish LysoTracker staining in HEK293T cells. All data represents the average of 30-40 cells. Unless otherwise stated, data was collected from HEK-GCaMP3-ML1 cells.
Figure 3.3 TPEN chelates ER Ca\textsuperscript{2+} and abolishes lysosome Ca\textsuperscript{2+} refilling. (A) TPEN treatment (2 min) chelates ER Ca\textsuperscript{2+} and blocks ATP induced Ca\textsuperscript{2+} release (compare to B). (B) Untreated HEK293T cells display robust responses to ATP, which release ER Ca\textsuperscript{2+} and GPN, which permeabilizes lysosome membranes. (C) TPEN does not change the response to GPN indicating it does not chelate lysosome Ca\textsuperscript{2+} (compare to B). (D) In HEK-GCaMP3-ML1 cells, application of TPEN during Ca\textsuperscript{2+} refilling abolishes the response to ML-SA1. (E) TPEN significantly (p=0.001) abolished Ca\textsuperscript{2+} refilling in HEK-GCaMP3-ML1 cells. (F) Long-term
treatment with TPEN (20 mins) abolished the lysosome and ER Ca\textsuperscript{2+} responses. All data represents the average of 30-40 cells. Unless otherwise noted, all experiments were performed in HEK293T cells.

Figure 3.4 Transfection of IP3R-LBD-ER chronically reduced ER Ca\textsuperscript{2+} and also affected lysosome Ca\textsuperscript{2+} refilling. (A) In HEK cells transfected with IP3R-LBD-ER, ER Ca\textsuperscript{2+} is significantly reduced and GPN responses are diminished. (B) In HEK-GCaMP3-ML1 cells transfected with IP3R-LBD-ER, responses to ML-SA1 are significantly reduced when compared to untransfected cells HEK-GCaMP3-ML1 on the same coverslip. Each panel represents the average 20-40 cells on one coverslip. (C) The 1\textsuperscript{st} (p=0.0014), 2\textsuperscript{nd} (p=0.0004), and 3\textsuperscript{rd} responses (p<0.0001) of GCaMP3-ML1 cells transfected with the IP3R-LBD-ER were significantly reduced compared to untransfected cells on the same coverslip.
**Figure 3.5 Lysosomes are in close apposition to ER tubules.** (A) Live cell imaging of Cos7 cells transfected with CFP-ER and LAMP1-mCherry show that the ER and lysosomes remain in close contact. It was impossible to find a lysosome that did not appear in contact with the ER. (B) Additional highlighted area from the cell in panel A, again showing the ER and lysosomes in close contact. (C) TEM of a smooth ER tubule in close apposition to a lysosome-like vesicle. Right panel shows ER highlighted in red and lysosome outlined in blue. Scale bar = 100 nm.
Figure 3.6 Model of ER-Lysosome Interaction. TRPML1 is a Ca$^{2+}$ channel that releases Ca$^{2+}$ from the lysosome into the cytosol. Baf-A and Con-A are inhibitors of the lysosome V-ATPase which maintains the proton gradient in the lysosome. did not affect lysosome Ca$^{2+}$. However, inhibiting SERCA pumps with TG and CPA inhibit SERCA pumps which maintain the ER Ca$^{2+}$ gradient, resulting in Ca$^{2+}$ leak from the ER down the concentration gradient. Chelating ER Ca$^{2+}$ stores with TPEN chelates ER Ca$^{2+}$ stores. Transfection with the IP3R-LBD-ER genetically reduces the ER Ca$^{2+}$ store by causing ongoing constitutive Ca$^{2+}$ release through IP3-receptors without increasing cytosolic Ca$^{2+}$. An unidentified Ca$^{2+}$ transporter or channel on the lysosome membrane serves to refill lysosome Ca$^{2+}$ stores.
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CHAPTER IV
THE IP3-RECEPTOR ON THE ENDOPLASMIC RETICULUM IS REQUIRED FOR REFILLING LYSOSOME CALCIUM STORES

ABSTRACT

Our findings suggested that ER Ca\(^{2+}\) is necessary to refill lysosome Ca\(^{2+}\) stores, so we next sought to test the hypothesis that blocking ER Ca\(^{2+}\) release through a specific channel or channels would abolish lysosome Ca\(^{2+}\) store refilling. We examined the two primary Ca\(^{2+}\) channels, IP3-receptors (IP3Rs) and ryanodine receptors (RYRs) on ER membranes. Pharmacological block of IP3Rs prevented Ca\(^{2+}\) refilling, as did blocking PLC to prevent the formation of IP3. Blocking RYRs did not significantly affect Ca\(^{2+}\) refilling. DT40-IP3R-TKO cells show Ca\(^{2+}\) refilling and normal acidification, but their Ca\(^{2+}\) store refilling isn’t affected by either IP3R or RYR antagonists. This suggests that an alternative mechanism of Ca\(^{2+}\) refilling exists in cells without IP3Rs. The finding that lysosomes in DT40 cells still have Ca\(^{2+}\) also supports the importance of Ca\(^{2+}\) stores to lysosome and cellular function. IP3Rs also transfer Ca\(^{2+}\) to mitochondria at ER-mitochondria contact sites. Our work supports the idea that one role of IP3Rs on the ER is to regulate Ca\(^{2+}\) transfer and store maintenance of other organelles in the cell. These findings also suggest that a subset of assumed to be IP3R mediated functions could potentially be mediated by lysosome Ca\(^{2+}\).

INTRODUCTION

Ca\(^{2+}\) ions are the most versatile and widely used signaling molecules within and between cells (Berridge et al., 2000). Ca\(^{2+}\) is highly buffered within the cytosol to keep cytosolic concentrations very low (100 nM), which allow for precise spatiotemporal control of Ca\(^{2+}\) signals that are involved everything from gene expression to cell death (Clapham, 2007). Indeed, at prolonged high concentrations, Ca\(^{2+}\) is known to be toxic to the cell. The two primary
methods of mobilizing Ca\(^{2+}\) for intracellular signaling are through the plasma membrane from the high Ca\(^{2+}\) in the extracellular environment, or from intracellular Ca\(^{2+}\) stores. Many intracellular vesicles are known to contain stores of Ca\(^{2+}\), the largest store being the endoplasmic reticulum (ER).

**ER Ca\(^{2+}\) Signaling**

The highly dynamic ER takes up about 10 – 40% of the cell volume depending on the cell type. \([\text{Ca}^{2+}]_{\text{ER}}\) is about 0.3-0.7 mM, making it the largest contiguous Ca\(^{2+}\) store in the cell. For comparison, lysosomes make up about 3 – 5% of cell volume and \([\text{Ca}^{2+}]_{\text{Ly}}\) is about 0.4-0.6 mM. The ER has many distinct domains and regions that are connected but have different functions and structures (Lynes and Simmen, 2011; English and Voeltz, 2013). Likewise, Ca\(^{2+}\) released from the ER seems to have segregated pools that are released by specific receptors triggered by distinct signals (Mozhayeva and Mozhayeva, 1996; Guerrero-Hernandez et al., 2010; Aulestia et al., 2011). An ER-centric view of the cell lends itself to the suggestion that the ER, particularly because it is known to make contact with the plasma membrane and all other organelles in the cell, is a master coordinator of all cell functions using Ca\(^{2+}\) as its most universal signaling tool. An understanding of the function of ER Ca\(^{2+}\) release channels may shed light on ER-lysosome Ca\(^{2+}\) interactions.

**IP3R and RYR Ca\(^{2+}\) Channels of the Endoplasmic Reticulum**

The ER has two primary Ca\(^{2+}\) release channels, ryanodine receptors (RYRs) and inositol-1,4,5-triphosphate receptors (IP3Rs). Because ER Ca\(^{2+}\) stores are implicated in lysosomal Ca\(^{2+}\) regulation (see Chapter 3), a basic understanding of the primary Ca\(^{2+}\) channels on the ER membrane is helpful in thinking about how they may provide Ca\(^{2+}\) to the lysosome. Because of this, important and relevant aspects of RYR and IP3R channel structure and function will be reviewed here briefly.

IP3Rs and RYRs are large, tetrameric channels located primarily on the ER. Both IP3Rs and RYRs have large cytoplasmic, mushroom shaped regions to detect a wide variety of signals from the cytosol and smaller stalk regions that extend into the ER lumen to detect intraluminal signals as well. These two Ca\(^{2+}\) release channels have structural and functional commonalities, as well as similar activation and gating mechanisms (Seo et al., 2012).
**Ryanodine Receptors**

RYR receptors are the largest known ion channels, almost twice the size of IP3Rs, composed of about 5000 amino acids (~2.2MDa) (Van Petegem, 2012). At concentrations around 10 nM ryanodine binds to RYRs and permits Ca\(^{2+}\) efflux, but at concentrations higher than 10 µM, ryanodine inhibits RYRs (Meissner, 1986). RYRs are best known for their involvement in excitation-contraction coupling in the sarcoplasmic reticulum (SR) of muscle cells. There are three known isoforms of RYRs, which have all been found in a variety of tissues although certain tissues contain predominately one isoform (Van Petegem, 2012).

The primary trigger for RYR opening is Ca\(^{2+}\), for which RYRs have multiple binding sites. Ca\(^{2+}\) modulation of RYRs can be plotted in a bell-shaped curve, where higher concentrations can cause channel closing (Meissner et al., 1986). RYRs are often found in clusters with other ion channels, and can be opened by the activity of other ion channels. In fact, RYRs can detect high luminal Ca\(^{2+}\) levels in the ER and open to prevent Ca\(^{2+}\) overloading in the ER. Furthermore, RYR channel opening is controlled by a wide array of post-translational modification events, proteins, small molecules, and binding proteins from the cytosol and ER/SR lumen, undoubtedly facilitated by the enormous surface area of the cytosolic portion of the channel (Van Petegem, 2012). More than 300 disease mutations of RYRs are known, most of which result in a gain of function that is found in skeletal muscle disorders, seizure disorders, and cardiac arrhythmias typical of RYR mutations (Van Petegem, 2012).

**Inositol Trisophosphate (IP3) Receptors**

If the ER is considered the largest Ca\(^{2+}\) store in the cell, then IP3R receptors are increasingly being appreciated as the master signal integrators to regulate the Ca\(^{2+}\) store (Patterson et al., 2004; Mikoshiba, 2007). IP3Rs themselves have the ability to integrate a wide variety of signals from kinases, nucleotides, proteins, and phosphatases, whereby the IP3R forms a scaffold to form macro-signaling complexes (Mikoshiba, 2007). This is supported by the fact that more than 50 structural, scaffolding, and motor proteins have already been identified to interact with IP3Rs (Parys and De Smedt, 2012). Adding to the complexity, kinases and phosphatases can regulate both the IP3R and its interacting proteins (Parys and De Smedt, 2012).
The IP3R is a very large protein with an open reading frame of more than 2700 amino acids (~1 MDa) (Patterson et al., 2004). Inositol 1,4,5-triphosphate (IP3) is a second messenger formed by phospholipase C metabolism of phosphoinositol-4,5-biphosphate (PIP2) as a result of stimulation of G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) (Berridge et al., 2000). IP3 binds to IP3Rs which transduces and/or amplifies cellular signals to rapidly release Ca\(^{2+}\) from the ER (Patterson et al., 2004). There are four presumed IP3 binding sites on the IP3R, about 2000 amino acids away from the channel pore, which are assumed to induce conformational changes to affect channel gating (Patterson et al., 2004). IP3Rs are also bi-phasically regulated by Ca\(^{2+}\), partly through a feedback mechanism whereby Ca\(^{2+}\) released inhibits further release (Patterson et al., 2004). Up to seven cytosolic Ca\(^{2+}\) binding sites on IP3Rs have been posited, and IP3R regulation has been shown to be conferred by both luminal ER Ca\(^{2+}\) and cytosolic Ca\(^{2+}\) levels (Parys and De Smedt, 2012).

There are three isoforms of the IP3R and a few splice variants. The three isoforms of IP3R display differing levels of sensitivity to IP3 (IP3R-2 > IP3R-1 > IP3R-3) (Newton et al., 1994) and different sensitivities to a variety of other regulatory mechanisms (Iwai et al., 2007; Parys and De Smedt, 2012). It has been difficult to deduce definitive and separate functions for each of these isoforms because almost all cell types contain more than one isoform. IP3R-2 and -3 isoform knockout mice display few phenotypic abnormalities. However, IP3R-1 knockout mice that survive past birth display severe defects, possibly because the cerebellum only expresses the IP3R-1 isoform. The cerebellum is the only region found to exhibit one isoform, and the three isoforms may on many levels exhibit functional redundancy to protect important functions within the cell (Newton et al., 1994; Patterson et al., 2004).

IP3Rs have been located in all regions of the ER, including the nuclear envelope or “neoplastic reticulum” (Echevarria et al., 2003), as well as to a much lower extent, the plasma membrane, Golgi, and secretory vesicles (Patterson et al., 2004). Whether IP3Rs are functional when they are on the plasma membrane is not known and remains controversial (Patterson et al., 2004), and perhaps they are a result of secretory vesicle fusion with the plasma membrane. IP3Rs are often found in clusters of 20-30, which plays a role in their regulation and the ability to produce localized microdomains of Ca\(^{2+}\) (Parys and De Smedt, 2012; Rahman, 2012).
The ability of IP3Rs to be regulated by such a wide variety of molecules allows for them to have many specific roles in Ca\textsuperscript{2+} release and exchange. For example, IP3Rs have been shown to provide Ca\textsuperscript{2+} to mitochondria at membrane contact sites, and may play larger signaling roles at these ER-mitochondrial junctions (Hayashi et al., 2009). Given the known role of IP3Rs in regulating ER-mitochondrial Ca\textsuperscript{2+} exchange, it is possible that they play a role in the regulation of membrane contact sites between the ER and the various other membranes, including the plasma membrane, Golgi, endosomes, lysosomes, lipid droplets, and perixosomes.

**Other ER Ca\textsuperscript{2+} Channels**

There are several other ion channels known to affect Ca\textsuperscript{2+} signaling in the ER that are not well characterized, but that undoubtedly affect ion homeostasis and Ca\textsuperscript{2+} release (Takeshima et al., 2014). For example, several non-selective cation channels exist that may function as Ca\textsuperscript{2+} leak channels including presenilins, pannexin channels, and several TRP family members, many of which are thought to affect the activity of IP3Rs and RYRs (Takeshima et al., 2014). There is data to support the existence of other putative exchangers and ion channels for K\textsuperscript{+}, H\textsuperscript{+}, Cl\textsuperscript{−}, Na\textsuperscript{2+}, and other ions, but many of these have yet to be definitively identified (Takeshima et al., 2014).

**Focus of the Present Study**

Our findings in Chapter 3 suggested that the ER Ca\textsuperscript{2+} store was important for lysosome Ca\textsuperscript{2+} store maintenance and refilling, so we next sought to test the hypothesis that blocking an ER Ca\textsuperscript{2+} channel or channels could abolish Ca\textsuperscript{2+} refilling to the lysosome. HEK cells possess both the primary Ca\textsuperscript{2+} release channels on the ER, IP3-receptors and ryanodine receptors (Querfurth et al., 1998; Jurkovicova et al., 2008), so we began by testing those (Fig 4.1A).

**RESULTS**

**IP3-Receptor antagonist Xestospongin blocks Ca\textsuperscript{2+} refilling of lysosomes.**

IP3-receptors on the ER provide Ca\textsuperscript{2+} to mitochondria through membrane contact sites at the mitochondrial associated membrane (MAM) (Hayashi et al., 2009). Many of the antagonists used in Chapter 3 to block ER Ca\textsuperscript{2+} release act on the pool of Ca\textsuperscript{2+} in the ER sensitive to IP3. Because of this, we first examined IP3-receptors to determine if they were involved in Ca\textsuperscript{2+} refilling to the lysosome.
We sought the most specific, fast-acting IP3 receptor blocker available, Xestospongicin-C (Xesto) (Gafni et al., 1997; Cardenas et al., 2010). Importantly, Xesto washes out rapidly. Xesto blocked the ATP induced Ca\(^{2+}\) response in HEK cells as expected (Fig. 4.2A). When applied during Ca\(^{2+}\) refilling to the lysosome, Xesto significantly blocked Ca\(^{2+}\) refilling to the lysosome (Fig. 4.2B,C). These findings suggest IP3-receptor involvement in Ca\(^{2+}\) refilling, but did not rule out ryanodine receptors.

\textbf{Ca}^{2+} \textbf{refilling to lysosomes is blocked by IP3-Receptor inhibitor Xestospongicin-C using the luminal Ca}^{2+} \textbf{indicator Fura-Dextran.}

We also tested the effects of the IP3R blocker Xesto in an alternative assay using the intraluminal Ca\(^{2+}\) indicator Fura-Dextran (see Chapter 2 for a detailed description and Fig. 2.7). After a pulse/chase protocol in cell medium, Fura-Dextran localizes to lysosomes (Fig. 4.5A). As we found in our GCaMP3-ML1 assay, Xesto completely blocked the decrease in lysosome luminal Ca\(^{2+}\), supporting the finding that IP3Rs are essential for lysosome Ca\(^{2+}\) store refilling (Fig. 4.3B).

\textbf{IP3-Receptors on the ER are required for Ca}^{2+} \textbf{refilling of lysosomes.}

The importance of IP3Rs in Ca\(^{2+}\) refilling was confirmed using additional pharmacological antagonists. This also allowed us to rule out any off-target effects of Xesto. 2-Aminoethoxydiphenyl borate (2-APB) rapidly inhibits IP3Rs (Maruyama et al., 1997). 2-APB may also affect Ca\(^{2+}\) refilling to the ER during store operated Ca\(^{2+}\) entry (SOCE) (Peppiatt et al., 2003), possibly because activation of IP3Rs is important for SOCE (Ma et al., 2000). 2-APB during Ca\(^{2+}\) refilling to the lysosome significantly blocked Ca\(^{2+}\) refilling (Fig. 4.4A,B). 2-APB can be difficult to washout (Peppiatt et al., 2003), which might be the cause of the diminished response to the third application of ML-SA1 that we observed (Fig. 4.4A).

U73122 is a PLC inhibitor that blocks the constitutive production of IP3 (Cardenas et al., 2010) and prevents IP3R-mediated Ca\(^{2+}\) release induced by the P2Y receptor agonist ATP (Fig. 4.5A). U73122 also completely prevented Ca\(^{2+}\) refilling of lysosomes (Fig. 4.5B,C). These findings may suggest that basal production of IP3 is essential for Ca\(^{2+}\) refilling of lysosomes, however the effects may simply be due to the fact that U73122 blocked IP3R Ca\(^{2+}\) release (Fig. 4.5A). Because U73122 also caused some Ca\(^{2+}\) leak from the ER, observed on our probe, it is
possible that it also acts as a SERCA inhibitor, which has been shown at least in smooth muscle (Hollywood et al., 2010).

**Ryanodine Receptors on the ER are not required for Ca\(^{2+}\) refilling to lysosomes.**

Ryanodine receptors (RYRs) are Ca\(^{2+}\) permeable channels on the ER membrane whose primary trigger for activation is Ca\(^{2+}\), although they are activated by a variety of other endogenous molecules (Van Petegem, 2012). Because they are Ca\(^{2+}\) activated, their involvement in Ca\(^{2+}\) refilling is plausible, given that Ca\(^{2+}\) released from lysosomes could be sensed by RYRs and trigger ER Ca\(^{2+}\) release to lysosomes. Low concentrations of ryanodine activate RYRs, but high (>10 µM) concentrations of ryanodine block the channel (Pessah and Zimanyi, 1991; Berridge, 2012). Application of a high concentration of ryanodine during Ca\(^{2+}\) refilling did not significantly affect Ca\(^{2+}\) refilling to the lysosome (Fig. 4A,C). An additional ryanodine receptor antagonist, 1,1’-diheptyl-4,4’-bipyridinium (DHBP) (Kang et al., 1994), also showed no effect to Ca\(^{2+}\) refilling of lysosomes (Fig. 4B,C). These results suggest that RYRs are not the primary Ca\(^{2+}\) release channel involved in Ca\(^{2+}\) refilling to lysosomes.

The close-apposition of the ER and lysosomes raised the possibility that the large ML-SA1 responses we observed might be mediated by lysosomal Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the ER. In certain cell types, releasing even small amounts of Ca\(^{2+}\) from lysosomes into the cytosol may trigger Ca\(^{2+}\) waves from the ER (Kilpatrick et al., 2013). Because of this concern, we co-applied IP3R and RYR blockers with ML-SA1 in all experiments with these antagonists (Figs. 4.1B, 4.2A, 4.4A,B). Co-application did not reduce the initial, naïve response to ML-SA1 before refilling, suggesting that Ca\(^{2+}\) release measured by GCaMP3-ML1 was not from RYRs or IP3Rs directly.

**DT40-IP3R-TKO chicken B cells exhibit Ca\(^{2+}\) refilling to lysosomes.**

We next sought a way to abolish IP3R Ca\(^{2+}\) release without using pharmacological methods. Transferring cells with the IP3R-LBD-ER ongoingly released Ca\(^{2+}\) and reduced ER Ca\(^{2+}\) stores (Varnai et al., 2005). The IP3R-LBD-ER also reduced lysosome Ca\(^{2+}\) stores and refilling (Fig. 3.4). However, the IP3-LBD-ER did not abolish IP3R activation, but instead reduced ER Ca\(^{2+}\) ongoingly through ongoing stimulation of the IP3 receptor. Inspired by studies examining the role of IP3R Ca\(^{2+}\) release in mitochondrial bioenergetics (Cardenas et al., 2010),
we obtained triple IP3R knockout DT40 cells (DT40-TKO). Both WT (Fig. 4.6A) and DT40-TKO (Fig. 4.6B) cells exhibited responses to GPN and lysosomal Ca\(^{2+}\) store refilling occurs in both WT (Fig. 4.6C and E) and TKO DT40 chicken B cells (Fig. 4.6D and E) that were transfected with GCaMP3-ML1. DT40 and DT40-TKO cells exhibited no overt differences in lysosome acidification, size, and number as measured by LysoTracker staining (Xu et al., 2014) (Fig. 4.6F).

In WT DT40 cells, the IP3R-specific antagonist Xesto completely blocked Ca\(^{2+}\) refilling (Fig. 4.7A and C). However, Xesto had no obvious blocking effect in DT40-TKO cells (4.7B and C). Refilling in DT40-TKO cells was not blocked by DHBP (Fig. 4.7D) or a combination of Dantrolene or Ditilizam (Fig. 4.7E). Results in DT40 cells are consistent with the notion that in normal conditions, IP3Rs are the primary source of Ca\(^{2+}\) refilling of lysosomes. When IP3Rs are genetically deleted, however, IP3R-independent mechanisms contribute to lysosomal Ca\(^{2+}\) refilling. The ER has multiple Ca\(^{2+}\) channels that could compensate for the lack of IP3Rs in DT40 cells and play the role of Ca\(^{2+}\) transfer to lysosomes (Takeshima et al., 2014).

**Ca\(^{2+}\) stores are released ongoingly for normal physiological processes.**

After examining refilling time (see Chapter 2 for details), we were also curious about ongoing Ca\(^{2+}\) release in lysosomes. Ca\(^{2+}\) store depletion in lysosomes could occur as a result of Ca\(^{2+}\) release for fusion and/or fission of vesicles, as a counterflux to other ion transporters in the lysosome, and for signaling to other vesicles about cell nutrient levels. To test whether ongoing Ca\(^{2+}\) release was occurring in HEK-GCaMP3-ML1 cells, we first allowed Ca\(^{2+}\) refilling to occur, then applied the IP3R blocker Xesto. After 2 mins, no effect on refilled Ca\(^{2+}\) stores was observed (Fig. 4.8A). Xesto treatment for 5 minutes after refilling showed a moderate decrease in Ca\(^{2+}\) released from lysosomes, below typical levels (Fig. 4.8B). After 10 minutes of Xesto treatment following store refilling, lysosome Ca\(^{2+}\) stores were significantly reduced (Fig 4.8C). These results suggest that slow, ongoing lysosomal Ca\(^{2+}\) release occurs under resting conditions that gradually depletes lysosome Ca\(^{2+}\) stores if refilling is prevented (Fig. 4.8D).

The results of Chapters 2 and 3 as well as those found in this chapter support the working model depicted in Figure 4.9. Within lysosomes and the ER, Ca\(^{2+}\) buffers regulate free and releasable Ca\(^{2+}\), and play a role in modulating the specificity of Ca\(^{2+}\) release by different
channels and triggers. \(Ca^{2+}\) is released from the lysosome by TRPML1 and other \(Ca^{2+}\) channels (not depicted). \(Ca^{2+}\) release and/or other signals to IP3Rs trigger IP3R \(Ca^{2+}\) release within the tight nanojunction between ER and lysosome membranes. This \(Ca^{2+}\) release recruits additional \(Ca^{2+}\) channels (possibly more IP3Rs) to refill lysosome \(Ca^{2+}\) stores. Tethers link ER membranes to lysosomes for a variety of purposes, and tethers possibly exist between IP3Rs and lysosomes to facilitate ER \(Ca^{2+}\) transfer to lysosomes. A putative, low-affinity \(Ca^{2+}\) pump or exchanger, located on the lysosome membrane that does not depend on the proton gradient, moves \(Ca^{2+}\) into lysosomes.

**DISCUSSION**

**IP3Rs on the ER refill lysosome \(Ca^{2+}\) stores.**

By measuring both \(Ca^{2+}\) release and luminal \(Ca^{2+}\) in the lysosome, we found evidence that normally functioning IP3Rs are required for lysosomal \(Ca^{2+}\) refilling, but when IP3Rs are lacking, alternative \(Ca^{2+}\) channels or mechanisms may refill lysosome \(Ca^{2+}\) stores. Regardless of whether IP3Rs were blocked using Xesto or 2-APB, or whether IP3 production was directly inhibited using U73122, \(Ca^{2+}\) refilling to the lysosome and subsequent \(Ca^{2+}\) release measured by the GCaMP3-ML1 probe was abolished when IP3R \(Ca^{2+}\) release was blocked. These findings were corroborated using Fura-Dextran, again showing that IP3R block with Xesto during refilling results in no \(Ca^{2+}\) refilling to lysosomes.

While IP3Rs reside predominately on the ER, they can be found to a lesser extent in the Golgi and secretory vesicles and sometimes have low expression in the plasma membrane (Vermassen et al., 2004). Chapter 3 uses pharmacological and genetic methods to show that ER \(Ca^{2+}\) is specifically required for \(Ca^{2+}\) refilling to lysosomes, which supports IP3R localization on the ER for lysosome refilling. We showed that extracellular \(Ca^{2+}\) depletion affects lysosome \(Ca^{2+}\) refilling, but also that it abolishes ER IP3R \(Ca^{2+}\) release, again suggesting that extracellular \(Ca^{2+}\) is not directly required for refilling \(Ca^{2+}\) to lysosomes. Furthermore, blocking plasma membrane \(Ca^{2+}\) channels, including IP3Rs (Palade et al., 1989), with \(La^{3+}\) did not affect \(Ca^{2+}\) refilling. Ablating Golgi using Brefeldin-A also showed little impact on \(Ca^{2+}\) refilling to lysosomes (Fig. 2.9). Taken together, these results suggest that IP3Rs residing on the ER membrane are responsible for \(Ca^{2+}\) refilling to lysosomes.
Although ER chelation using TPEN, disrupting ER Ca\(^{2+}\) store maintenance using SERCA inhibitors, and blocking IP3 production using U73122 all completely abolished Ca\(^{2+}\) refilling, very slight levels of Ca\(^{2+}\) remained when using IP3R blockers Xesto and 2-APB. It is possible that an incomplete block of IP3Rs, or one particular IP3R subtype, was responsible for this, particularly because low concentrations of each drug were used intentionally to avoid off-target effects. Perhaps less likely but worth considering, IP3R Ca\(^{2+}\) release may be initially required for refilling but then additionally recruit other Ca\(^{2+}\) release channels to aid in lysosome Ca\(^{2+}\) store refilling. Because blocking RYR channels increased Ca\(^{2+}\) refilling to lysosomes, it seems unlikely that RYRs play this role.

**Ryanodine Receptors are not required for Ca\(^{2+}\) refilling.**

Blocking RYRs did not prevent Ca\(^{2+}\) refilling to lysosomes, suggesting they are not the primary Ca\(^{2+}\) channel on the ER for lysosome Ca\(^{2+}\) refilling. Notably, blocking RYRs increased Ca\(^{2+}\) refilling to lysosome slightly, especially with Ryanodine, although this increase was not significant. Blocking RYRs is known to increase Ca\(^{2+}\) in the ER (Ong et al., 2010), which may explain why refilling increased slightly. This increase parallels the decrease in lysosome Ca\(^{2+}\) that was observed when ER Ca\(^{2+}\) levels are reduced using IP3R-LBD-ER (Chapter 3). These findings suggest that ER Ca\(^{2+}\) refilling to lysosomes occurs continuously and the amount refilled is affected by the available store of Ca\(^{2+}\) in the ER.

**DT40-IP3R-TKO cells have alternative lysosome Ca\(^{2+}\) refilling mechanisms.**

DT40-IP3R-TKO lysosomes transfected with GCaMP3-ML1 released and refilled Ca\(^{2+}\) and were acidified similarly when compared to WT DT40 cells (Fig 4.6). Both DT40-TKO and WT cells responded to GPN, albeit their responses were smaller than those compared to HEK293T or Cos7 cells. While Xesto blocked refilling in WT DT40 cells, Xesto had no effect on refilling in DT40-TKO cells (Fig 4.7A-C). These results rule out potential off-target effects of Xesto because Xesto only blocks Ca\(^{2+}\) refilling in cells with functioning IP3Rs. Block of RYRs using three different antagonists in DT40-TKO cells did not block refilling (Fig 4.7D,E). These findings suggest that an alternative mechanism exists to support Ca\(^{2+}\) store refilling when the IP3R is unable to. That lysosomes in DT40-TKO cells still have luminal Ca\(^{2+}\) stores that refill highlights the importance of the lysosome Ca\(^{2+}\) store to proper lysosome function and cellular
health. Ca\textsuperscript{2+} refilling in DT40-TKO cells also raises the important question of what alternative mechanisms may exist to refill Ca\textsuperscript{2+} stores in these cells.

DT40-WT cells transfected with GCaMP3-ML1 exhibited similar Ca\textsuperscript{2+} store refilling when compared to other cell types in that their second response to ML-SA1 was slightly higher than the first, and the third response was slightly reduced. However, Ca\textsuperscript{2+} refilling in DT40-TKO cells transfected with GCaMP3-ML1 was slightly different than in WT, showing a reduced second response and a further reduced third response, although these effects were not statistically significant. These findings support the possibility that different mechanisms exist to refill Ca\textsuperscript{2+} stores in cells lacking IP3Rs, although the kinetics of refilling were not closely examined. Mitochondria-ER contact sites still exist in DT40-TKO cells (Csordas et al., 2006), and even dramatically altering ER morphology in healthy cells does not abolish the contact sites with mitochondria (Friedman et al., 2011). Thus, contact sites still exist and can likely respond dynamically to facilitate Ca\textsuperscript{2+} exchange by alternative mechanisms.

There are two main possibilities for alternative Ca\textsuperscript{2+} store refilling mechanisms in DT40-TKO cells. First, lysosomes in DT40-TKO cells may refill their Ca\textsuperscript{2+} stores via ER independent mechanisms. Second, and more likely, is that lysosomes in DT40-TKO cells may refill Ca\textsuperscript{2+} stores using alternative Ca\textsuperscript{2+} channels on the ER membrane.

If DT40 cells refill Ca\textsuperscript{2+} stores by a mechanism other than ER Ca\textsuperscript{2+} transfer, what could this mechanism be? The most feasible explanation is that fusion with other intracellular vesicles through membrane trafficking, including autophagosomes or transport vesicles from the trans-Golgi network, could provide Ca\textsuperscript{2+} to lysosomes in DT40 cells. Fusion with other Ca\textsuperscript{2+} containing intracellular vesicles may provide less Ca\textsuperscript{2+} to the lysosome than transfer from the ER, however. Supporting this possible mechanism, DT40 WT and IP3R-TKO cells may have smaller Ca\textsuperscript{2+} stores compared to other cell types. GPN responses were small in DT40 WT and IP3R-TKO cells. However, this possibility should be more closely examined with BAPTA-AM during GPN application to determine how much of the GPN response was due to pH versus Ca\textsuperscript{2+}. Differences in the size of Ca\textsuperscript{2+} stores are not possible to elucidate with GCaMP3-ML1 which does not directly measure the amount of Ca\textsuperscript{2+} released. While the amplitude of Ca\textsuperscript{2+} stores were smaller in both DT40 WT and TKO cells transfected with GCaMP3-ML1, it is difficult to tell if this is due to negative consequences of transient transfection and/or the manipulations required.
to ensure DT40 cells adhere to glass coverslips (DT40 cells normally exist in suspension culture). Thus, luminal Ca\textsuperscript{2+} indicators should also be used to directly measure Ca\textsuperscript{2+} stores in DT40 cells. If Ca\textsuperscript{2+} stores are smaller in DT40 cells, mechanisms of Ca\textsuperscript{2+} store refilling that do not involve the ER could support their store maintenance because less Ca\textsuperscript{2+} would be required to refill their stores.

An alternative possibility, and one that is more likely, is that a Ca\textsuperscript{2+} channel other than IP3Rs may be involved in Ca\textsuperscript{2+} store refilling, and that this channel may not be the RYR. Given the redundancy of Ca\textsuperscript{2+} release channels (Marder and Goaillard, 2006) and numerous other potential Ca\textsuperscript{2+} channels on the ER (Takekuma et al., 2014) compensation by an alternative Ca\textsuperscript{2+} channel in DT40-IP3R-TKO cells seems like a real possibility. It is known that ion channels of different identities can produce similar phenomenon in order to maintain homeostasis, particularly when normal homeostasis is disrupted by the ablation or blockade of a normally occurring ion channel (Marder and Goaillard, 2006). Additionally, studies have shown vast differences between short-term pharmacological blockade of ion channels vs. long-term deletions of the same current (Marder and Goaillard, 2006). While acute inhibition with antagonists often reduces or abolishes ion release, knockout of particular ion channels often do not show any physiological phenotype (Marder and Goaillard, 2006). For example, a seminal study showed that long-term reductions in Na\textsuperscript{+} currents had little effect on patterns of electrical activity in purkinje neurons when compared with short-term blockade (Swensen and Bean, 2005). Similar effects have been found for Ca\textsuperscript{2+} channels, where knock-out of the P/Q type Ca\textsuperscript{2+} channel had little effect on synaptic transmission due to compensatory changes in the mRNA expression levels of a vast array of genes (Piedras-Renteria et al., 2004). DT40 cells are easy to manipulate genetically because they have the highest rate of homologous recombination of any mammalian cell line, with a ratio of targeted to random integration of DNA of 1:2 (Winding and Berchtold, 2001). Thus, particularly in DT40 cells, the possibility of compensatory mechanisms to upregulate the expression of alternative Ca\textsuperscript{2+} channels to refill lysosome Ca\textsuperscript{2+} stores seems likely.

One possible candidate for an alternative ER Ca\textsuperscript{2+} channel to provide Ca\textsuperscript{2+} to the lysosome in DT40-TKO cells is Alzheimer’s (AD)-causing presenilin. Presenilin is a proposed ER Ca\textsuperscript{2+} leak channel whose mutations result in impaired lysosomal Ca\textsuperscript{2+} homeostasis (Coen et
al., 2012). However, numerous other ER Ca$^{2+}$ releasing channels and factors aiding in Ca$^{2+}$ release exist and have unknown functions, and more are likely to be discovered (Takeshima et al., 2014). Thus, the identity of the compensatory ion channel is possible to speculate but may not yet be known.

Constitutive Ca$^{2+}$ release from lysosomes may explain bidirectional interaction between ER and lysosome Ca$^{2+}$ stores.

Several groups have recently suggested interactions between ER and lysosome Ca$^{2+}$ stores, but these observations were based on indirect measurements of lysosome or acidic vesicle Ca$^{2+}$ derived from cytosolic signals. For example, lysosomes have been suggested to buffer ER Ca$^{2+}$, which was based on the observation that larger cytosolic Ca$^{2+}$ signals from the ER were observed after inhibiting the lysosome V-ATPase (Lopez-Sanjurjo et al., 2013). Shortly thereafter, the same group suggested that Ca$^{2+}$ is rapidly recycled between the ER and lysosomes, again based on the size of cytosolic Ca$^{2+}$ signals (Lopez Sanjurjo et al., 2014). Other groups have shown bidirectional Ca$^{2+}$ signaling between acidic organelles in sea urchin eggs (Morgan et al., 2013). Another found that osmotic permeabilization of lysosome membranes using GPN could provoke cytosolic Ca$^{2+}$ signals they suggested to be from the ER (Kilpatrick et al., 2013). These findings and others suggesting the involvement of the ER in lysosome Ca$^{2+}$ stores (Haller et al., 1996b; Haller et al., 1996a) could be due to the fact that lysosomes are constitutively releasing Ca$^{2+}$ for ongoing signaling in the cell and require ER Ca$^{2+}$ transfer to maintain their stores.

Tethers may exist to functionally link ER-lysosome Ca$^{2+}$ transfer microdomains.

The idea that IP3Rs are responsible for providing Ca$^{2+}$ to the lysosome is also supported by the extensive research showing that IP3Rs play a significant role in mitochondrial Ca$^{2+}$ homeostasis (Hayashi et al., 2009). Tethering protein GRP-75 links IP3Rs directly to mitochondrial VDAC channels (Szabadkai et al., 2006) presumably for the purpose of providing Ca$^{2+}$ to mitochondria to regulate cellular bioenergetics (Cardenas et al., 2010). However, there are numerous tethering proteins linking the ER to the mitochondria, and others that have been specifically shown to regulate mitochondrial Ca$^{2+}$ (De Vos et al., 2012). ER-mitochondrial contact is surprisingly
resilient, as is contact between the ER and plasma membrane. Treatments to alter ER morphology or knock-down of particular tethers do not abolish these contact sites.

Similar tethers may also link IP3Rs with the putative lysosomal Ca\(^{2+}\) transporter specifically for the purpose of Ca\(^{2+}\) store refilling (see Fig. 4.9). ER-endosome contact has been shown to increase as endosomes mature, and up to 99% of late-endosomes are in contact with the ER (Friedman et al., 2013). Most of the known ER–late-endosome contact sites involve cholesterol sensors and transporters, highlighting the important role of cholesterol/sterol transport between late-endosomes/lysosomes and the ER.

Although the ER is in constant contact with lysosomes, transient contact sites have also been observed. The ER has been shown to contact endosomes at constriction sites that form restricted cargo domains within endosomes prior to endosome fission events. Elegant confocal microscopy studies reveal that the ER appears to define the timing and positioning of endosome fission, and these contact sites may provide precise triggers, including Ca\(^{2+}\) to trigger fission (Rowland et al., 2014).

**Cholesterol transport between the ER and lysosomes may be affected by Ca\(^{2+}\) transfer.**

Cholesterol loading of late endosomes and lysosomes seems to impact lysosome Ca\(^{2+}\) levels (Lloyd-Evans et al., 2008; Shen et al., 2012), and may be due to decreased lysosome-ER contact. RYR blockers have been suggested to ameliorate lipid storage in NPC disease fibroblasts (Yu et al., 2012), possibly because they increase ER Ca\(^{2+}\) (Ong et al., 2010) which could then promote additional Ca\(^{2+}\) transfer to lysosomes.

**Limitations**

The major limitation of the aforementioned findings is that they are mostly pharmacological manipulations of ER Ca\(^{2+}\) release. Off-target effects of any pharmacological manipulation should be considered. While we use multiple pharmacological manipulations to examine each receptor, additional results using deletion of IP3Rs would be ideal. SIRNA deletion or CRISPR/Cas9 gene knockout experiments would lend additional support to the idea that IP3Rs play an important role in Ca\(^{2+}\) refilling. It is also possible that IP3Rs are required for Ca\(^{2+}\) refilling but are not the primary Ca\(^{2+}\) channel to refill lysosome stores. Theoretically, stimulation
of IP3Rs could trigger an additional Ca\(^{2+}\) release channel on the ER that primarily refills lysosome Ca\(^{2+}\) stores.

There are many alternative explanations for why DT40 IP3R TKO cells still have Ca\(^{2+}\) refilling to lysosomes. In support of our hypothesis, lysosome Ca\(^{2+}\) stores may be so important to cell health that compensation using another ER Ca\(^{2+}\) channel or an alternative mechanism altogether serves to ensure lysosome stores are refilled. Ryanodine receptors may function to refill Ca\(^{2+}\) stores in DT40 IP3R TKO cells but may not be blocked by our pharmacological manipulations. Further characterization of RYR Ca\(^{2+}\) release in DT40 IP3R TKO cells should be performed to determine if this is possible. Additional pharmacological characterization of ER Ca\(^{2+}\) stores in DT40 cells should be performed using SERCA inhibitors, TPEN, and other IP3R and RYR inhibitors to further confirm that ER Ca\(^{2+}\) release is still the mechanism of Ca\(^{2+}\) store refilling in DT40-TKO cells. The kinetics of DT40 IP3R TKO Ca\(^{2+}\) refilling should be tested to determine whether they differ from WT cells. This is possible given the different kinetics of Ca\(^{2+}\) release from Ca\(^{2+}\) channels on the ER other than IP3Rs and RYRs (Takeshima et al., 2014).

**Future Directions**

Knowing that ER Ca\(^{2+}\) is aberrant in many lysosome storage disorders, in Chapter 5 we test the hypothesis that blocking Ca\(^{2+}\) transfer from the ER to lysosomes would have negative consequences on lysosome function or cell health.

**METHODS AND MATERIALS**

**Molecular biology.** Genetically-encoded Ca\(^{2+}\) indicator GCaMP3 was fused directly to the N-terminus of ML1 (GCaMP3-ML1) as described previously (Shen et al., 2012). LAMP1-mCherry was made by fusing mCherry with the C terminus of LAMP1.

**Mammalian Cell Culture.** HEK293 cells stably expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells) were generated using the Flip-In T-Rex 293 cell line (Invitrogen). DT40-WT and IP3R-TKO cells were a generous gift from Dr. Darren Boehning (The University of Texas Health Sciences Center at Houston). We also thank Serena Lynn Clark (The University of Texas Health Sciences Center at Houston) for her guidance on best practices for DT40 cell culture. All
cells were cultured in a 37°C incubator with 5% CO₂. HEK293T cells, Tet-On HEK293 cells stably expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells), Cos-7 cells, and human fibroblasts were cultured in DMEM F12 (Invitrogen) supplemented with 10% (vol/vol) FBS or Tet-free FBS. DT40 cells were kept in suspension in RPMI 1640 (Invitrogen) supplemented with 450 µL β-mercaptoethanol, 2 mM L-glutamine, 10% FBS, and 1% chicken serum (Varnai et al., 2005; Cardenas et al., 2010).

DT40 cells were transiently transfected using the Invitrogen Neon electroporation kit (1200V, 1 pulse, 30s). HEK293T cells and HEK-GCaMP3-ML1 cells were transfected using Lipofectamine 2000 (Invitrogen). All cells were used for experiments 24-48 hrs after transfection.

**Confocal imaging.** Live imaging of cells was performed on a heated and humidified stage using a Spinning Disc Confocal Imaging System. The system includes an Olympus IX81 inverted microscope, a 100X Oil objective NA 1.49 (Olympus, UAPON100XOTIRF), a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor). MetaMorph Advanced Imaging acquisition software v.7.7.8.0 (Molecular Devices) was used to acquire and analyze all images. LysoTracker (50 nM; Invitrogen) was dissolved in culture medium and loaded into cells for 30 min before imaging. MitoTracker Coverslips were washed 3 times with Tyrode’s and imaged in Tyrode’s.

**GCaMP3-ML1 Ca²⁺ imaging.** GCaMP3-ML1 expression was induced in Tet-On HEK-GCaMP3-ML1 cells 20-24h prior to experiments using 0.01μg/mL doxycycline. GCaMP3-ML1 fluorescence was monitored at an excitation wavelength of 470 nm (F₄₇₀) using a EasyRatio Pro system (PTI). Cells were bathed in Tyrode’s solution containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Glucose, and 20 mM Hepes (pH 7.4). Lysosomal Ca²⁺ release was measured in a zero Ca²⁺ solution containing 145 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 10 mM glucose, 1 mM EGTA, and 20 mM HEPES (pH 7.4). Ca²⁺ concentration in the nominally free Ca²⁺ solution is estimated to be 1–10 μM. With 1 mM EGTA, the free Ca²⁺ concentration is estimated to be < 10 nM based on the Maxchelator software (http://maxchelator.stanford.edu/). Experiments were carried out 0.5 to 6 hrs after plating.

**Fura-2 Ca²⁺ imaging.** Cells were loaded with Fura-2 (3 µM) and Pluronic-F127 (3 µM) in the culture medium at 37°C for 60 min. Florescence was recorded using the EasyRatio Pro system.
(PTI) at two different wavelengths (340 and 380 nm) and the ratio (F_{340}/F_{380}) was used to calculate changes in intracellular [Ca^{2+}]. All experiments were carried out 1.5 to 6 hrs after plating.

**Reagents.** All reagents were dissolved and stored in DMSO or water and then diluted in Tyrode’s and 0 Ca^{2+} solutions for experiments. 2-APB, ATP, Doxycycline, and DHBP were from Sigma; GPN and U73122 were from Santa Cruz; Ryanodine was from Abcam; LysoTracker, Fura-2, Plurionic F-127, and Fura-Dextran were from Invitrogen; ML-SA1 was from Chembridge; and Xestospongin-C was from Cayman Chemical, AG Scientific, and Enzo.

**Data analysis.** Data are presented as mean ± SEM. All statistical analyses were conducted using GraphPad Prism. Paired t-tests were used to compare the average of three or more experiments between treated and untreated conditions. A value of P <0.05 was considered statistically significant.
Figure 4.1 HEK cells have ryanodine receptors (RYRs) and IP3-receptors (IP3Rs). (A) Caffeine stimulates Ca$^{2+}$ release through RYRs and ATP stimulates Ca$^{2+}$ release through IP3Rs in HEK293T cells loaded with Fura-2. Representative traces are shown from an experiment that contained 30-40 cells.
Figure 4.2 Specific IP3R antagonist Xestospongion-C prevented Ca$^{2+}$ refilling to lysosomes. (A) Xestospongion-C (Xesto) application (5 min, 25 µM) blocked ER Ca$^{2+}$ release stimulated by ATP in HEK293 cells loaded with Fura-2. Importantly, Xesto washes out rapidly. (B) The IP3-receptor (IP3R) antagonist Xesto (10 µM) prevented Ca$^{2+}$ refilling of lysosomes in HEK-GCaMP3-ML1 cells. Note that Xesto was co-applied with ML-SA1. (C) Xesto significantly reduced lysosomal Ca$^{2+}$ refilling in HEK-GCaMP3-ML1 cells (n=5; p=0.007). Panels A and B show the average response of 30-40 cells from one representative experiment.
Figure 4.3 Xesto abolishes Ca$^{2+}$ refilling to lysosomes measured with luminal Ca$^{2+}$ indicator Fura-Dextran. (A) Fura-Dextran was pulse/chased into HEK293T cells transfected with Lamp1-mCherry. Fura-Dextran dyes were co-localized well with Lamp1-mCherry after 12h pulse and 4h chase, although not all lysosomes were loaded with the dye, evidenced by many Lamp1-mCherry vesicles without Fura-Dextran co-localization. Scale bar = 5 µm. (B) Xesto (25 µM) treatment for 5 minutes prevented Ca$^{2+}$ refilling to lysosomes. Right panels show the zoomed-in images of ML-SA1-induced responses before and after Xesto treatment. Data represent the average of 30-40 cells from a representative experiment.
Figure 4.4 IP3R antagonist 2-APB inhibits lysosomal Ca\textsuperscript{2+} refilling. (A) IP3R antagonist 2-APB (200 µM) blocked lysosomal Ca\textsuperscript{2+} refilling in HEK-GCaMP3-ML1 (p=0.013). Panel shows the average of 36 cells from one representative experiment. (B) Quantification of the responses to ML-SA1 in HEK-GCaMP3-ML1 cells after treatment with 2-APB (mean ± SEM; n=5).
Figure 4.5 PLC inhibitor U73122 blocked Ca\(^{2+}\) refilling to the lysosome. (A) PLC inhibitor U73122 (10 µM) blocked Ca\(^{2+}\) release from IP3Rs stimulated by ATP. (B) U73122 treatment abolished Ca\(^{2+}\) refilling of lysosomes. (C) Quantification of the responses to ML-SA1 in HEK-GCaMP3-ML1 cells after treatment with u-73122 (n=5; p=0.0070). The data represents mean ± SEM.
Ryanodine receptor blockers did not affect Ca\(^{2+}\) refilling to lysosomes. (A) Ryanodine (100 µM), which blocks Ryanodine receptors at high concentrations, did not block Ca\(^{2+}\) refilling to lysosomes. Note that Ryanodine was co-applied with ML-SA1. (B) Ryanodine receptor blocker DHBP (50 µM) did not block Ca\(^{2+}\) refilling of lysosomes. (C) Quantification of the responses to ML-SA1 in HEK-GCaMP3-ML1 cells after treatment with Ryanodine (Ry) (p=0.8293) and DHBP (p=0.0682). Data in panel C represents mean ± SEM. Panels A and B show the average response of 30-40 cells from one representative experiment.
Figure 4.7 DT40 IP3R-TKO cells show typical responses to GPN, normal acidification, and release and refill Ca\(^{2+}\) like WT DT40 cells. (A) DT40-WT cells respond to GPN (400 μM) in Fura-2. (B) DT40-IP3R-TK0 cells respond to GPN (400 μM) in Fura-2. (C) DT40 WT cells transiently transfected with GCaMP3-ML1 show Ca\(^{2+}\) refilling. (D) DT40 IP3R triple KO
(TKO) cells transiently transfected with GCaMP3-ML1 also show Ca\(^{2+}\) refilling. (E) Quantification of the 1\(^{st}\), 2\(^{nd}\) and 3\(^{rd}\) ML-SA1 responses in GCaMP3-ML1-transfected WT and IP3R-TKO DT40 cells (mean ± SEM). Note that the 2\(^{nd}\) and 3\(^{rd}\) responses between each WT (p=0.1221) and DT40-TKO (p=0.1927) response were not significantly different, although they did decrease. There were no significant differences between WT and TKO 2\(^{nd}\) (p=0.0548) and 3\(^{rd}\) responses (p=0.6036). (F) DT40-WT and DT40-IP3R-TKO cells showed similar LysoTracker staining patterns. Panels C and D show the average of 30-40 cells form one experiment.
Figure 4.8 Xestospongin-C blocked lysosome Ca\textsuperscript{2+} store refilling in DT40-WT but not IP3R-TKO cells. (A) IP3R antagonist Xesto completely blocked Ca\textsuperscript{2+} refilling of lysosomes in DT40 WT cells (p=0.0278). (B) Xesto did not block Ca\textsuperscript{2+} refilling of lysosomes in IP3R-TKO cells (p=0.9646). (C) Quantification of ML-SAl responses with or without Xesto in WT and
IP3R-TKO DT40 cells (mean ± SEM; n=5). (D) GCaMP3-ML1-transfected DT40 IP3R-TKO cells still showed refilling after 5 min of DHBP application to block RYRs. (E) A combination of RYR blockers Diltiazem and Dantrolene did not block Ca\(^{2+}\) refilling to GCaMP3-ML1-transfected DT40 IP3R-TKO cells. Panels A, B, D, and E, are the average of 30-40 cells from one representative experiment.
Figure 4.9 Lysosome Ca\(^{2+}\) stores are emptied and refilled ongoingly. (A) After 5 min of refilling, which is expected to fully refill the lysosomal Ca\(^{2+}\) stores, acute treatment of Xesto (10 \(\mu\)M) for 2 min did not significantly reduce lysosomal Ca\(^{2+}\) release. Lysosomal Ca\(^{2+}\) release was induced by ML-SA1 in HEK-GCaMP3-ML1 cells. (B) After 5 min of refilling of lysosomal...
Ca$^{2+}$ stores, subsequent acute treatment of Xesto (10 µM) for 5 min reduced lysosomal Ca$^{2+}$ release. (C) After 5 min of refilling of lysosomal Ca$^{2+}$ stores, acute treatment of Xesto (10 µM) for 10 min abolished lysosomal Ca$^{2+}$ release. (D) Time-dependent depletion of lysosomal Ca$^{2+}$ stores by pharmacological inhibition of IP3-receptors.
Figure 4.10 A proposed model of Ca\(^{2+}\) transfer from the ER to lysosomes. The ER is a Ca\(^{2+}\) store with \([\text{Ca}^{2+}]_{\text{ER}} \sim 0.3-0.7 \text{ mM}\); lysosomes are acidic (pH\(_{\text{Ly}} \sim 4.6\)) Ca\(^{2+}\) stores (\([\text{Ca}^{2+}]_{\text{Ly}} \sim 0.5 \text{ mM}\)). IP3Rs on the ER release Ca\(^{2+}\) to produce a local high Ca\(^{2+}\) concentration, from which an unknown low-affinity Ca\(^{2+}\) transport mechanism refills Ca\(^{2+}\) to the lysosome. Unidentified tether proteins may link the ER membrane proteins to lysosomal membrane proteins to maintain contact sites of 20-30 nm for purposes of Ca\(^{2+}\) transfer. Ca\(^{2+}\) released from lysosomes or a reduction/depletion in \([\text{Ca}^{2+}]_{\text{Ly}}\) may regulate ER-lysosome interaction and trigger Ca\(^{2+}\) refilling from the ER to lysosomes. Xesto and 2APB are IP3R blockers; U73122 is a PLC inhibitor that blocks the production of IP3; DHBP and Ryanodine (> 10 μM) are specific RyR blockers.
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CHAPTER V

INHIBITION OF IP3-RECEPTORS ON THE ER CAUSES A LYSOSOME STORAGE PHENOTYPE

ABSTRACT

Endoplasmic reticulum (ER) Ca\(^{2+}\) deregulation is found in lysosome storage disorders (LSDs) and neurodegenerative disorders. We discovered that maintenance of lysosome Ca\(^{2+}\) stores is disrupted by inhibiting IP3Rs on the ER and sought to determine whether this could impact lysosome storage phenotypes. Antagonising IP3Rs and ER Ca\(^{2+}\) chelation increased lysosome associated membrane protein-1 (LAMP-1). LAMP-1 upregulation is a phenotype of lysosome dysfunction found in LSDs and neurodegenerative disorders. Previous studies have shown that the relative volume of lysosomal compartments is a valid biomarker for lysosome storage when measured using LysoTracker. After inhibition of IP3Rs, we found increased LysoTracker staining, suggesting that inhibiting ER IP3Rs results in lysosome dysfunction. Finally, we observed significantly increase accumulation of lipofuscin-like non-degraded materials in lysosomes after inhibiting IP3Rs. These findings suggest that impairing ER Ca\(^{2+}\) transfer to lysosomes has negative consequences on lysosome function, resulting in increased lysosome storage. Aberrant ER Ca\(^{2+}\) transfer to lysosomes may be responsible for altered Ca\(^{2+}\) homeostasis and the accumulation of toxic materials in LSDs and common neurodegenerative disorders.
INTRODUCTION

Aberrant lysosome Ca\textsuperscript{2+} store maintenance in LSDs and neurodegeneration

Lysosomal Ca\textsuperscript{2+} is important to lysosomal function and membrane trafficking (Cribbs and Strack 2007; Lloyd-Evans, Morgan et al. 2008; Shen, Wang et al. 2012). Lysosome Ca\textsuperscript{2+} levels are often reduced in lysosome storage diseases (LSDs) (Lloyd-Evans and Platt 2011). For example, Ca\textsuperscript{2+} levels have been shown to be reduced by 70\% in Niemann-Pick Disease, type C (NPC) cells (Lloyd-Evans, Morgan et al. 2008). Some LSDs result from the inability of lysosomes to release Ca\textsuperscript{2+}, as is the case with NPC1 (Shen, Wang et al. 2012) and Mucolipidosis type IV, which results in a mutation in the lysosome Ca\textsuperscript{2+} channel TRPML1 (Cheng, Shen et al. 2010). Interestingly, impaired ER Ca\textsuperscript{2+} homeostasis is associated with a variety of other LSDs including Gaucher’s disease, Sandhoff Disease, Niemann-Pick A disease, and GM1 gangliosidosis (Vitner, Platt et al. 2010). Although the underlying cause of Ca\textsuperscript{2+} alterations varies, Ca\textsuperscript{2+} dyshomeostasis is a commonality of many LSDs.

The presence of Ca\textsuperscript{2+} dyshomeostases in Alzheimer’s disease (AD) is clear, although precise mechanisms altering Ca\textsuperscript{2+} remain to be elucidated. Most of the focus on Ca\textsuperscript{2+} in AD has been on ER Ca\textsuperscript{2+} stores, although lysosome Ca\textsuperscript{2+} stores have recently been appreciated as an important site of Ca\textsuperscript{2+} deregulation as well (Bezprozvanny 2012; McBrayer and Nixon 2013).

Additional biomarkers of lysosome storage

Lysosomal dysfunction is often associated with a compensatory increase of lysosome biogenesis, manifested as increased expression of essential lysosomal genes (Settembre, Fraldi et al. 2013). For example, the expression of LAMP1, a house-keeping gene in the lysosome, is elevated in most LSDs (Cribbs and Strack 2007; Shen, Wang et al. 2012). The Coordinated Lysosomal Expression and Regulation (CLEAR) gene network regulates lysosomal biogenesis and function, and is dysregulated in LSDs as well as in neurodegenerative diseases (Settembre, Fraldi et al. 2013). Lysosome enlargement and an increase in lysosome number are also characteristic of LSDs (Cribbs and Strack 2007). The accumulation of undigested materials is often found in LSDs, which can further impair lysosome function and other cellular processes including lysosome acidification, lysosome Ca\textsuperscript{2+} homeostasis, vesicular trafficking, autophagy, exocytosis, endocytosis, and synaptic release (Schultz, Tecedor et al. 2011).
Altered lysosome function in Alzheimer’s disease

Lysosomal dysfunction and altered autophagy are clearly present in AD and other neurodegenerative diseases (Nixon, Yang et al. 2008). However, there are few mechanistic links between AD and lysosomal and autophagy defects (Bezprozvanny 2013). The efficiency of protein degradation decreases with age (Butler, Nixon et al. 2006) and endocytic dysfunction is also found early in AD (Nixon and Cataldo 2006). It is possible that ER Ca\textsuperscript{2+} transfer to endosomes and lysosomes is dysfunctional, resulting in the early pathologies of AD and influencing disease progression. Indeed, recent evidence links presenilin loss of function in AD with ER and lysosome Ca\textsuperscript{2+} homeostasis (Coen, Flannagan et al. 2012).

Focus of the current study

Our findings suggest that the ER provides Ca\textsuperscript{2+} to lysosomes through IP3Rs. Thus we sought to test the hypothesis that blocking ER Ca\textsuperscript{2+} or antagonizing IP3Rs would impact lysosome function, which is the focus of the following experiments.

RESULTS

LAMP-1 is upregulated after IP3R inhibition

The size and number of lysosomes can increase dramatically as a result of lysosome dysfunction, which causes the accumulation of undigested materials in lysosomal compartments (Appelqvist, Waster et al. 2013). Increases in LAMP1 are used as diagnostic marker of lysosome storage (Meikle, Brooks et al. 1997), as LAMP1 is the most abundant lysosomal membrane protein (Eskelinen, Tanaka et al. 2003). We first sought to determine if inhibiting ER Ca\textsuperscript{2+} or IP3Rs affected LAMP1 levels. We treated cells with low concentrations of ER inhibitors for 24h and then measured the amount of LAMP1 using western blotting when compared to DMSO treated controls. LAMP1 expression was significantly elevated in cells treated with IP3R blockers 2-APB and Xesto, as well as the ER Ca\textsuperscript{2+} chelator TPEN. The RYR blocker DHBP did not affect LAMP1 expression significantly (Fig. 5.1A,B). Because lysosomal membrane proteins are relatively stable, these effects are likely due to transcriptional upregulation of LAMP1, however this possibility was not examined further.

Lysotracker staining is increased after blocking Ca\textsuperscript{2+} transfer from the ER to lysosomes
Lysosomal dysfunction is also often associated with lysosomal enlargement due to the accumulation of various incompletely digested biomaterials (Cribbs and Strack 2007; Dong, Cheng et al. 2008; Shen, Wang et al. 2012). LysoTracker is a red fluorescent dye that contains a weak base that is only weakly protonated at neutral pH. Thus, LysoTracker can permeate live cell membranes, and it is highly selective for acidic vesicles within the cell. LysoTracker staining is used as a phenotypic assay to screen for enlarged lysosomes in LSDs (Xu, Liu et al. 2014) and is considered an additional biomarker of lysosome storage (te Vruchte, Speak et al. 2014). Consistent with our western blot analyses, LysoTracker staining was significantly increased in cells treated with Xesto for 24h, but not DHBP (Fig. 5.2A,B). These results suggest that either lysosome size or number increases, although this possibility was not investigated further.

**IP3R inhibitor Xesto increases accumulation of lipofuscin-like non-degradable material in lysosomes**

In addition to the enlargement of lysosomal compartments after abolishing Ca\(^{2+}\) transfer to lysosomes, we noticed that in unstained cells treated with IP3R blockers, lysosomal compartments displayed a high level of autofluorescence during our live cell imaging experiments. Autofluorescence was significantly increased in cells treated with Xesto when compared to those treated with DMSO (Fig. 5.3A,B,D). These findings are suggestive of the presence of non-degradable, lipofuscin-like materials accumulated within lysosomes (Schroder, Elsasser et al. 2007) (Fig. 5.3A,B). Notably, Xesto treated cells are reminiscent of cells with defective lysosomal Ca\(^{2+}\) release as shown with TRPML1 KO cells (Dong, Cheng et al. 2008) (Fig. 5.3C).

**DISCUSSION**

The lysosome compartment increases when Ca\(^{2+}\) stores are not maintained

Here we show that long-term (24h) application of IP3R inhibitors and ER Ca\(^{2+}\) chelators results in a lysosome storage phenotype in the cell, demonstrated using three different assays of lysosome storage. A plausible mechanism to account for our findings is that impaired Ca\(^{2+}\) transfer from the ER to lysosomes is responsible. If so, these findings suggest that Ca\(^{2+}\) transfer from the ER to lysosomes is an unappreciated phenomenon that, if altered or impaired, has a significant impact on lysosome function. We found that lysosome resident protein LAMP1 is
upregulated when IP3Rs and ER Ca\(^{2+}\) is inhibited, and that the overall volume of lysosomes was significantly enlarged. Interestingly, there were no obvious impairments in lysosome acidification, demonstrated by LysoTracker staining. Finally, we found undegraded, lipofusin-like material accumulates within cells that have been treated with IP3R antagonist Xesto. These findings highlight the importance of Ca\(^{2+}\) to lysosome function and suggest that it is not just H\(^+\) ions that are required for normal lysosome degradation and/or export of unwanted materials in the cell.

The increase in lysosome membrane proteins, number, and volume could be due to several factors. First, lysosomes may have increased fusion with other vesicles resulting in their enlargement. Lysosome fusion has been shown to be Ca\(^{2+}\) dependent, however, so this possibility is unlikely. Second, lysosome reformation through fission of larger lysosomes into smaller ones may be decreased. Lysosome fission has been shown to be Ca\(^{2+}\) dependent, making this possibility more likely (Durchfort, Verhoef et al. 2012; Zou, Hu et al. 2015).

**Lipofuscin accumulation increases in lysosome storage**

Lysosome storage material, summarized by the term lipofuscin, characterizes aged cells because its presence increases as aging progresses. The presence of lipofuscin has been found to be increased in many aging related diseases including Parkinson’s and Alzheimer’s diseases, macular degeneration, and a variety of other neurodegenerative diseases (Seehafer and Pearce 2006). Although lipofuscin is also termed the “aging pigment,” accumulation of lipofuscin occurs to a lesser degree even in healthy cells (Schroder, Wrocklage et al. 2010). Inhibition of lysosomal enzymes in young rat brains for one to two weeks induced lipofuscin accumulation, suggesting that loss of lysosomal enzymatic activity can result in lipofuscin accumulation (Ivy, Schottler et al. 1984). Analysis of accumulated lipofuscin-like materials in cells has revealed that these inclusions contain ~27-121 different proteins, many of which originate in other organelles (Schutt, Ueberle et al. 2002; Warburton, Southwick et al. 2005; Schroder, Elsasser et al. 2007). These findings support the idea that accumulated lipofuscin results from the buildup of materials from autophagic turnover of organelles and other unwanted materials in the cell (Schroder, Wrocklage et al. 2010). This may explain why lipofuscin is characterized by a wide range of fluorophores, typically between 320 to 480 nm and 460 to 630 nm (Seehafer and Pearce 2006; Schroder, Wrocklage et al. 2010). The buildup of accumulated materials further impairs
lysosomal degradative functions (Seehafer and Pearce 2006; Schroder, Wrocklage et al. 2010). Our findings that merely inhibiting ER IP3Rs for 18-24 h results in accumulated lipofuscin-like material supports the idea that ER Ca\(^{2+}\) transfer to lysosomes is essential to normal lysosome degradative function.

**Lysosome Ca\(^{2+}\) homeostasis does not impact lysosome pH, but does affect lysosome function**

Increased lysotracker staining suggests that lysosomes remain acidified after prolonged IP3R block. This suggests that Ca\(^{2+}\) entry into lysosomes may not be directly required for lysosome acidification, or that altered ion homeostasis can compensate for lack of Ca\(^{2+}\) to ensure lysosome acidification. Normal acidification is often the only factor examined when determining lysosome health and function, but our results suggest that Ca\(^{2+}\) is also required for lysosomal digestion. It is possible that Ca\(^{2+}\) activated enzymes exist in the lysosome, and this may be one way the ER communicates intracellular needs to the lysosome to match cellular degradation and recycling to production of necessary substrates in the cell.

**Altered ER Ca\(^{2+}\) homeostasis in LSDs**

Ca\(^{2+}\) homeostasis is altered in a variety of LSDs. ER Ca\(^{2+}\) is important for protein folding of key lysosomal enzymes so that they may be properly trafficked and delivered to the lysosome. Improper delivery of lysosomal enzymes results in the accumulation of that enzyme’s substrates within lysosomal membranes. For example, Gaucher’s disease, the most common LSD, is characterized by the buildup of glucosylceramide within lysosomes as a result of lysosomal glucocerebrosidase deficiency (Ong, Mu et al. 2010). Several LSDs result from specific enzyme deficiency and benefit from enzyme replacement therapies. Interestingly, in Gaucher’s disease, accumulated glucosylceramide in lysosomes in turn increases agonist induced Ca\(^{2+}\) release from RYR receptors on the ER (Korkotian, Schwarz et al. 1999; Lloyd-Evans, Pelled et al. 2003; Pelled, Trajkovic-Bodennec et al. 2005). Even more, ER morphology is significantly altered in Gaucher’s disease (Korkotian, Schwarz et al. 1999).

In light of our results, it is possible to speculate that signals from the lysosome that modulate ER Ca\(^{2+}\) release are altered when lysosomes have accumulated undigested contents. Indeed, decreased IP3 production has been observed when increases in glucosylceramide are
induced in cell culture (Mahdiyoun, Deshmukh et al. 1992). This may result in upregulation of RYRs to ensure ER Ca$^{2+}$ levels are stable (Korkotian, Schwarz et al. 1999), but then may result in aberrant Ca$^{2+}$ release and decreased Ca$^{2+}$ transfer to organelles like lysosomes and mitochondria. Similar mechanisms may also play a role in the alterations in lysosomal Ca$^{2+}$ storage and release seen in NPC1 cells (Lloyd-Evans, Morgan et al. 2008; Shen, Wang et al. 2012), as accumulated sphingomyelins have been shown to alter Ca$^{2+}$ release from TRPML1 (Shen, Wang et al. 2012). This suggests that the benefits seen by abolishing RYR Ca$^{2+}$ release and thereby increasing ER Ca$^{2+}$ in Gaucher’s disease and other LSDs may extend beyond just protein folding in the ER (Ong, Mu et al. 2010).

Some LSDs also have mitochondrial Ca$^{2+}$ defects. Pompe disease is characterized by profound mitochondrial Ca$^{2+}$ dyshomeostasis indicated by Ca$^{2+}$ overload, decreased in membrane potential, decreased ATP production, an increase in reactive oxygen species, and increased apoptosis (Lim, Li et al. 2015). It is possible that increased ER Ca$^{2+}$ transfer to mitochondria diverts Ca$^{2+}$ transfer to lysosomes and contributes to the disease pathogenesis. Alternatively, reduced ER-lysosome Ca$^{2+}$ transfer may result in the Ca$^{2+}$ overload seen in mitochondria to ensure ER Ca$^{2+}$ store homeostasis is maintained, as mitochondria do buffer ER Ca$^{2+}$, at least in polarized cells (Rizzuto, De Stefani et al. 2012). Increased ER-mitochondrial connectivity has also been shown in Alzheimer’s disease (Schon and Area-Gomez 2013), and it is possible that Ca$^{2+}$ in this case is diverted from lysosomes to reduce lysosome fusion and digestion and facilitate the accumulation of toxic proteins.

**Altered ER Ca$^{2+}$ homeostasis in Alzheimer’s disease**

Missense mutations in presenilins are responsible for early-onset AD. Presenilins provide catalytic substrates for various γ-secretases, but more recently additional roles for presenilins have been discovered including Ca$^{2+}$ homeostasis and protein trafficking (De Strooper, Iwatsubo et al. 2012). Presenilins are primarily localized to the ER membrane (Stutzmann 2007; Honarnejad and Herms 2012), and there is compelling evidence that presenilin depletion disrupts the ER Ca$^{2+}$ store, but the mechanisms by which this occurs are disputed (Bezprozvanny and Mattson 2008; De Strooper, Iwatsubo et al. 2012). Presenilins themselves may act as Ca$^{2+}$ leak channels on the ER (Tu, Nelson et al. 2006; Nelson, Tu et al. 2007; Bezprozvanny 2013). Presenilins have also been suggested to modify the activity of existing ER
Ca\textsuperscript{2+} channels. Mutations in presenilin have been shown to increase Ca\textsuperscript{2+} leak from the ER in part by increasing RYR expression (Chan, Mayne et al. 2000). Presenilins have also been shown to increase the activity of RYR channels through a direct interaction between the two proteins (Hayrapetyan, Rybalchenko et al. 2008). Presenilins have been suggested to modify Ca\textsuperscript{2+} signaling through IP3R channels as well (Leissring, Parker et al. 1999; Leissring, Paul et al. 1999), possibly by altering gating to increase Ca\textsuperscript{2+} signaling (Cheung, Shineman et al. 2008; Cheung, Mei et al. 2010). There is also some evidence to support diminished ER SERCA activity that normally maintains the ER Ca\textsuperscript{2+} as a result of presenilin loss of function (McBrayer and Nixon 2013). Whatever the final mechanism by which presenilins modify ER Ca\textsuperscript{2+}, it is clear that ER Ca\textsuperscript{2+} is affected by loss of presenilin function and this is a common pathology in AD.

Recently, presenilins have been implicated as the mechanistic link between ER and lysosome Ca\textsuperscript{2+} store alterations in AD (Bezprozvanny 2012). Lysosome dysfunction and autophagy defects are clear hallmarks of Alzheimer’s disease, but a cause for these defects has been elusive (Schultz, Tecedor et al. 2011). Presenilins were originally suggested to play a role in lysosomal acidification (Lee, Yu et al. 2010), however several groups have since thoroughly demonstrated normal acidification of lysosomes in cells lacking presenilins (Neely, Green et al. 2011; Coen, Flannagan et al. 2012; Zhang, Garbett et al. 2012; Bezprozvanny 2013). Presenilin deficient cells have enlarged lysosomes and decreased lysosomal Ca\textsuperscript{2+}, measured with GPN as well as a low-affinity Rhodamine-2 dextran Ca\textsuperscript{2+} probe (Bezprozvanny 2012; Coen, Flannagan et al. 2012). Notably, Coen et al’s GPN measurements of lysosome Ca\textsuperscript{2+} stores are consistent with a small GPN signal remaining due to pH in presenilin deficient cells (Coen, Flannagan et al. 2012). In agreement with Coen et al’s suggestion, this decrease in lysosomal Ca\textsuperscript{2+} may result in the impairments seen previously in lysosome fusion in presenilin deficient cells, which may be the cause of increased autophagy markers in these cell (Coen, Flannagan et al. 2012). Taken together, these findings may suggest that ER Ca\textsuperscript{2+} transfer to lysosomes may play a role in AD pathology.

Another interesting possibility is that in disorders where altered Ca\textsuperscript{2+} homeostasis is a hallmark, unknown tethers between organelles may be altered or abolished, thereby reducing the ability or efficiency of ER Ca\textsuperscript{2+} transfer to lysosomes. This may be a cause of increased Ca\textsuperscript{2+}
transfer to mitochondria in LSDs and AD, and may also be responsible for increased cytosolic 
Ca^{2+} release in AD that would instead be diverted to lysosomes in healthy circumstances.

**ER to lysosome Ca^{2+} transfer as a potential therapeutic target**

The possibilities for enhanced ER Ca^{2+} transfer to lysosomes as a potential therapeutic 
target are interesting to consider. For example, TFEB enhances lysosomal exocytosis and 
therefore lysosome clearance (Medina, Fraldi et al. 2011), which has been shown to require 
lysosomal Ca^{2+} (Samie, Wang et al. 2013) and the TRPML1 Ca^{2+} channel (Medina, Di Paola et 
al. 2015). While inducing exocytosis itself may be dangerous to cells (Schultz, Tecedor et al. 
2011), enhancing lysosome Ca^{2+} to ensure effective exocytosis could be equally efficacious.
Given that RYR antagonism increases ER Ca^{2+} (Ong, Mu et al. 2010), and even our results 
suggest that it may increase lysosomal Ca^{2+} and degradation, this may be one route to consider. 
Given that the master regulator of lysosomes, TFEB, requires Ca^{2+} release from lysosomes 
(Medina, Di Paola et al. 2015), one can imagine a whole host of unexplored negative 
consequences of reduced lysosome Ca^{2+}.

**Limitations**

Pharmacological studies are limited by potential off-target effects of each antagonist used. In the 
case of the aforementioned findings, it is possible that impairing ER Ca^{2+} release may impair ER 
function. Impaired ER function may affect lysosome degradative functions not because of 
impaired Ca^{2+} transfer, but for an alternative reason. While these possibilities are difficult to 
separate, we showed that both chelation of ER Ca^{2+} and block of Ca^{2+} release through IP3Rs 
both resulted in impaired lysosome degradation.

**METHODS AND MATERIALS**

**Western blotting.** Standard western blotting protocols were used. A Bradford assay was 
performed prior to adding DTT in order to ensure the loading of equal loading. HEK293T cells 
were treated every 4 hrs for 24 hrs with IP3R antagonists 2-APB and Xestospongin-C, ER Ca^{2+} 
chelator TPEN, and RyR antagonist DHBP. LAMP1 and tubulin antibodies were from 
Developmental Studies Hybridoma Bank (Iowa). LAMP1 was normalized to tubulin amounts to 
ensure quantifications were not affected by loading concentrations.
Mammalian Cell Culture. All cells were cultured in a 37°C incubator with 5% CO₂. HEK293T cells and human fibroblasts were cultured in DMEM F12 (Invitrogen) supplemented with 10% (vol/vol) FBS or Tet-free FBS.

Confocal imaging. Live imaging of cells was performed on a heated and humidified stage using a Spinning Disc Confocal Imaging System. The system includes an Olympus IX81 inverted microscope, a 100X Oil objective NA 1.49 (Olympus, UAPON100XOTIRF), a CSU-X1 scanner (Yokogawa), an iXon EM-CCD camera (Andor). MetaMorph Advanced Imaging acquisition software v.7.7.8.0 (Molecular Devices) was used to acquire and analyze all images. LysoTracker (50 nM) was dissolved in culture medium and loaded into cells for 30 min before imaging. Coverslips were washed 3 times with Tyrode’s and imaged in Tyrode’s. LysoTracker staining was quantified as described previously, using Image J.

Reagents. All reagents were dissolved and stored in DMSO or water and then diluted in cell culture medium for experiments. 2-APB, DHBP, and TPEN were from Sigma; LysoTracker, was from Invitrogen; and Xestospongion-C was from Cayman Chemical, AG Scientific, and Enzo.

Data analysis. Data are presented as mean ± SEM. All statistical analyses were conducted using GraphPad Prism. Paired t-tests were used to compare the average of three or more experiments between treated and untreated conditions. A value of P <0.05 was considered statistically significant.
Figure 5.1 LAMP1 increased significantly after inhibiting ER Ca\(^{2+}\) and IP3Rs. (A) Western blotting analyses of LAMP1 in HEK293T cells treated with 2-APB (50 µM), TPEN (0.1 µM), Xesto (10 µM), and DHBP (5 µM) compared to DMSO for 24 hrs (n=4 separate experiments using one dish of cells and separate protein extractions for each condition). (B) Treating HEK293T cells with 2-APB (p=0.05) and Xesto (p=0.012), as well as TPEN (p=0.02), significantly increased LAMP1 expression. DHBP did not significantly change LAMP1 expression (p=0.23).
Figure 5.2 LysoTracker staining increased, suggestive of a LSD phenotype, in cells treated with IP3R blocker Xesto. (A) The effects of Xesto (10 µM, 18 h; p= 0.0001) and DHBP (50 µM, 18 h; p= 0.063) treatment compared to DMSO on the lysosomal compartments detected by LysoTracker staining in HEK293T cells (average of 20-30 cells in each of 3 experiments). Scale bar = 15 µm. (B) The effect of Xesto (10 µM, 18 h) treatment on accumulation of the autofluorescent lipofuscin materials in non-transfected HEK293T cells.
Figure 5.3 Autofluorescent, lipofuscin-like accumulation in cells treated with IP3R blocker Xesto mimics TRPML1 KO cells. (A) Human fibroblasts treated with DMSO for 18h. (B) Human fibroblasts treated with Xesto (2.5 µM) for 18h. (C) ML1 KO MEFs are shown for comparison. (D) Quantification of DMSO and Xesto (p<0.0001) treated cells. Autofluorescence was observed in a wide spectrum but shown at two excitation wavelengths (488 and 561 nm). Scale bar = 15 µm.
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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

INTRODUCTION

Lysosomes are significant Ca\textsuperscript{2+} stores in the cell, with higher Ca\textsuperscript{2+} than all other organelles except the endoplasmic reticulum (ER). How the ER maintains and refills its Ca\textsuperscript{2+} store has been the subject of intense study for over a decade beginning around 2000, though store operated Ca\textsuperscript{2+} entry (SOCE) has been appreciated since the early 1980’s. How lysosomes acquire their Ca\textsuperscript{2+} stores has been misattributed to their pH gradient for over a decade. Because Ca\textsuperscript{2+} stores are integral to lysosome membrane trafficking and degradative functions, understanding how lysosomes acquire their Ca\textsuperscript{2+} answers a basic question of cell biology. Furthermore, it highlights new potential therapeutic targets for lysosome storage disorders (LSDs) and neurodegenerative disorders, both of which have disrupted Ca\textsuperscript{2+} stores.

This research shows that the lysosomal V-ATPase and pH gradient are not required for the refilling of lysosome Ca\textsuperscript{2+} stores (Chapter 2). We developed a new assay to measure lysosome Ca\textsuperscript{2+} store refilling that allows us to release lysosome Ca\textsuperscript{2+} stores in a physiological manner that is uninhibited by low lysosome pH. This assay allows us to apply pharmacological agonists and antagonists during refilling to directly assess the source of Ca\textsuperscript{2+} to the lysosome. This assay has numerous advantages over previously used methods to study lysosomes in that it does not affect ion homeostasis or disrupt lysosome membranes.

We found that a commonly used lysosome disrupting reagent, GPN, induces a signal on GCaMP3 and Fura-2 Ca\textsuperscript{2+} indicators that is not due to Ca\textsuperscript{2+}, which has likely obscured the conclusions of many studies examining lysosome Ca\textsuperscript{2+} stores. Because GPN has been used as the primary method to examine lysosome Ca\textsuperscript{2+} stores, our findings suggest that many studies may have misattributed effects of GPN on pH to the release of Ca\textsuperscript{2+}. This is also likely the reason that many studies have failed to appreciate the effects of ER Ca\textsuperscript{2+} on lysosome Ca\textsuperscript{2+}. Our
findings support the need to use the Ca\textsuperscript{2+} chelator BAPTA with all studies examining lysosome Ca\textsuperscript{2+} to ensure the effects are due to Ca\textsuperscript{2+}.

We showed for the first time that Ca\textsuperscript{2+} stores in the lysosome require ER Ca\textsuperscript{2+} stores to be maintained (Chapter 3). Using SERCA inhibitors to prevent ER Ca\textsuperscript{2+} store maintenance, we showed that lysosome stores are no longer refilled when ER Ca\textsuperscript{2+} stores are abolished. Chelating ER Ca\textsuperscript{2+} had the same effect, preventing lysosome Ca\textsuperscript{2+} refilling. Genetically reducing ER Ca\textsuperscript{2+} stores also prevented lysosome Ca\textsuperscript{2+} store refilling. We corroborate the findings of others showing that the ER and lysosomes are in close contact, which could facilitate Ca\textsuperscript{2+} transfer from the ER to lysosomes through membrane contact sites.

More specifically, we found that the IP3-receptor (IP3R) on the ER membrane is a candidate for transferring Ca\textsuperscript{2+} to the lysosome (Chapter 4). Using three different IP3R inhibitors, we show that lysosome Ca\textsuperscript{2+} stores do not refill when ER IP3R Ca\textsuperscript{2+} release is blocked. Ryanodine receptors are not required for lysosome Ca\textsuperscript{2+} store refilling, because their block had no impact on Ca\textsuperscript{2+} store refilling. We found that IP3R blockers prevented Ca\textsuperscript{2+} refilling in DT40-WT cells, but not in DT40-IP3R-TKO cells, which may suggest that a compensatory mechanism accounts for Ca\textsuperscript{2+} refilling in cells without IP3Rs.

Finally, disrupting lysosome Ca\textsuperscript{2+} store maintenance by blocking ER Ca\textsuperscript{2+} release through IP3Rs results in an upregulation of lysosome associated membrane protein 1 (LAMP1), which is a marker for lysosome dysfunction. IP3R antagonism also results in an increase in lysosome volume, another biomarker of lysosome storage disorders. Finally, an accumulation of lipofuscin results from blocking ER IP3Rs. These results highlight the importance of ER Ca\textsuperscript{2+} transfer to lysosome Ca\textsuperscript{2+} store maintenance and proper lysosome function. Because Ca\textsuperscript{2+} store dysregulation is found in lysosome storage disorders and neurodegeneration, these findings suggest that increasing ER Ca\textsuperscript{2+} release to lysosomes could improve or prevent lysosome pathogenesis.

**Limitations**

Studies of lysosome Ca\textsuperscript{2+} stores are difficult because of the high acidity of the lysosome lumen, which is known to affect a number of Ca\textsuperscript{2+} indicators (Rudolf et al., 2003). Indeed, many of our experiments confirmed this. Our lysosome-membrane targeted GCaMP3-ML1 probe avoids pH
effects of the lysosome lumen and allows us to measure \( \text{Ca}^{2+} \) release from lysosomes. A disadvantage of this probe is that it does not directly measure the amount of \( \text{Ca}^{2+} \) in the lysosome lumen and it does not allow us to observe \( \text{Ca}^{2+} \) refilling in real-time. Additionally, overexpression of a \( \text{Ca}^{2+} \) channel is not ideal, as it likely results in an increase in \( \text{Ca}^{2+} \) release when compared to typical \( \text{Ca}^{2+} \) release from lysosomes. This may increase the amount of \( \text{Ca}^{2+} \) refilled to lysosomes, increase the time it takes to refill \( \text{Ca}^{2+} \) stores, and may change signaling between lysosomes and the ER.

Many of our manipulations of ER \( \text{Ca}^{2+} \) are pharmacologic, which has significant limitations due to the potential of off-target effects of each treatment. While we utilized different treatments with the same target, future studies should attempt to genetically deplete IP3Rs further support their importance in \( \text{Ca}^{2+} \) store refilling.

The finding that DT40 IP3R TKO cells still have lysosome \( \text{Ca}^{2+} \) stores even without IP3Rs does not support our hypothesis. While we believe a compensatory mechanism is at play, this possibility should be further examined in future studies.

We attempted to measure ER \( \text{Ca}^{2+} \) levels during lysosome \( \text{Ca}^{2+} \) store refilling with two different luminal ER \( \text{Ca}^{2+} \) indicators, but we had difficulty with the sensitivity of these probes and were thus unable to observe all but the largest changes in ER \( \text{Ca}^{2+} \) due to complete depletion of ER \( \text{Ca}^{2+} \) stores with SERCA inhibitors. Future studies should use newly developed ER luminal \( \text{Ca}^{2+} \) indicators to observe ER \( \text{Ca}^{2+} \) decreases during lysosome \( \text{Ca}^{2+} \) refilling. Most of our studies are performed in HEK cells, which may limit their application to other cell types. It is possible that lysosomes function differently in different cell types.

**Future Directions**

These results form the basis for a stronger understanding of lysosome biology, but raise many yet to be answered questions, which will be discussed below.

**Additional Assays of Lysosomal \( \text{Ca}^{2+} \)**

While our GCaMP3-ML1 probe has advantages for measuring lysosomal \( \text{Ca}^{2+} \) over previously used methods, there are disadvantages as well. In cases where TRPML1 is inhibited, as in NPC (Shen et al., 2012), lysosome \( \text{Ca}^{2+} \) store refilling cannot be accurately measured. Recently,
several new \( \text{Ca}^{2+} \) sensors have been developed that should be used to confirm our findings. A new endosomal \( \text{Ca}^{2+} \) indicator to measure luminal \( \text{Ca}^{2+} \) levels should be tested in lysosomes (Albrecht et al., 2015). Calcium orange is a newly used \( \text{Ca}^{2+} \) dye for acidic compartments that should also be tested (Zou et al., 2015).

**How is \( \text{Ca}^{2+} \) Transfer from the ER to Lysosomes Regulated?**

The existence of a \( \text{Ca}^{2+} \) sensor dedicated to sensing lysosome \( \text{Ca}^{2+} \) stores and regulating their refilling remains to be shown. In the ER, STIM1 senses when \( \text{Ca}^{2+} \) stores are being depleted using an EF-hand-like motif, and relocates when stores are reduced to trigger \( \text{Ca}^{2+} \) channel opening on the plasma membrane (Luik et al., 2008). This suggests that a similar molecule may exist on lysosomes to trigger ER \( \text{Ca}^{2+} \) transfer to lysosomes.

Phosphinositides are known to be regulated at membrane contact sites with the ER and plasma membrane (Stefan et al., 2011). Because phosphinositides can activate/regulate ion channels (Dong et al., 2010; Wang et al., 2012), it is possible that phosphinositide localization plays a role in \( \text{Ca}^{2+} \) exchange between the ER and lysosomes.

The question remains as to what other triggering factors are required for \( \text{Ca}^{2+} \) refilling to lysosomes. Is \( \text{Ca}^{2+} \) release from lysosomes sensed by the ER which leads to tighter contact and \( \text{Ca}^{2+} \) refilling? Our results showing that refilling is mediated by IP3Rs, but not RYRs, suggest that ER \( \text{Ca}^{2+} \) release induced by lysosomal \( \text{Ca}^{2+} \) release may not operate through \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release, as RyRs are better suited for this role than IP3Rs. This suggests that other signalling molecules may be involved in triggering IP3R \( \text{Ca}^{2+} \) release to refill lysosome stores.

\( \text{Ca}^{2+} \) exchange between the ER and lysosomes could also be continuous to maintain the lysosome \( \text{Ca}^{2+} \) store at a constant level. In mitochondria, constitutive \( \text{Ca}^{2+} \) transfer to mitochondria prevents autophagy, and when IP3R \( \text{Ca}^{2+} \) signaling is decreased, autophagy is induced (Cardenas et al., 2010). \( \text{Ca}^{2+} \) transfer from ER to mitochondria is therefore involved in ER coordination of cellular bioenergetics, which could also be true for the lysosome.

**How do Membrane Contact Sites Regulate ER-Lysosome \( \text{Ca}^{2+} \) Transfer?**

Our time lapse imaging and TEM findings corroborate previous findings that membrane contact sites exist between the ER and lysosomes (Kilpatrick et al., 2013). Nanojunctions of 50 nm or
less are ideal for Ca$^{2+}$ exchange (Fameli et al., 2014), making these sites between the ER and lysosomes ideal to serve as “nano-domains” of Ca$^{2+}$ exchange.

Linker proteins have been shown to exist to facilitate ER-lysosome contact sites (Rocha et al., 2009; Alpy et al., 2013), but similar to ER-mitochondrial junctions, there may be many proteins that create contact sites, and abolishing just one may not impact Ca$^{2+}$ exchange (Lynes and Simmen, 2011). It has been hypothesized that different distances between membranes are required for lipid, protein, and Ca$^{2+}$ exchange and that different subsets of contact sites may exist to facilitate different functions (Csordas et al., 2010). Whether specific signalling molecules exists to increase contact sites or decrease contact length to facilitate the ER-lysosome nanojunction, or to trigger ER Ca$^{2+}$ release to lysosomes is also a mystery.

ER-endosome membrane contact, although currently still difficult to study, is proposed to facilitate cholesterol transport from endosomes to the ER (Rocha et al., 2009; Du et al., 2011; Du et al., 2012; van der Kant and Neefjes, 2014). Given the established role of lysosomal Ca$^{2+}$ release in cholesterol transport (Shen et al., 2012), lysosomal Ca$^{2+}$ may have a direct role in regulating this ER-lysosome interaction. For example, cytosolic cholesterol sensor ORP1L detects cholesterol in late endosomes which subsequently facilitates a membrane contact site using VAP complexes on the ER and the Rab7-RILP complex on late endosomes. Low cholesterol levels increases contact sites, whereas high cholesterol decreases these contact sites, and positioning of late-endosomes is more peri-nuclear when cholesterol levels are higher (Rocha et al., 2009).

Cholesterol binding protein NPC1 on late-endosome and lysosome membranes has been shown to interact with and be required for ER-localized sterol carrier protein ORP5 to transport cholesterol out of late-endosomes and lysosomes (Du et al., 2011). In fact, ORP proteins can simultaneously bind two membranes at once, suggesting that membranes must be in close contact for its function as a sterol transporter (Schulz et al., 2009). Separately, Hrs-Vps27 regulates cholesterol transfer from late-endosomes/lysosomes independently of NPC1 and NPC2 (Du et al., 2012).

Late endosome/lysosome lipid binding proteins STARD3 and STARD3NL, using a conserved FFAT-like motif, contact ER proteins VAP-A and VAP-B to form membrane contact

166
sites that separate the ER and late-endosomes by about 8.3 nm (Alpy et al., 2013). It may be that different subpopulations of late-endosomes and lysosomes use different cholesterol transporters, as has been suggested (van der Kant et al., 2013), that these contact sites have different purposes that are not yet clear, or that there is functional redundancy for cholesterol transport at these membrane contact sites.

Notably, VAP-A and VAP-B on the ER are involved in membrane contact sites between the ER and mitochondria (De Vos et al., 2012) and the ER and golgi (Kawano et al., 2006), in addition to late-endosomes (Rocha et al., 2009), as discussed above. Although siRNA knock-down of ER VAP proteins does not completely abolish membrane contact sites (Alpy et al., 2013), it does reduce them, and therefore ER VAP proteins could be a starting point of future studies examining ER-lysosome Ca\(^{2+}\) transfer.

**Do Specific Isoforms of IP3Rs Transfer Ca\(^{2+}\) to Lysosomes?**

These results also raise the question of whether specific IP3R isoforms contribute Ca\(^{2+}\) to the lysosome for different purposes and whether some are more involved in Ca\(^{2+}\) refilling than others. This question may prove difficult to answer, however. All three IP3R isoforms can transmit Ca\(^{2+}\) to mitochondria, and knock-down of a particular isoform only reduces but does not abolish Ca\(^{2+}\) transfer to mitochondria (Mendes et al., 2005). Also, knocking down IP3Rs does not abolish ER-mitochondria contact, which may suggest that other Ca\(^{2+}\) channels can transfer Ca\(^{2+}\) from the ER to mitochondria as well.

GRP75 tethers the ligand binding domain of the type I IP3R isoform on the ER to the VDAC on the OMM of mitochondria (Szabadkai et al., 2006). Both type I and III IP3R isoforms are involved in Ca\(^{2+}\) signaling in apoptosis, although the type III isoform seems to play a larger role. The type III IP3R isoform has been suggested to be enriched at the MAM as well (Mendes et al., 2005).

Making it more complicated, IP3R subtypes are dynamically regulated in the cell and their expression levels and localization seem to depend on the physiological state of the cell (Vermassen et al., 2004). Even though MAMs are a high Ca\(^{2+}\) release site, many studies have found that IP3Rs are not specifically enriched on the MAM of the ER compared to other parts of the ER membrane (Lynes and Simmen, 2011). This may suggest that ER Ca\(^{2+}\) buffering within
the ER membrane may function to more directly regulate ER Ca\textsuperscript{2+} release rather than the location of IP3Rs. Enrichment and spatiotemporal regulation of Ca\textsuperscript{2+} buffering proteins has been shown for ER Ca\textsuperscript{2+} buffering proteins calreticulin and calsequestrin (Means et al., 2006; Lynes and Simmen, 2011).

IP3Rs are known to cluster into groups of 20-30, and clustering has also been shown to be dynamically regulated (Rahman and Taylor, 2009; Rahman, 2012). It is possible that increase in Ca\textsuperscript{2+} release seen between the first and second responses in HEK-GCaMP3-ML1 cells is due to an increase in or formation of IP3R clusters. Alternatively, it is probable that overexpression of the TRPML1 channel in HEK cells stably expressing GCaMP3-ML1 increases the amount of Ca\textsuperscript{2+} released by lysosomes. This may result in an increased transfer of Ca\textsuperscript{2+} back to lysosomes, particularly because many Ca\textsuperscript{2+} channels on the ER, including IP3Rs, are regulated by Ca\textsuperscript{2+}.

Possible Subdomains of Ca\textsuperscript{2+} Release in the ER to Regulate Membrane Contact Sites?

Subdomains of Ca\textsuperscript{2+} release, or pools of Ca\textsuperscript{2+} released by different factors, are beginning to be understood within the contiguous ER (Orci et al., 2009; Aulestia et al., 2011; Penny et al., 2014; Petersen, 2015). These findings raise the question of whether a separate pool of Ca\textsuperscript{2+} exists in the ER that is releasable for refilling to organelles or even lysosomes specifically. Specific membrane domains of the ER for specialized functions are also being more closely examined (Lynes and Simmen, 2011).

It is possible that local subdomains of ions regulate site specific processes even in smaller vesicles. Given that lysosomes make transient and on-going membrane contact sites with a variety of different organelles (Penny et al., 2014), intraluminal ion subdomains would facilitate appropriate fusion and communication between each of these vesicles and allow for multiple membrane contact sites on each organelle. These spatiotemporal sites are likely regulated in part by membrane lipids that can in turn regulate ion channels on vesicular membranes like lysosomes (Dong et al., 2010; Wang et al., 2012; Zhang et al., 2012). The balance of ions and thus tightly regulated membrane potential may also indicate lysosomal function and health to other organelles.
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