Vacuolar Proteases of *Saccharomyces cerevisiae*: Characterization of an Overlooked Homolog Leads to New Functional Insights

by

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Dedication

To my family, with love.

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Table of Contents

Dedication ii

Acknowledgements iii

List of Figures vii

List of Tables viii

Abstract ix

Chapter I: Vacuolar hydrolysis and efflux: Current knowledge and unanswered questions 1

Introduction 1

Nucleic Acids 3

Lipids 4

Polyphosphate 6

Carbohydrates 9

Organelles 12

Proteins 15

Vacuolar proteins of unknown function 27

Conclusions and goals for this dissertation 28

References 29

Chapter II: An overview of autophagy: Morphology, mechanism and regulation 41

Abstract 41

Introduction 41

I. Microautophagy 43

II. Chaperone-mediated autophagy (CMA) ... 43
III. Macroautophagy ... 45
Conclusions ... 57
References ... 67

Chapter III: A newly characterized vacuolar serine carboxypeptidase, Atg42/Ybr139w, is required for normal vacuole function and the terminal steps of autophagy in the yeast *Saccharomyces cerevisiae* ... 77

Abstract ... 77
Introduction ... 78
Results ... 80
Discussion ... 87
Materials and Methods ... 89
References ... 101

Chapter IV: Conclusions and Future Directions ... 105

Comparative studies of Atg42/Ybr139w and Prc1 ... 105
Zymogen activation cascade ... 108
Lysis of autophagic bodies and effects on substrate degradation ... 109
Characterization of additional proteases ... 110
Final Perspectives ... 111
References ... 112

List of Figures

Figure II.1. Three types of autophagy in mammalian cells. 59

Figure II.2. Morphology of macroautophagy. 60

Figure II.3. The induction complex consists of ULK1/2, ATG13, RB1CC1, and C12orf44. 61

Figure II.4. The activity of the class III PtdIns3K complex is regulated by subunit composition. 62

Figure II.5. ATG12–ATG5-ATG16L1 conjugation complex. 63

Figure II.6. The LC3 conjugation system. 64

Figure II.7. Regulation of macroautophagy. 65

Figure II.8. Two mechanisms of mitophagy. 66

Figure III.1. Ybr139w is a soluble vacuolar glycoprotein. 92

Figure III.2. Vacuolar function is impaired in cells lacking *PRC1* and *YBR139W*. 93

Figure III.3. Ybr139w is a serine carboxypeptidase. 94

Figure III.4. Cells lacking *PRC1* and *YBR139W* are defective in the terminal steps of autophagy. 95

Supplemental Figure III.S1. Reintroduction of *PRC1* or *YBR139W* complements protein processing defects in *prc1*Δ *ybr139w*Δ mutants. 98

Supplemental Figure III.S2. Prc1, but not Ybr139w, is required for Carboxypeptidase Y activity. 99

Supplemental Figure III.S3. Reintroduction of *PRC1* or *YBR139W* complements autophagy defects in *prc1*Δ *ybr139w*Δ mutants. 100

List of Tables

Table III.1. Strains used in this study. 96

Table III.2. Plasmids used in this study. 97

Abstract

Hydrolysis within the vacuole in yeast and lysosome in mammals is required for the degradation and recycling of a vast array of substrates. In humans, defects in lysosomal hydrolysis and efflux contribute to a class of diseases referred to as lysosomal storage disorders that affect 1/8000 live births. Despite the importance of these processes, many of the proteins and regulatory mechanisms involved in hydrolysis and efflux are poorly understood, especially those involved in proteolysis.

Using the yeast *Saccharomyces cerevisiae* as a model, I employed a combination of molecular and cellular biological techniques to characterize a previously overlooked homolog of the protease Prc1 (carboxypeptidase Y), Ybr139w. I demonstrated that these two homologous serine carboxypeptidases are required for proper functioning of the vacuole; cells lacking Prc1 and Ybr139w exhibit defects in zymogen activation, amino acid recycling, and degradation of autophagic bodies delivered to the vacuole via macroautophagy (hereafter autophagy). Based on its function in the terminal steps of autophagy, I have proposed that Ybr139w be renamed as Atg42.

This work expands our understanding of vacuolar proteases and encourages improved characterization of these proteins together with potential homologs. Such an undertaking will enable further dissection of the mechanisms of proteolytic activation of zymogens and the terminal steps of autophagy, including lysis of the autophagic body, degradation of the cargo, and efflux of the resultant macromolecules.

Chapter I

Vacuolar hydrolysis and efflux: Current knowledge and unanswered questions[1]

Introduction

The yeast vacuole (lysosome in humans) is a key center for metal ion homeostasis, nutrient storage, and cellular detoxification (Li and Kane, 2009). Perhaps its best-known function is as a degradative organelle; in yeast, the vacuole accounts for approximately 40% of protein degradation during growing conditions, which increases to 85% when cells are starved of nutrients (Teichert *et al.*, 1989). Substrates destined for the vacuole can be delivered there by a variety of trafficking mechanisms including the vacuolar protein sorting pathway, endocytosis, the cytoplasm-to-vacuole targeting (Cvt) pathway, and direct transport across the vacuole membrane (Li and Kane, 2009; Feyder *et al.*, 2015). A major trafficking pathway whereby proteins and other substrates are delivered to the vacuole during stress conditions is autophagy (Wen and Klionsky, 2016). A detailed review of the mechanism of autophagy in mammals is presented in chapter II. Here, I will provide a brief overview of the process in *Saccharomyces cerevisiae*.

In yeast, there are two primary types of autophagy, selective and non-selective. Either of these processes can occur through microautophagy or macroautophagy. Microautophagy involves internalization of cargo into the vacuolar lumen through invagination or protrusion of the vacuolar limiting membrane. The membrane scissions off into the vacuolar lumen, after which the resultant intralumenal vesicles are lysed and the cargo degraded (Reggiori and

[1] A modified version of this chapter has been submitted for publication in *Autophagy*.

Klionsky, 2013; Wen and Klionsky, 2016). Macroautophagy, the more extensively studied form of autophagy, is characterized by *de novo* formation of a double-membrane structure that encapsulates cargo away from the vacuole. Under conditions of nutrient starvation, macroautophagy, hereafter autophagy, is induced at the perivacuolar phagophore assembly site (PAS) by the Atg1 kinase complex (Suzuki *et al.*, 2001; Mizushima, 2010). Following induction, nucleation and membrane expansion lead to formation of a transient double-membrane phagophore that forms *de novo* and gradually expands to surround cargo. These processes involve the transmembrane protein Atg9 and phosphatidylinositol 3-kinase (PtdIns3K) complex I, as well as two ubiquitin-like (Ubl) conjugation systems that include the Ubl proteins Atg12 and Atg8 (Reggiori and Klionsky, 2013; Wen and Klionsky, 2016).

Eventually, the ends of the expanding phagophore join to form a completed double-membrane vesicle called an autophagosome. The mature autophagosome travels to the vacuole, where the outer membrane of the autophagosome fuses with the limiting membrane of the vacuole, releasing the cargo bound by the autophagosome inner membrane into the vacuolar lumen, where it is now termed an autophagic body. Once inside the vacuole, the autophagic body must be lysed, its contents degraded, and the breakdown products transported back into the cytoplasm for reuse (Reggiori and Klionsky, 2013).

The vast majority of autophagy research has focused on induction, regulation, membrane recruitment and autophagosome formation, cargo recognition, and fusion of autophagosomes with the vacuole, whereas the intravacuolar steps of degradation and efflux of substrates have been largely neglected. Although generally glossed over in discussions of autophagic processes, these terminal events are critically important for completion of autophagy and maintenance of cellular health. In humans, there is an entire class of more than fifty diseases, the lysosomal

storage disorders, involving accumulation of different substrates in the lysosome resulting from defects in lysosomal hydrolases and their activators as well as vacuolar transporters (Greiner-Tollersrud *et al.*, 2005; Boustany, 2013). Altogether, lysosomal storage disorders occur in approximately 1/8000 live births (Meikle *et al.*, 1999; Poorthuis *et al.*, 1999), varying widely in age of onset, severity, substrate(s) accumulated, and organ system(s) affected.

In this chapter, I will discuss several major classes of vacuolar substrates, current knowledge regarding their degradation and efflux, effects of defects in these processes, and unanswered questions that require further study.

Nucleic Acids

The vast majority of studies on RNA degradation have focused on nuclear and cytoplasmic RNA decay and quality control pathways. However, RNA degradation also occurs in the vacuole in an autophagy-dependent manner, which contributes to cellular RNA homeostasis and regulation of translational fidelity (Frankel *et al.*, 2017). T2 RNases are a highly-conserved family of endoribonucleases that cleave single-stranded RNA, resulting in mono- or oligonucleotides with a terminal 3' phosphate group (Irie, 1999). To date, Rny1 is the only known vacuolar RNase (MacIntosh *et al.*, 2001). This enzyme converts RNA in the vacuole to 3' mononucleotides, and cells lacking Rny1 accumulate free RNA in the vacuole following autophagy induction by nitrogen starvation (Huang *et al.*, 2015). The vacuolar phosphatase Pho8 then converts the 3' mononucleotides into nucleosides, which are released into the cytoplasm for further processing (Huang *et al.*, 2015). Similar to several autophagy-related proteins, Rny1 levels increase significantly during nitrogen starvation, as do Pho8 levels, albeit not significantly (Müller *et al.*, 2015).

Various animal studies have highlighted the physiological importance of RNA degradation in the context of neurodegenerative diseases. Loss of the zebrafish ortholog of Rny1, Rnaset2, leads to accumulation of rRNA in neuronal lysosomes (Haud *et al.*, 2011). Loss-of-function mutations in the human ortholog, RNASET2, are associated with the neurological disease cystic leukoencephalopathy (Henneke *et al.*, 2009).

Cells lacking the two major vacuolar proteases, Pep4 (proteinase A) and Prb1 (proteinase B), fail to display an increase in nucleoside levels upon nitrogen starvation (Huang *et al.*, 2015). As will be discussed later in this chapter, Pep4 and Prb1 are required for proteolytic processing and activation of several vacuolar hydrolases, including Pho8 (Klionsky and Emr, 1989), as well as lysis of autophagic bodies within the vacuole (Takeshige *et al.*, 1992). It is unknown whether it is the failure to lyse autophagic bodies, failure to activate Pho8, or both that accounts for the impaired RNA degradation in *pep4*Δ *prb1*Δ cells. In yeast, it is still unclear which RNA species undergo autophagy-dependent degradation. Also unknown is the efflux mechanism for the resultant nucleosides and the identity of any transporter(s) involved in this process. The nucleoside transporter Fun26 is likely involved, due to its localization to the vacuolar membrane (Vickers *et al.*, 2000; Wiederhold *et al.*, 2009; Lu and Lin, 2011; Boswell-Casteel *et al.*, 2014).

Lipids

Non-polar, or "neutral", lipids serve a variety of purposes in eukaryotic cells; they can be used as precursors for membrane biogenesis and participate in energy production during starvation (Barbosa and Siniossoglou, 2017). Cells defective in the production of neutral lipids exhibit a block in autophagy at the early stages of autophagosome formation (Li *et al.*, 2015; Shpilka *et al.*, 2015). In the cytoplasm, neutral lipids are stored within specialized organelles called lipid droplets (LDs) that can also sequester toxic fatty acids that may be harmful to cells.

LDs consist of a phospholipid monolayer and associated proteins surrounding a hydrophobic core of neutral lipids, mostly consisting of triacylglycerol (TAG) and steryl esters (SE) (Wang, 2015; Barbosa and Siniossoglou, 2017). Lipids can be liberated from LDs in several ways, including both a cytoplasmic process and an autophagy-related process called lipophagy.

In mammalian cells, lipophagy occurs through macroautophagy (Singh *et al.*, 2009). In yeast, however, lipophagy occurs through microautophagy and can be induced in response to nitrogen starvation (van Zutphen *et al.*, 2014), stationary phase (Wang *et al.*, 2014), and lipid imbalances resulting from inhibition of phosphatidylcholine biosynthesis (Vevea *et al.*, 2015). Lipophagy induced by nitrogen starvation or stationary phase requires the core autophagy machinery (van Zutphen *et al.*, 2014; Wang *et al.*, 2014). The role of the autophagic machinery in lipid stress-induced lipophagy is unclear, but *ATG7* at least is not required (Vevea *et al.*, 2015). Once inside the vacuole, turnover of LDs is largely dependent on the lipase Atg15 (van Zutphen *et al.*, 2014).

Studies in mice and humans have indicated that functional lipophagy is important for regulating fat content in the liver. For example, in mice, lipophagy is involved in the generation of free fatty acids to be used in very-low-density lipoprotein production. Furthermore, there is strong correlative evidence linking autophagy to the prevention of nonalcoholic fatty liver disease in humans (Martinez-Lopez and Singh, 2015).

Despite the importance of lipophagy, much remains unknown about the terminal steps within the vacuole. As mentioned above, vacuolar lipase activity in yeast is not completely abrogated in Atg15-deficient cells (van Zutphen *et al.*, 2014). What accounts for this residual activity? Are there other as yet unidentified vacuolar lipases? Once broken down, how are lipids then recycled?

In addition to these broader issues, many questions still remain about Atg15 itself. Atg15 is a vacuolar phospholipase that has activity primarily towards phosphotidylserine and, to a lesser extent, cardiolipin and phosphatidylethanolamine (Epple *et al.*, 2001; Ramya and Rajasekharan, 2016). As previously mentioned, Atg15 is required for efficient turnover of LDs within the vacuole (van Zutphen *et al.*, 2014). Atg15 is also required for the breakdown of autophagic and Cvt bodies (the single-membrane intravacuolar vesicles that form via the Cvt pathway) (Epple *et al.*, 2001; Teter *et al.*, 2001). These functions of Atg15 are critical for cell survival, as cells lacking Atg15 lose viability within six days of nitrogen starvation, whereas wild-type cells maintain robust viability at this time point (Teter *et al.*, 2001). The regulation of Atg15 activity is poorly understood, but proteolytic processing is hypothesized to play a role in Atg15 activation, similar to many other vacuolar hydrolases (Klionsky *et al.*, 1990; Teter *et al.*, 2001). If and how Atg15 is proteolytically activated in the vacuole remains to be elucidated.

Polyphosphate

Due to its structural incorporation into nucleic acids and phospholipids as well as roles in protein modification and signal transduction, phosphorus is an essential element for sustaining life (Yang *et al.*, 2017). In yeast, phosphorus is primarily stored as chains of inorganic polyphosphate (polyP), most of which is retained in the vacuole (Kornberg, 1999; Saito *et al.*, 2005; Gerasimaitė and Mayer, 2016; Yang *et al.*, 2017). Aside from acting as a phosphate storage mechanism, polyP functions in metal chelation in yeast, whereas in mammals it serves diverse functions ranging from activation of inflammatory responses and blood clotting to regulation of bone calcification (Gerasimaitė and Mayer, 2016).

In yeast, polyP is simultaneously synthesized and translocated across the vacuolar membrane by the VTC (vacuolar transporter chaperone) complex, which consists of two

proposed regulatory subunits, Vtc2 and Vtc3, as well as Vtc1 and the catalytic subunit Vtc4 (Cohen *et al.*, 1999; Gerasimaitė *et al.*, 2014; Gerasimaitė and Mayer, 2016). The VTC complex can form two distinct subcomplexes; the first consists of Vtc4, Vtc3, and Vtc1, and localizes primarily to the vacuolar membrane, whereas the second, consisting of Vtc4, Vtc2, and Vtc1, localizes to the cell periphery, but can be found at the vacuole during phosphate starvation (Gerasimaitė and Mayer, 2016). Vtc4 synthesizes polyP from ATP in a metal ion-dependent manner, with Mn^{2+} being the most effective cofactor. This enzyme is highly stimulated by inorganic pyrophosphate (PPi), which is also thought to be a primer for polyP polymerization (Hothorn *et al.*, 2009). The subunits of the VTC complex form a channel that allows for translocation of polyP across the membrane into the vacuolar lumen in a process dependent on a proton gradient established by the vacuolar-type proton-translocating ATPase (V-ATPase) (Hothorn *et al.*, 2009; Gerasimaitė *et al.*, 2014). A fifth subunit of the VTC complex, Vtc5, was recently characterized; overexpression of Vtc5 enhances polyP synthesis, whereas deletion of the corresponding gene decreases it (Desfougères *et al.*, 2016). The mechanistic details of how Vtc5 regulates polyP synthesis remain to be determined.

PolyP within the vacuole can be broken down by the polyphosphatase Ppn1 (Gerasimaitė and Mayer, 2016). Expression of *PPN1* increases during phosphate starvation (Ogawa *et al.*, 2000). Ppn1 is delivered to the vacuole via the multivesicular body (MVB) pathway, after which the N-terminal transmembrane domain is cleaved, releasing the soluble enzyme into the vacuole lumen (Reggiori and Pelham, 2001). Activation of Ppn1 is dependent on vacuolar proteases and involves both N-terminal and C-terminal cleavage events (Sethuraman *et al.*, 2001; Shi and Kornberg, 2005). Following release from polyP chains, inorganic phosphate (Pi) is then exported from the vacuole into the cytoplasm (Gerasimaitė and Mayer, 2016). It is suggested that the

phosphate transporter Pho91 is responsible for this activity, as it localizes to the vacuole membrane (Hürlimann *et al.*, 2007).

Extracts from cells lacking Ppn1 and the other major yeast polyphosphatase, Ppx1, which localizes to the cytosol and mitochondria, still have detectable polyphosphatase activity (Lichko *et al.*, 2008; Gerasimaitė and Mayer, 2016). Very recently, Gerasimaitė and Mayer investigated Ynl217w, a previously uncharacterized vacuolar protein (Huh *et al.*, 2003), and suggested that it may be the remaining vacuolar polyphosphatase, thus the name Ppn2 was proposed. Purified vacuolar lysates from cells lacking Vtc4, Ppn1, and Ppn2 have no phosphatase activity *in vitro* (Gerasimaitė and Mayer, 2017). Ppn2 was shown to be an endopolyphosphatase delivered to the vacuole via the MVB pathway (Gerasimaitė and Mayer, 2017).

No Ppn1 activity was detected in cells lacking Pep4, Prb1, and a third major vacuolar protease, Prc1 (carboxypeptidase Y) (Sethuraman *et al.*, 2001), but as will be discussed later in this chapter, these three proteases are involved in activation of several other zymogens. Which vacuolar protease(s) are directly involved in the activation of Ppn1? Is Ppn2 also proteolytically activated and, if so, how? Also, polyP is a storage molecule localized to the same cellular compartment as the enzymes that disassemble it, Ppn1 and Ppn2. How are these phosphatases regulated to only be active when appropriate?

Several connections between phosphate metabolism and autophagy have been suggested that require further study. It has been shown that nitrogen starvation increases Vtc1, Vtc3, and Vtc4 localization to the vacuole membrane and that these proteins are required for microautophagy (Uttenweiler *et al.*, 2007). How the VTC complex directly participates in microautophagy remains to be determined, as well as how this may or may not connect to phosphate levels and metabolism. It has recently been demonstrated that phosphate starvation

can induce autophagy, albeit to a much lower level than autophagy induced by nitrogen or carbon starvation (Yokota *et al.*, 2017). Does polyphosphate serve as a phosphate source under these conditions?

Carbohydrates

Several types of carbohydrates, including oligosaccharides and storage carbohydrates, can undergo vacuolar degradation. Ams1 (α-mannosidase) is a peripheral membrane protein associated with the lumenal face of the vacuole membrane (Van der Wilden *et al.*, 1973; Opheim, 1978; Yoshihisa *et al.*, 1988; Yoshihisa and Anraku, 1990). In the vacuole, it is involved in the degradation of free oligosaccharides generated as a result of newly synthesized, but misfolded, glycoproteins undergoing ER-associated protein degradation (ERAD) (Chantret *et al.*, 2003). Ams1 expression increases during both nitrogen and glucose starvation (Müller *et al.*, 2015; Umekawa *et al.*, 2016), similar to many other hydrolases and autophagy-related genes (Van Den Hazel *et al.*, 1996; Cebollero and Reggiori, 2009). Expression also increases in response to treatment of cells with rapamycin, a TORC1 inhibitor, which indicates involvement of the TORC1 signaling pathway in the regulation of Ams1 levels (Umekawa *et al.*, 2016). The reason behind this TORC1-dependent regulation is currently unclear, but it suggests that Ams1 may have a role in digestion of glycoproteins during autophagic recycling, as TORC1 is also a repressor of autophagy (Reggiori and Klionsky, 2013).

S. cerevisiae can store glucose as either trehalose or glycogen. Trehalose is a glucose disaccharide that accumulates during entry into stationary phase or in nutrient-poor conditions (Lillie and Pringle, 1980). It has many intracellular functions; in addition to serving as a source of carbon and energy, trehalose can protect cells from stresses including desiccation, temperature extremes, and oxidative and osmotic stress (Elbein *et al.*, 2003; Eleutherio *et al.*, 2015).

Trehalose can be degraded in the cytosol by the neutral trehalase Nth1 or in the vacuole by the acid trehalase Ath1 (François and Parrou, 2001). Ath1 is a resident vacuolar protein and has optimal activity under acidic pH (Keller *et al.*, 1982; Mittenbühler and Holzer, 1988; Huang *et al.*, 2007); however, Ath1 has also been detected at the cell periphery (Jules *et al.*, 2004; He *et al.*, 2009). Mutations in *ATH1* lead to higher levels of intracellular trehalose and increased resistance to stresses such as dehydration, freezing, and toxic levels of ethanol (Kim *et al.*, 1996), as well as an inability to grow using trehalose as a carbon source (Nwaka *et al.*, 1996).

Glycogen is a larger polymer of extensively-branched glucose chains (François and Parrou, 2001; Wilson *et al.*, 2010). When nutrients such as carbon, nitrogen, phosphorous, or sulfur are depleted, glycogen is synthesized in the cytoplasm from glucose donated from UDP-glucose molecules (Lillie and Pringle, 1980; François and Parrou, 2001). Glycogen synthesis is regulated by many of the same signaling pathways as autophagy. Snf1 and Pho85, which are positive and negative regulators of autophagy, respectively (Wang *et al.*, 2001), also exert the same types of control on glycogen synthesis (François and Parrou, 2001). Furthermore, inhibition of TORC1 by rapamycin increases glycogen synthesis (Barbet *et al.*, 1996), and cells lacking Tor1 hyperaccumulate glycogen (Wilson *et al.*, 2002).

Glucose can be liberated from glycogen via two distinct mechanisms. The first occurs in the cytoplasm, where the glycogen phosphorylase Gph1 releases glucose-1-phosphate from the ends of the glycogen chains and the debranching enzyme, Gdb1, removes glucose at the branch points (Hwang *et al.*, 1989; Teste *et al.*, 2000). The second glycogen degradation pathway occurs in the vacuole; the vacuolar glucoamylase Sga1 releases glucose from glycogen by hydrolysis (Colonna and Magee, 1978; Yamashita and Fukui, 1985; Pugh *et al.*, 1989). Cells lacking Sga1 show decreased glycogen degradation in late stationary phase (Wang *et al.*, 2001).

Degradation of vacuolar/lysosomal glycogen stores is crucial in maintaining cell health. In mice lacking the lysosomal α-glucosidase (GAA), glycogen overaccumulates in lysosomes in multiple muscle groups (Fukuda *et al.*, 2006). In humans, mutation of GAA leads to Pompe disease (glycogen storage disease type II); the infantile form, which is most severe, is characterized by cardiomegaly, hypotonia, and respiratory distress, and life expectancy is less than 1 year of age (van den Hout *et al.*, 2003; Kishnani *et al.*, 2006).

It is unclear why there are two distinct pools of glycogen and how glycogen is transported into the vacuole, but it is suggested that autophagy has a role in glycogen transport and storage in the vacuole, as *atg1*Δ cells have reduced glycogen storage (Wang *et al.*, 2001). A model has been proposed whereby some of the glycogen synthesized in the cytoplasm is transported by autophagy to the vacuole for storage, where it is protected from cytoplasmic degradation by Gph1 and Gdb1. Later in starvation, vacuolar glycogen can be degraded by Sga1 (Wang *et al.*, 2001). This model is highly speculative and warrants extensive testing. If vacuolar glycogen is delivered via autophagy, does this occur in a selective or non-selective manner? If it is selective, what are the receptor and adaptor protein(s) involved in cargo recognition? Additionally, what is the nutritional or intracellular cue to trigger degradation of the vacuolar pool of glycogen?

Many questions about vacuolar glycogen storage, degradation, and efflux remain. As with polyP storage and degradation, both glycogen and its degradative enzyme, Sga1, are localized in the same intracellular compartment. How is Sga1 activity regulated so that vacuolar glycogen is only degraded at the appropriate time? Does Sga1 undergo proteolytic activation by proteases? In a screen for mutants affecting glycogen storage, deletion of 11 of the 17 V-ATPase subunits and assembly factors that were screened results in elevated glycogen accumulation,

indicating that vacuolar acidification is required for the degradation of glycogen (Wilson *et al.*, 2002), but the reason for this is currently unknown.

Additionally, how glucose or mannose exit the vacuole is unclear, as no vacuolar hexose exporter has been identified. Ybr241c may be worth investigating in this regard, as it is a putative transporter of the sugar porter family that localizes to the vacuole membrane (Huh *et al.*, 2003; Palma *et al.*, 2007).

Organelles

As discussed above, various smaller cargoes are delivered to the vacuole through autophagy. However, several types of selective autophagy can deliver larger portions of organelles to the vacuole. For example, mitochondria, peroxisomes, and ribosomes can be degraded by autophagic processes termed mitophagy, pexophagy, and ribophagy, respectively (Suzuki, 2013). Depending on the cargo and nurient conditions, this can occur by either macroautophagy or microautophagy. Numerous studies have been devoted to characterizing the induction of these processes, identification of target organelles by the autophagy machinery, and receptors and adaptors involved in phagophore engulfment of these organelles (Suzuki, 2013). However, how these organelles are broken down once inside the vacuole remains a mystery. Two illustrative examples I will discuss here are mitophagy and micronucleophagy (also termed piecemeal microautophagy of the nucleus).

Under mitophagy-inducing conditions, such as nitrogen starvation following growth in non-fermentable carbon sources that induce proliferation of mitochondria, the cytosolic N terminus of the mitochondrial outer membrane protein Atg32 is phosphorylated (Kanki *et al.*, 2015). This facilitates its interaction with the cytosolic selective autophagy scaffold protein Atg11, which recruits the mitochondrion to the PAS (Aoki *et al.*, 2011; Kanki *et al.*, 2015). At

the PAS, phosphorylated Atg32 binds to Atg8, which, in its phosphatidylethanolamine-conjugated form, associates with the expanding phagophore (Ichimura *et al.*, 2000; Farré *et al.*, 2013). Following phagophore membrane expansion and autophagosome completion, the autophagosome travels to the vacuole and membrane fusion occurs, releasing the autophagic body with its mitochondrial cargo into the vacuolar lumen.

As with all macroautophagic cargoes, whether selective or non-selective, breakdown of autophagic bodies must then occur to allow cargo access to the degradative environment of the vacuolar lumen. As previously mentioned, this process is defective in cells lacking Pep4, Prb1, or Atg15 (Takeshige *et al.*, 1992; Epple *et al.*, 2001; Teter *et al.*, 2001). Perhaps not surprisingly then, autophagic bodies containing mitochondria are observed in cells lacking Pep4 and Prb1 (Okamoto *et al.*, 2009). Mitochondria are not the only selective autophagy cargo for which this is true. Peroxisomes also fail to be degraded in cells lacking Pep4 or Atg15 (Epple *et al.*, 2003), presumably due to the autophagic body remaining intact.

Mitophagy is an important process for degrading superfluous or damaged mitochondria (Kanki *et al.*, 2015). During erythrocyte maturation in mammals, for example, mitophagy clears mitochondria from these cells as part of their proper development (Ashrafi and Schwarz, 2013). Reactive oxygen species (ROS) produced as a natural byproduct of mitochondrial oxidative phosphorylation cause damage to mitochondrial proteins and mtDNA. This damage then leads to increased ROS production, spawning a vicious cycle of oxidative damage and further mitochondrial dysfunction. Over time, this damage can contribute to aging, cancer, and neurodegenerative diseases (Wallace, 2005). One pathway by which dysfunctional mitochondria are identified and targeted for mitophagy in mammals involves the proteins PINK1/PARK6 and PRKN/PARK2/Parkin. In depolarized mitochondria, the mitochondrial protein PINK1

accumulates on the mitochondrial outer membrane. There, it is recognized by PRKN, an E3 ubiquitin ligase, which then ubiquitinates several mitochondrial outer membrane proteins, targeting the damaged mitochondrion for degradation via mitophagy (Kanki *et al.*, 2015; Pickrell and Youle, 2015). Mutations in *PINK1* and *PRKN* are associated with autosomal recessive familial Parkinson disease (Kitada *et al.*, 1998; Valente *et al.*, 2004; Rodolfo *et al.*, 2017), underscoring the important role of mitophagy in clearing dysfunctional mitochondria.

Selective autophagy can also occur by microautophagy. Micronucleophagy is a microautophagic process and, as such, occurs by invagination of the vacuolar membrane rather than delivery to the vacuole by autophagosomes. In response to nitrogen or carbon starvation, non-essential portions of the nucleus become anchored to the vacuole membrane through interaction of the nuclear membrane protein Nvj1 with the vacuolar membrane protein Vac8 to form nucleus-vacuole (NV) junctions (Pan *et al.*, 2000; Roberts *et al.*, 2003; Kvam and Goldfarb, 2007). Invagination of the vacuolar membrane and extrusion of the nucleus then occur at these sites before the membranes pinch off, releasing small vesicles into the vacuolar lumen, where they are degraded (Kvam and Goldfarb, 2007). Breakdown of these vesicles depends on Pep4 and Atg15, and is also inhibited when cells are treated with the Prb1 inhibitor PMSF (Roberts *et al.*, 2003; Krick *et al.*, 2008), similar to the breakdown of autophagic bodies resulting from macroautophagy (Takeshige *et al.*, 1992; Epple *et al.*, 2001; Teter *et al.*, 2001).

Both of these examples of specific autophagy illustrate large gaps in our knowledge of the vacuolar degradation of specific organellar autophagic cargo. First, how does Atg15 differentiate between microautophagic vesicles derived from the vacuolar membrane and the vacuolar membrane itself? What happens during microautophagy to mark the invaginated membrane as distinct from its source? Concerning both macroatuophagy and microautophagy,

which hydrolases are responsible for organellar breakdown following lysis of the autophagic bodies or microautophagic vesicles by Atg15? Does Atg15 also disrupt the organelle membranes, or are there other lipases involved? How are other non-lipid components of the organelles degraded and recycled within the vacuole?

Proteins

Introduction to proteases

The yeast vacuole is home to a vast array of proteases and peptidases. Whereas synthesis and trafficking of these enzymes have been extensively studied and characterized, this section will focus on what is known about their activities within the context of the vacuole.

The two major proteases in the vacuole are the endoproteases Pep4 and Prb1. Pep4 is an aspartyl endoprotease related to mammalian CTSD (cathepsin D) and cleaves preferentially between hydrophobic amino acids (Ammerer *et al.*, 1986; Woolford *et al.*, 1986; Dreyer, 1989). Prb1 is a subtilisin-like serine endoprotease with fairly broad substrate specificity similar to porcine chymotrypsin C and trypsin (Lenney *et al.*, 1974; Kominami *et al.*, 1981; Moehle *et al.*, 1987). During vegetative growth conditions, the vacuole is responsible for 40% of cellular proteolysis, which increases to 85% during nutrient starvation (Teichert *et al.*, 1989). Pep4 and Prb1 are critical in this process through their own proteolytic activities, as well as through proteolytic activation of other proteases, which will be discussed below (Knop *et al.*, 1993; Van Den Hazel *et al.*, 1996). When both *PEP4* and *PRB1* are mutated, protein degradation is severely impaired during nitrogen starvation and sporulation is almost completely abolished (Zubenko and Jones, 1981; Teichert *et al.*, 1989).

Prc1 is a broad specificity vacuolar serine carboxypeptidase that prefers cleavage between hydrophobic residues and is thought to contribute to general protein/peptide turnover in

the vacuole; however, specific biological substrates have not been defined (Hayashi, 1976; Stennicke *et al.*, 1996; Van Den Hazel *et al.*, 1996; Jung *et al.*, 1999; Huh *et al.*, 2003). Ybr139w is a vacuolar protein that is predicted to be a serine carboxypeptidase based on a high degree of amino acid sequence similarity with Prc1 (Nasr *et al.*, 1994; Huh *et al.*, 2003; Baxter *et al.*, 2004). The substrate specificity of Ybr139w is currently unknown. Both Prc1 and Ybr139w are involved in the synthesis of phytochelatins, peptides that bind heavy metal ions (Wünschmann *et al.*, 2007), suggesting that there may be at least some functional overlap between these two proteins and that Ybr139w may be a functional homolog of Prc1, which is the basis of the work presented in chapter III.

Cps1 (carboxypeptidase S) is a vacuolar carboxypeptidase that is predicted to belong to a family of zinc metalloproteases (Spormann *et al.*, 1992; Hecht *et al.*, 2014). Although its intracellular function remains unclear, it is likely to participate in hydrolysis of leucine from the C terminus of proteins along with Prc1, as Cps1 is required for growth in Prc1-deficient strains when a synthetic dipeptide with leucine as the C-terminal amino acid is provided as the sole source of nitrogen (Wolf and Weiser, 1977; Spormann *et al.*, 1991).

There are three known resident vacuolar aminopeptidases. Ape3 (aminopeptidase Y) is a broad-specificity vacuolar protease able to cleave N-terminal Lys, Arg, Leu, Met, Ala, Ser, Phe, Tyr, and Pro residues with varying efficiency and accounts for most of the aminopeptidase activity in the vacuole (Yasuhara *et al.*, 1994); however, its biological function remains unknown. Another vacuolar aminopeptidase, Ape1 (aminopeptidase I), is activated by Zn^{2+}, cleaves N-terminal leucine residues, and may play a role in glutathione metabolism as discussed below (Metz and Röhm, 1976; Frey and Röhm, 1978; Trumbly and Bradley, 1983; Adamis *et al.*, 2009). The third resident aminopeptidase, Ape4 (aspartyl aminopeptidase), belongs to the same

family of metalloproteases as Ape1, is similar to mammalian aspartyl aminopeptidase, and cleaves the acidic residues Asp and Glu from the N terminus of substrates (Wilk *et al.*, 1998; Yokoyama *et al.*, 2006); however, like Ape3, its biological substrates are unknown (Yuga *et al.*, 2011). Ape4 resides in the vacuole during vegetative growth, although an increase in vacuolar localization of Ape4 occurs when cells are starved for nutrients, perhaps in order to assist with autophagic protein turnover (Yuga *et al.*, 2011).

Dap2 (dipeptidyl aminopeptidase B) is an integral membrane protein of the vacuole annotated as a serine hydrolase and, while its biological function remains unknown, it bears homology to Ste13, which cycles between the *trans*-Golgi network and endosomal system in a phosphorylation-dependent manner and is involved in proteolytic activation of the yeast α-factor (Fuller *et al.*, 1988; Roberts *et al.*, 1989; Baxter *et al.*, 2004; Johnston *et al.*, 2005).

Zymogen activation cascade

Many vacuolar hydrolases are synthesized as inactive zymogens that undergo Pep4- and/or Prb1-dependent proteolytic processing in the vacuole that leads to activation (Klionsky *et al.*, 1990; Van Den Hazel *et al.*, 1996; Hecht *et al.*, 2014).

Pep4 undergoes self-mediated processing in the vacuole to remove its N-terminal propeptide and mature into the active enzyme (Rupp *et al.*, 1991). It was originally thought that Pep4 maturation would be induced by the acidic environment of the vacuole, as most vacuolar hydrolases have an acidic pH optimum (Li and Kane, 2009). Additionally, mutations that impair activity of the V-ATPase, which is required for vacuolar acidification, show accumulation of autophagic bodies and protein degradation defects following nitrogen starvation (Nakamura *et al.*, 1997; Kane, 2006). However, the maturation of Pep4 and several other Pep4-dependent zymogens is fairly normal in these mutants, albeit somewhat slower (Yamashiro *et al.*, 1990;

Sørensen *et al.*, 1994; Nakamura *et al.*, 1997), indicating that there are other vacuolar factors involved in Pep4 activation. These factors, as well as how premature activation of Pep4 is prevented, are currently unknown. Once activated, Pep4 initiates further zymogen activation through Prb1. Prb1 undergoes two C-terminal processing events in the vacuole; the first is mediated by Pep4 and the second was thought to be catalyzed by Prb1 itself (Mechler *et al.*, 1988; Moehle *et al.*, 1989; Nebes and Jones, 1991); however, I present evidence in chapter III that other proteases are involved in this second processing step.

Prc1 is processed to its mature form through sequential N-terminal propeptide cleavage events mediated by Pep4 and Prb1 (Hecht *et al.*, 2014); mutations in the *PEP4* gene result in accumulation of a Prc1 precursor (Hemmings *et al.*, 1981), purified Pep4 can process prPrc1 to an intermediate form *in vitro* (Sørensen *et al.*, 1994), and an intermediate form of Prc1 is seen in Prb1-deficient cells (Mechler *et al.*, 1987). It is currently unknown whether the predicted Prc1 homolog, Ybr139w, is proteolytically processed. Ape3 is also processed and activated in a Prb1-dependent manner; vacuolar extracts from cells lacking Pep4, Prb1, Prc1, and Cps1 show no Ape3 enzymatic activity; however, addition of purified Prb1 allows for cleavage of the N-terminal propeptide and an increase in Ape3 activity (Yasuhara *et al.*, 1994).

Other targets of proteolytic activation are Pho8 and Ppn1, which participate in vacuolar RNA and polyP degradation, respectively. Cleavage of the Pho8 C-terminal propeptide is Pep4-dependent (Klionsky and Emr, 1989); in this case, however, Pep4 may be acting through Prb1, as overexpression of Prb1 increases activation of Pho8 (Merz and Wickner, 2004), but it remains to be determined whether activation by Pep4 and Prb1 is direct or indirect. Ppn1 is delivered to the vacuole by the multivesicular body pathway, after which the transmembrane domain is cleaved to release soluble Ppn1 into the vacuole lumen (Reggiori and Pelham, 2001). There is no Ppn1

activity in *pep4*Δ *prb1*Δ *prc1*Δ cells (Sethuraman *et al.*, 2001), and sequencing of the mature enzyme indicates a C-terminal cleavage event (Shi and Kornberg, 2005). It is also suggested that Ppn2 is delivered to the vacuole by the MVB pathway (Gerasimaitė and Mayer, 2017), but whether or not it is proteolytically activated upon arrival is yet to be determined.

Not all vacuolar proteases undergo proteolytic activation. While Prb1 does process Cps1 from a membrane-bound to a soluble enzyme within the vacuole (Spormann *et al.*, 1992), this is not an activating event; Cps1 activity is independent of both Pep4 and Prb1 (Bordallo *et al.*, 1991; Spormann *et al.*, 1991). Processing of precursor Ape1 (prApe1) to its mature form by cleavage of the N-terminal propeptide is also Pep4- and Prb1-dependent (Klionsky *et al.*, 1992; Seguí-Real *et al.*, 1995); however, this may not be required for its activity, as prApe1 is enzymatically active in *pep4*Δ cells (Andrei-Selmer *et al.*, 2001). Similarly, processing of Ape4 is defective in *pep4*Δ *prb1*Δ cells (Yuga *et al.*, 2011), but both full-length and cleaved forms of Ape4 exhibit enzymatic activity (Yokoyama *et al.*, 2006). Another protease independent of Pep4 is Dap2; disruption of the *PEP4* gene has no effect on either the apparent molecular weight or *in vitro* enzymatic activity of Dap2 (Roberts *et al.*, 1989).

Although many proteins undergo Prb1- and/or Pep4-dependent cleavage and activation, it is still unclear in most cases whether this is direct or indirect and whether the activation cascade is more complex. If it is indirect, which proteases function downstream of Prb1 to facilitate the processing of zymogens?

<u>Glutathione catabolism</u>

Whereas many specific functions of vacuolar protease are unknown, there is evidence for the involvement of several vacuolar proteases in the catabolism of glutathione (GSH, L-γ-glutamyl-L-cysteinylglycine), a tripeptide that performs many functions in the cell including

detoxification of toxic metabolites and protection from oxidative stress (Meister and Anderson, 1983; Penninckx and Elskens, 1993). GSH is broken down within the vacuole in two steps; first the γ-glutamyltranspeptidase Ecm38 hydrolyzes the N-terminal glutamate, followed by degradation of the CysGly dipeptide by an as yet unidentified cysteinylglycine dipeptidase (Jaspers *et al.*, 1985; Penninckx and Jaspers, 1985). Ecm38 is associated with the vacuolar membrane (Jaspers and Penninckx, 1984), with its active site facing the vacuolar lumen (Mehdi *et al.*, 2001). It has been proposed, though not experimentally demonstrated, that Ape1 may act in the second step to degrade the CysGly dipeptide in the vacuole (Adamis *et al.*, 2009); however, the ability of Ape1 to cleave N-terminal Cys residues has not been previously described.

In *Arabidopsis thaliana*, GSH catabolism can proceed by a second pathway, during which phytochelatin synthase (PCS) can remove the C-terminal Gly from GSH, resulting in a γ-GluCys dipeptide (Beck *et al.*, 2003; Grzam *et al.*, 2006; Blum *et al.*, 2007). Using fluorescent glutathione-*S*-bimane (GS-bimane) conjugates, it was shown that *S. cerevisiae* can generate both CysGly-bimane and γ-GluCys-bimane from GS-bimane, indicating that this second degradation pathway also occurs in yeast (Wünschmann *et al.*, 2010). While *S. cerevisiae* does not have a PCS homolog, the vacuolar serine carboxypeptidases Prc1 and Ybr139w are required for phytochelatin synthesis in yeast (Wünschmann *et al.*, 2007), as well as the conversion of GS-bimane to γ-GluCys-bimane, with Ybr139w having a larger role than Prc1 (Wünschmann *et al.*, 2010). Cells lacking Ecm38 accumulate γ-GluCys-bimane, while *ecm38*Δ *prc1*Δ *ybr139w*Δ cells show no breakdown of GS-bimane (Wünschmann *et al.*, 2010). Whether Prc1 and Ybr139w cleave the C-terminal Gly from GSH directly or indirectly, perhaps through proteolytic activation of the actual protease, remains to be determined. Also, it is still unclear how γ-GluCys

or CysGly dipeptides are broken down in the final step in either GSH degradation pathway, but there is no shortage of canditate vacuolar amino-, carboxy-, and dipeptidases available for future study.

There is some evidence that GSH metabolism and autophagy may be connected; nitrogen starvation causes a migration of the majority of cellular GSH to the vacuole (Mehdi and Penninckx, 1997). The pool of GSH increases for ~2 h, followed by a decrease (Mehdi and Penninckx, 1997). When GSH biosynthesis is blocked during nitrogen starvation, cell growth is impaired (as measured by dry weight), suggesting that GSH may possibly be used as a source of nitrogen (Mehdi and Penninckx, 1997). After 3-4 h of starvation, the specific activity of Ecm38 also increases (Mehdi and Penninckx, 1997). Both nitrogen starvation and treatment of cells with the TORC1 inhibitor rapamycin induce expression of Ecm38 (Springael and Penninckx, 2003). Ecm38 is also derepressed in sulphate starvation, during which GSH can be used as a source of sulfur and cysteine (Elskens *et al.*, 1991). Another observation is that in cells defective in GSH synthesis, there is an increase in mitophagy during nitrogen starvation as compared to wild-type cells, and addition of a cell-permeable GSH derivative reduces mitophagy under these same conditions (Deffieu *et al.*, 2009).

There are many questions remaining about the roles of autophagy and vacuolar proteases in GSH metabolism. Is the increased influx of GSH into the vacuole during nitrogen starvation solely dependent on the vacuolar glutathione-*S*-conjugate transporters Ycf1 and Bpt1 (Szczypka *et al.*, 1994; Li *et al.*, 1996; Rebbeor *et al.*, 1998; Klein *et al.*, 2002; Sharma *et al.*, 2002), or is at least some of it autophagy dependent? If the latter, is this process selective or non-selective and if it is selective, what are the scaffold and receptor proteins involved in cargo recognition? Additionally how is Ecm38 activity towards GSH regulated? Which vacuolar proteases are

responsible for the breakdown of γ-GluCys and CysGly dipeptides in the second step of GSH catabolism? What is the mechanism whereby GSH metabolism regulates mitophagy? As GSH provides an alternative nitrogen source during nitrogen starvation, will blocking GSH catabolism via *ECM38* mutation also impair cell viability during prolonged nitrogen starvation?

Importance of proteases in cell survival

Many vacuolar proteases are required for the terminal steps of autophagy and cell survival under nitrogen starvation conditions. Similar to many autophagy-related genes (Cebollero and Reggiori, 2009), most of the known vacuolar proteases are upregulated in response to nitrogen starvation or rapamycin treatment, including Ape1, Prc1, Cps1, Pep4, Prb1, and Ybr139w (Gasch *et al.*, 2000; Scherens *et al.*, 2006; Müller *et al.*, 2015). Ape3, and Dap2 are also upregulated, but not significantly (Müller *et al.*, 2015). Pep4 and Prb1 are especially important in cell survival and the autophagic response, as Pep4- and Prb1-deficient cells accumulate autophagic bodies in the vacuole during nitrogen starvation (Takeshige *et al.*, 1992), and *pep4*Δ cells lose viability in nitrogen starvation after approximately 8 days, whereas wild-type cells still show 90% viability at this time (Teter *et al.*, 2001). Prc1-deficent cells do not accumulate autophagic bodies (Takeshige *et al.*, 1992), but as Ybr139w may be a homolog of Prc1, this observation could be due to reduncancy and compensatory effects, which is the basis of the work presented in chapter III.

Human diseases related to lysosomal protease defects

In humans, defects in lysosomal proteolysis can have serious effects on health and disease. Cathepsins, of which there are 15 in humans, are a class of lysosomal proteases (Ketterer *et al.*, 2017). Mutations or defects in the cathepsin-encoding genes result in a variety of pathologies, including several lysosomal storage disorders.

Neuronal ceroid lipofuscinoses (NCLs) are a group of neurodegenerative lysosomal storage disorders characterized by progressive visual failure, seizures, and dementia (Mole *et al.*, 2005). There are fourteen different subtypes of NCLs based on the gene affected, although for several, the function of the causative gene is relatively unknown. Five subtypes are due to mutations in lysosomal enzymes, three of which are proteases (two cathepsins and a tripeptidyl peptidase). CLN2 is caused by mutations in *TPP1* (tripeptidyl peptidase I), which is not a cathepsin, CLN10 is caused by mutations in *CTSD*, and CLN13 is caused by mutations in *CTSF* (cathepsin F) (Mole and Cotman, 2015). Yeast Pep4 is related to mammalian CTSD (Parr *et al.*, 2007). In its most severe form, which involves a complete lack of CTSD activity, CLN10 is characterized by severe neuronal loss, microcephaly, seizures, and death within hours to weeks after birth (Barohn *et al.*, 1992; Steinfeld *et al.*, 2006). CLN13 is markedly less severe than CLN10; symptoms include progressive loss of mental and motor function, but do not manifest until late adulthood (Ketterer *et al.*, 2017).

Mutations in the gene encoding CTSA (cathepsin A), a serine carboxypeptidase that is structurally similar to yeast Prc1 (and likely Ybr139w as well), lead to a different type of lysosomal storage disorder called galactosialidosis (Hiraiwa, 1999). CTSA stabilizes a multienzyme complex of GLB1/β-galactosidase and NEU1 (neuraminidase 1), protecting them from degradation in the lysosome (Potier *et al.*, 1990). Mutations in *CTSA* result in deficiency of GLB1 and NEU1, leading to accumulation of glycoproteins in the vacuole (Bonten *et al.*, 2014; Ketterer *et al.*, 2017). In its most severe form, galactosialidosis can cause death within the first year of life (Ketterer *et al.*, 2017).

Not all diseases involving cathepsin defects are classified as lysosomal storage diseases. For example, loss-of-function mutations in the gene coding for CTSH (cathepsin H) are

associated with myopia (Ketterer *et al.*, 2017). Pycnodysostosis, which is a disease of the bones characterized by osteopetrosis, short stature, and skull deformities, is caused by mutations in the *CTSK* (cathepsin K) gene (Andren *et al.*, 1962; Maroteaux and Lamy, 1962; Ketterer *et al.*, 2017). Papillon-Lefèvre syndrome, which is caused by mutations in the *CTSC* (cathepsin C) gene, primarily affects the teeth and skin (Ketterer *et al.*, 2017); aggressive periodontitis causes loss of both deciduous and permanent teeth, and palmoplantar hyperkeratosis causes thickening of the skin and scaly lesions that crack and fissure (Gorlin *et al.*, 1964; Haneke, 1979; Sreeramulu *et al.*, 2015).

Whereas the transcriptional regulation, synthesis, trafficking, and proteolytic processing of vacuolar proteases have been extensively studied, surprisingly little is known about their substrates and intracellular functions. However, it is clear from the wide range of human diseases related to protease defects that vacuolar/lysosomal proteases are critically important in cellular function and survival. A significant effort must be made to better characterize and fully appreciate the vast array of proteases within the yeast vacuole.

Efflux of amino acids

Following protein breakdown, amino acids generated in the vacuole can be exported back into the cytoplasm. The AVT family of proteins in *S. cerevisiae* consists of 7 predicted membrane-spanning proteins related to vesicular transporters belonging to the amino acid/auxin permease (AAAP) family in higher eukaryotes (Sekito *et al.*, 2008). Avt1 imports glutamine, asparagine, leucine, isoleucine, and tyrosine into vacuoles for storage (Russnak *et al.*, 2001; Sekito *et al.*, 2008). The substrate(s), localization, and direction of transport of Avt2 and Avt5 are unknown (Sekito *et al.*, 2008), but in *Schizosaccharomyces pombe*, the Avt5 homolog localizes to the vacuole membrane and is involved in amino acid uptake into vacuoles

(Chardwiriyapreecha *et al.*, 2010), which may give clues as to its function in *S. cerevisiae*. The remaining four AVT family members, Avt3, Avt4, Avt6, and Avt7 are all indicated to be involved in amino acid efflux from the vacuole. Avt3 and Avt4 export glutamine, leucine, isoleucine, asparagine, and tyrosine from vacuoles into the cytoplasm (Russnak *et al.*, 2001). Avt4 can also export the basic amino acids arginine, lysine, and histidine (Sekito *et al.*, 2014). Avt3 may additionally export proline, as vacuolar proline levels are higher in *avt3*Δ cells than wild-type cells (Nishida *et al.*, 2016). Atg6 exports glutamate and aspartate from vacuoles (Russnak *et al.*, 2001), whereas recent work has demonstrated that Avt7 may be involved in efflux of glutamine and proline (Tone *et al.*, 2015).

Another protein involved in vacuolar amino acid efflux is Atg22. Atg22 is a vacuolar integral membrane protein that is indicated to transport tyrosine, leucine, and isoleucine (Yang *et al.*, 2006). Although biochemical methods have yet to confirm the transport activity of Atg22, the observation that cells lacking Atg22 accumulate more vacuolar tyrosine, leucine, and isoleucine as compared to wild-type cells supports this function (Yang *et al.*, 2006).

Several proteins of the PQ-loop family are also proposed to be involved in vacuolar amino acid efflux. The first, Ers1, is similar to human CTNS (cystinosin, lysosomal cystine transporter), which exports cystine, a disulfide-linked form of cysteine resulting from lysosomal degradation of proteins, from lysosomes (Kalatzis *et al.*, 2001; Gao *et al.*, 2005; Sekito *et al.*, 2008). Ers1 localizes to the vacuole membrane and while Ers1-deficient yeast cells show sensitivity to the antibiotic hygromycin B, expression of CTNS in these cells can complement the hygromycin B sensitivity and confer resistance (Gao *et al.*, 2005). Recently, it was demonstrated that Ers1 can transport cystine, although intracellular cystine does not increase in *ers1*Δ cells (Simpkins *et al.*, 2016), possibly indicating the presence of redundant, as yet

unidentified, cystine transporters. Additional PQ-loop family members Ypq1, Ypq2, and Rtc2 also localize to the vacuole membrane, and it is proposed that they export basic amino acids (Jézégou *et al.*, 2012).

In humans, mutations in CTNS lead to the disease cystinosis, which is characterized by accumulation of cystine crystals in the lysosome. In its most severe and common form, the infantile nephropathic form, it leads to the development of renal Fanconi syndrome by 6-12 months of age and progressive loss of kidney function, growth retardation, neuromuscular dysfunction, hypothyroidism, and vision problems if left untreated (Elmonem *et al.*, 2016).

Efflux of vacuolar amino acids generated by autophagy is critically important to support protein synthesis and continued cell survival during starvation conditions. Similar to many other autophagy-related genes (Cebollero and Reggiori, 2009), Atg22 expression and protein level increase during nitrogen starvation (Gasch *et al.*, 2000; Yang *et al.*, 2006). Additionally, microarray data show that Atg22, Avt1, Avt4, and Avt7 are upregulated in response to rapamycin treatment (Scherens *et al.*, 2006).

Free amino acids generated by autophagy are required to support increased synthesis of several proteins during nitrogen starvation, including Ape1 and Prc1 (Onodera and Ohsumi, 2005). In autophagy-deficient *atg7*Δ cells, synthesis of Ape1 and Prc1 is severely impaired compared to wild-type cells in nitrogen starvation conditions (Onodera and Ohsumi, 2005). Similarly, when the genes encoding the leucine transporters Atg22, Avt3, and Avt4 are deleted, synthesis of Ape1 and Prc1 is reduced in leucine-starvation conditions (Yang *et al.*, 2006), suggesting that efflux of amino acids generated by autophagic degradation back into the cytoplasm is necessary to support protein synthesis. The lack of these efflux permeases also affects the ability of cells to survive in starvation conditions; when starved for nitrogen, wild-

type cells maintain robust viability over an extended time course, whereas cells lacking Atg22 lose viability at 12 days, cells lacking Avt3 and Avt4 lose viability at 5-6 days, and cells lacking all three lose viability at 4 days, similar to an autophagy-defective *atg1*Δ mutant (Yang *et al.*, 2006).

Although many amino acid efflux transporters have been identified and characterized, further work must be done to determine the transport mechanism of amino acids for which transporters have not yet been identified.

Vacuolar proteins of unknown function

In addition to the subset of vacuolar hydrolases discussed herein, there are many known or predicted vacuolar proteins for which a function has not yet been described; more than 200 of the approximately 6000 open reading frames in the yeast genome are annotated as having vacuolar localization, at least under some conditions (Li and Kane, 2009). Many of these putative proteins are just beginning to be characterized and some have not been characterized at all. In Uniprot, search results for vacuolar hydrolases include Pff1 (*YBR074W*), Ecm14 (*YHR132C*), *YHR202W*, and *YNL115C* (The UniProt Consortium, 2017), among others.

Pff1 and Ecm14 are predicted to be proteases. Pff1 is a predicted metalloprotease that localizes to the vacuole membrane; it is a multipass integral membrane protein, and topology studies indicate that the protease domain faces the vacuole lumen (Hecht *et al.*, 2013). Protease activity and substrate specificity have yet to be shown, as well as determination of biological function and regulation. Ecm14 localizes to the vacuole (Huh *et al.*, 2003) and is predicted to be a zinc-dependent carboxypeptidase (The UniProt Consortium, 2017).

GFP-tagged Yhr202w exhibits vacuolar localization (Huh *et al.*, 2003) and may have phosphatase and/or nucleotidase activity (Finn *et al.*, 2017). Ynl115c localizes to the vacuolar

membrane (Huh *et al.*, 2003) and possesses an α/β-hydrolase fold (Finn *et al.*, 2017). Another protein of interest is Yol019w, a protein of unknown function that localizes to the vacuole and is transcriptionally upregulated in cells treated with rapamycin (Huh *et al.*, 2003; Scherens *et al.*, 2006).

Conclusions and goals for this dissertation

While decades of study have greatly increase our knowledge of vacuolar substrate degradation and efflux, there are still many gaps in this knowledge, especially concerning protein substrates. Many known vacuolar proteases have indeterminate substrate specificities and intracellular functions, while many predicted proteases remain to be characterized. In addition, for many vacuolar zymogens it is still unknown whether activation via Pep4 and/or Prb1 occurs directly or indirectly through other proteases. In the case of indirect activation, what are the additional proteases involved in the activation and processing cascade? Vacuolar proteases are especially important during nutrient starvation when autophagy is induced, as the vacuole is almost entirely responsible for cellular protein degradation under these conditions (Teichert *et al.*, 1989). In addition, many human diseases are associated with defects in lysosomal proteolysis and efflux; therefore, it is critical that we increase our understanding of these vacuolar/lysosomal events.

The goal of this dissertation is to begin to fill in the gaps in our knowledge of vacuolar proteases in *S. cerevisiae*, especially with regard to their roles in the terminal events of autophagic degradation of proteins, beginning with the study of serine carboxypeptidases, specifically Prc1 and its predicted homolog Ybr139w.

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Chapter II

An overview of autophagy: Morphology, mechanism and regulation[2]

Abstract

Significance: Autophagy is a highly conserved eukaryotic cellular recycling process. Through the degradation of cytoplasmic organelles, proteins, and macromolecules, and the recycling of the breakdown products, autophagy plays important roles in cell survival and maintenance. Accordingly, dysfunction of this process contributes to the pathologies of many human diseases. **Recent Advances:** Extensive research is currently being done to better understand the process of autophagy. In this review, we describe current knowledge of the morphology, molecular mechanism, and regulation of mammalian autophagy. **Critical Issues:** At the mechanistic and regulatory levels, there are still many unanswered questions and points of confusion that have yet to be resolved. **Future Directions:** Through further research, a more complete and accurate picture of the molecular mechanism and regulation of autophagy will not only strengthen our understanding of this significant cellular process, but will aid in the development of new treatments for human diseases in which autophagy is not functioning properly.

Introduction

Autophagy is a cellular degradation and recycling process that is highly conserved in all eukaryotes. In mammalian cells, there are three primary types of autophagy: microautophagy,

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macroautophagy, and chaperone-mediated autophagy. While each is morphologically distinct, all three culminate in the delivery of cargo to the lysosome for degradation and recycling (Figure II.1) (Yang and Klionsky, 2010). During microautophagy, invaginations or protrusions of the lysosomal membrane are used to capture cargo (Mijaljica *et al.*, 2011). Uptake occurs directly at the limiting membrane of the lysosome, and can include intact organelles. Chaperone-mediated autophagy differs from microautophagy in that it does not use membranous structures to sequester cargo, but instead uses chaperones to identify cargo proteins that contain a particular pentapeptide motif; these substrates are then unfolded and translocated individually directly across the lysosomal membrane (Massey *et al.*, 2004). In contrast to microautophagy and chaperone-mediated autophagy, macroautophagy involves sequestration of the cargo away from the lysosome. In this case, *de novo* synthesis of double-membrane vesicles—autophagosomes—is used to sequester cargo and subsequently transport it to the lysosome (Yorimitsu and Klionsky, 2005).

Of the three types of autophagy, macroautophagy is the best studied. Macroautophagy occurs at a low level constitutively and can be further induced under stress conditions, such as nutrient or energy starvation, to degrade cytoplasmic material into metabolites that can be used in biosynthetic processes or energy production, allowing for cell survival (Yorimitsu and Klionsky, 2005). Under normal growing conditions, macroautophagy aids in cellular maintenance by specifically degrading damaged or superfluous organelles (Yang and Klionsky, 2010). Thus, macroautophagy is primarily a cytoprotective mechanism; however, excessive self-degradation can be deleterious. Accordingly, autophagic dysfunction is associated with a variety of human pathologies, including lung, liver, and heart disease, neurodegeneration, myopathies, cancer, ageing, and metabolic diseases such as diabetes (Wirawan *et al.*, 2012).

This review provides an overview of the current state of knowledge of autophagy, with an emphasis on the morphology, molecular mechanism, regulation, and selectivity of mammalian macroautophagy.

I. Microautophagy

Microautophagy refers to a process by which cytoplasmic contents enter the lysosome through an invagination or deformation of the lysosomal membrane (Marzella *et al.*, 1981). In one early study, isolated rat liver lysosomes were shown by electron microscopy to engulf Percoll particles *in vitro* by way of protrusions or cup-like invaginations of the lysosomal membrane, forming vesicles within the lysosome. Some of these particles were seen free-floating within the lysosomal lumen, presumably through rupture/lysis of the vesicles (Marzella *et al.*, 1980). A very recent study presented evidence that a microautophagy-like process called endosomal microautophagy transports soluble cytosolic proteins to the vesicles of late endosomal multivesicular bodies (Sahu *et al.*, 2011). Due to the limited number of tools available for the study of microautophagy, we know relatively little about this process, including its regulation and possible roles in human health and disease (Mijaljica *et al.*, 2011).

II. Chaperone-mediated autophagy (CMA)

A second type of autophagy, which has so far only been described in mammalian cells, is chaperone-mediated autophagy (CMA). Unlike microautophagy and macroautophagy, which can both non-specifically engulf bulk cytoplasm, CMA is highly specific; common to all CMA substrates is a pentapeptide targeting motif biochemically related to KFERQ (Dice, 1990). Based on sequence analysis and immunoprecipitation experiments, it is estimated that approximately 30% of cytosolic proteins contain such a sequence (Chiang and Dice, 1988). Target proteins containing the KFERQ consensus motif are unfolded through the action of cytosolic chaperones

and translocated directly across the lysosomal membrane where they are degraded in the lumen (Orenstein and Cuervo, 2010). CMA degrades a wide range of substrate proteins, including certain glycolytic enzymes, transcription factors and their inhibitors, calcium and lipid binding proteins, proteasome subunits, and proteins involved in vesicular trafficking (Arias and Cuervo, 2011).

During CMA, the KFERQ motif is recognized by the heat shock 70kDa protein 8 (HSPA8/HSC70), as well as other co-chaperones (Figure II.1) (Chiang *et al.*, 1989). HSPA8 can then deliver the substrate to the lysosomal membrane, where it likely assists in substrate unfolding (Agarraberes and Dice, 2001). At the lysosomal membrane, the substrate binds to monomers of the CMA substrate receptor, lysosomal-associated membrane protein 2A (LAMP2A) (Cuervo and Dice, 1996). This substrate-receptor binding leads to the multimerization of LAMP2A (Cuervo and Dice, 1996; Bandyopadhyay *et al.*, 2008). As the multimeric translocation complex forms, subunits of the complex are stabilized on the lumenal side of the lysosomal membrane by HSP90 (Bandyopadhyay *et al.*, 2008). Following translocation of the substrate into the lysosomal lumen—in part through the action of lumenal HSPA8—the translocation complex is actively disassembled by cytosolic HSPA8, and LAMP2A returns to a monomeric state where it can bind new substrate and initiate a new round of translocation (Bandyopadhyay *et al.*, 2008).

Regulation of the translocation process occurs at the level of substrate binding to LAMP2A, which is rate-limiting for CMA (Cuervo and Dice, 2000a). Changes in LAMP2A levels at the lysosomal membrane modulate the level of CMA activity and primarily result from changes in degradation and organization of LAMP2A rather than synthesis of the protein (Cuervo and Dice, 2000a, b; Bandyopadhyay *et al.*, 2008). Some data support the idea that

redistribution of LAMP2A between fluid regions of the lysosomal membrane and lipid-enriched microdomains influences the degradation of LAMP2A (Kaushik *et al.*, 2006). While much is known about translocation regulation, far less is clear about overall CMA regulation (Arias and Cuervo, 2011). Mild oxidative stress (Kiffin *et al.*, 2004), protein-damaging toxins (Cuervo *et al.*, 1999), and extended periods of nutrient deprivation all upregulate CMA (Auteri *et al.*, 1983; Cuervo *et al.*, 1995), but the intracellular signaling pathways that facilitate this change are not fully understood (Arias and Cuervo, 2011).

It is suggested that HSPA8 and LAMP2A also participate in a type of macroautophagy called chaperone-assisted selective autophagy (CASA). During this process, chaperones aid in the clearance of selectively ubiquitinated organelles and protein complexes (Kirkin *et al.*, 2009c). Association of these ubiquitinated targets with receptors such as SQSTM1/p62 and NBR1, and with enzymes including HDAC6 allows for recognition by the macroautophagy machinery, delivery to the lysosome, and degradation (Kirkin *et al.*, 2009a; Kirkin *et al.*, 2009c; Lamark *et al.*, 2009).

III. Macroautophagy

III.A Basic morphological progression

As stated above, macroautophagy is distinct from microautophagy and CMA in part because the initial site of sequestration occurs away from the limiting membrane of the lysosome, and involves the formation of cytosolic vesicles that transport the cargo to this organelle. The morphological feature that makes macroautophagy unique from other intracellular vesicle-mediated trafficking processes is that the sequestering vesicles, termed autophagosomes, form *de novo* rather than through membrane budding; that is, the autophagosome forms by expansion, and does not bud from a preexisting organelle, already containing cargo (Yang and

Klionsky, 2009). Upon induction of macroautophagy in yeast, formation of autophagosomes begins at a single perivacuolar site called the phagophore assembly site (PAS) (Chen and Klionsky, 2011). In mammalian systems, autophagosome generation is initiated at multiple sites throughout the cytoplasm rather than at a single PAS (Itakura and Mizushima, 2010; Chen and Klionsky, 2011). Several studies suggest that endoplasmic reticulum-associated structures called omegasomes may serve as initiation sites in mammals (Hayashi-Nishino *et al.*, 2009; Ylä-Anttila *et al.*, 2009).

Following initiation, the membrane begins to expand. At this stage, it is called a phagophore, which is the primary double-membrane sequestering compartment (Figure II.2) (He and Klionsky, 2009). The source of membrane that makes up the phagophore is highly debated, but various studies have implicated the plasma membrane (Ravikumar *et al.*, 2010a; Ravikumar *et al.*, 2010b), endoplasmic reticulum (ER) (Hayashi-Nishino *et al.*, 2009; Ylä-Anttila *et al.*, 2009), Golgi complex (Takahashi *et al.*, 2011), and mitochondria (Hailey *et al.*, 2010) as possible sources (Mizushima *et al.*, 2011; Weidberg *et al.*, 2011). As the phagophore expands, the membrane bends to ultimately generate a spherical autophagosome. The factors that drive curvature of the membrane during nonspecific macroautophagy are not known. In the case of selective macroautophagy, the membrane appears to essentially wrap around the cargo, thus adjusting to fit the specific target (Mijaljica *et al.*, 2012). Upon completion, the phagophore fully surrounds its cargo and fuses to form the double-membrane autophagosome. The size of the autophagosome varies based on organism and cargo type. For example, the diameter of autophagosomes ranges from approximately 0.4 to 0.9 μm in yeast, and 0.5 to 1.5 μm in mammals (Pfeifer, 1978; Schworer *et al.*, 1981; Takeshige *et al.*, 1992; Mizushima and Klionsky, 2007).

Once the autophagosome is formed, it must deliver its cargo to the lysosome in mammals or the functionally related vacuole in yeast and plants. As it reaches its destination, the outer membrane of the autophagosome will fuse with the lysosomal/vacuolar membrane. In yeast and plants, due to the relatively large size of the vacuole, this releases a single-membrane autophagic body into the vacuolar lumen. Fusion between autophagosomes and lysosomes in mammals, however, does not generate autophagic bodies (Devenish and Klionsky, 2012). The product of fusion between an autophagosome and lysosome in mammalian cells is referred to as an autolysosome (Yang and Klionsky, 2009). Exposed to the acidic lumen and resident hydrolases of the lysosome/vacuole, the autophagosome inner membrane and, subsequently, the autophagic cargo are degraded and the component parts are exported back into the cytoplasm through lysosomal permeases for use by the cell in biosynthetic processes or to generate energy (Yorimitsu and Klionsky, 2005). In mammals, macroautophagy often converges with the endocytic pathway. Hence, prior to fusion with lysosomes, autophagosomes may also fuse with early or late endosomes to form amphisomes, which then fuse with lysosomes to become autolysosomes (Tooze *et al.*, 1990; Berg *et al.*, 1998).

III.B Macroautophagy machinery

III.B.1 Induction

In yeast macroautophagy, induction of autophagosome formation is regulated by the Atg1-Atg13-Atg17 kinase complex (He and Klionsky, 2009). In mammalian cells this complex is made up of an Atg1 homolog from the Unc-51-like kinase family (either ULK1 or ULK2), the mammalian homolog of Atg13 (ATG13), and RB1-inducible coiled-coil 1 (RB1CC1/FIP200), which is required for the induction of macroautophagy and may be an ortholog of yeast Atg17 (Figure II.3) (Hara *et al.*, 2008; Ganley *et al.*, 2009; Hosokawa *et al.*, 2009a; Jung *et al.*, 2009).

Also in this complex is C12orf44/ATG101, which binds directly to ATG13, is essential for macroautophagy, and has no known yeast homolog (Hosokawa *et al.*, 2009b; Mercer *et al.*, 2009). The mammalian ULK1/2-ATG13-RB1CC1 complex is stable and forms regardless of nutrient status (Hosokawa *et al.*, 2009a; Jung *et al.*, 2009).

The association of the mechanistic target of rapamycin complex 1 (MTORC1) with the induction complex *is*, however, influenced by nutrient status. Under nutrient-rich conditions, MTORC1 associates with the complex, but dissociates upon nutrient starvation (Hosokawa *et al.*, 2009a). When MTORC1 is complex-associated, it phosphorylates ULK1/2 and ATG13, inactivating them. However when cells are treated with rapamycin or starved for nutrients, MTORC1 dissociates from the induction complex, resulting in dephosphorylation at these sites and induction of macroautophagy (Hosokawa *et al.*, 2009a; Jung *et al.*, 2009). The phosphatases responsible at this stage are as yet unknown. The involvement of MTORC1 in the regulation of macroautophagy is an active area of research and will be discussed in greater detail below as well as in another review in this forum series.

III.B.2 Nucleation

The next complex recruited to the putative site of autophagosome formation is the ATG14-containing class III phosphatidylinositol 3-kinase (PtdIns3K) complex (Itakura and Mizushima, 2010). The PtdIns3K complex generates PtdIns3P, which is required for macroautophagy in both yeast and mammals (Burman and Ktistakis, 2010). This complex is involved in the nucleation of the phagophore and consists of PIK3C3/VPS34, PIK3R4/p150 (Vps15 in yeast), and BECN1 (Vps30/Atg6 in yeast) (Figure II.4) (Liang *et al.*, 1999; Kihara *et al.*, 2001; Furuya *et al.*, 2005; Itakura *et al.*, 2008; Yan *et al.*, 2009). As in yeast, this complex can either function in macroautophagy by associating with ATG14 or in the endocytic pathway through an interaction

with UVRAG (an ortholog of yeast Vps38) (Liang *et al.*, 2006; Itakura *et al.*, 2008; Sun *et al.*, 2008). While some data suggest that the UVRAG-associated PtdIns3K complex is involved in autophagosome formation (Liang *et al.*, 2006), other reports suggest that it may act in later stages of autophagosome development (Liang *et al.*, 2008). Another study found that siRNA knockdown of UVRAG in HeLa cells does not affect macroautophagy (Itakura *et al.*, 2008). It is clear that further work is required to fully understand the role of UVRAG in the endocytic and macroautophagic pathways.

Regulation of the PtdIns3K complex occurs largely through proteins that interact with BECN1, which is essential for macroautophagy (Liang *et al.*, 1999; Zeng *et al.*, 2006). The antiapoptotic protein BCL2 binds BECN1 and prevents its interaction with PIK3C3, thus inhibiting macroautophagy (Liang *et al.*, 1998; Furuya *et al.*, 2005; Pattingre *et al.*, 2005). Another BECN1-binding protein, KIAA0226/Rubicon, inhibits PIK3C3 activity in UVRAG-associated PtdIns3K complexes (Figure II.4) (Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). Two positive regulators of the PtdIns3K complex are AMBRA1 (which directly binds BECN1) and SH3GLB1/Bif-1 (which interacts with BECN1 through UVRAG, and may be involved in generating membrane curvature) (Fimia *et al.*, 2007; Takahashi *et al.*, 2007; Takahashi *et al.*, 2009). Very little is known, however, about upstream events regulating the constituents of the various PtdIns3K complexes.

In yeast, there are several proteins that bind to PtdIns3P generated by the Vps34 complex. Of these, Atg18 and Atg21 have a role in macroautophagy and localize to the PAS (Krick *et al.*, 2008). Mammalian cells express two Atg18 orthologs, WIPI1 and WIPI2, which are also involved in macroautophagy and associate with phagophores during amino acid starvation by binding to PtdIns3P (Jeffries *et al.*, 2004; Proikas-Cezanne *et al.*, 2004; Polson *et al.*, 2010).

Another PtdIns3P-binding protein in mammalian cells is the zinc finger, FYVE containing 1 (ZFYVE1/DFCP1), which associates with PtdIns3P-enriched omegasomes (Axe *et al.*, 2008). The precise functions of WIPI1/2 and ZFYVE1 in macroautophagy are still unknown.

III.B.3 Elongation

The ubiquitin-like enzymes

In both yeast and mammals, there are two conjugation systems involving ubiquitin-like (UBL) proteins that contribute to the expansion of the phagophore (Weidberg *et al.*, 2011). The first system involves formation of the Atg12–Atg5-Atg16 complex. In yeast, the UBL protein Atg12 is covalently conjugated to Atg5 in a manner dependent on the E1 activating enzyme Atg7 and the E2 conjugating enzyme Atg10 (Kim *et al.*, 1999; Shintani *et al.*, 1999; Ohsumi, 2001). This process differs from ubiquitination in that the conjugation of Atg12 to Atg5 is irreversible and does not require an E3 ligase enzyme (Geng and Klionsky, 2008). Following Atg12–Atg5 conjugation, Atg16 binds to Atg5 noncovalently and dimerizes to form a larger complex (Kuma *et al.*, 2002). Mammalian orthologs of this system, ATG5, ATG12 and ATG16L1, have been identified, and function as in yeast (Figure II.5) (Ohsumi, 2001; Mizushima *et al.*, 2003). The mammalian ATG12–ATG5-ATG16L1 complex associates with the phagophore membrane, but dissociates following autophagosome completion (Mizushima *et al.*, 2001; Mizushima *et al.*, 2003). One way in which this complex is regulated is through the Golgi protein RAB33A, which can bind to and inhibit ATG16L1 (Itoh *et al.*, 2008). Additionally, ATG5, ATG7, and ATG12 are inhibited through acetylation by the acetyltransferase KAT2B/p300 (Lee and Finkel, 2009).

The second UBL system involved in phagophore expansion is the Atg8/LC3 system. This conjugation pathway in yeast begins with processing of Atg8 by the cysteine protease Atg4 to expose a glycine residue at the C terminus of Atg8 (Kirisako *et al.*, 2000). The E1-like enzyme

Atg7 activates the processed Atg8 and transfers it to the E2-like enzyme Atg3 (Ichimura *et al.*, 2000). Finally, the C-terminal glycine of Atg8 is covalently conjugated to the lipid phosphatidylethanolamine (PE). The Atg12–Atg5 conjugate, which may act as an E3 ligase, facilitates this final step (Ichimura *et al.*, 2000; Hanada *et al.*, 2007; Fujita *et al.*, 2008). Atg8–PE is membrane-associated, but can be released from membranes as a result of a second Atg4-mediated cleavage (Kirisako *et al.*, 2000). The mechanism of regulation of the second Atg4-dependent processing event, referred to as deconjugation, is not known; however, this appears to be an important step in macroautophagy because defects in cleavage result in partial autophagic dysfunction (Nair *et al.*, 2012).

Mammalian homologs of the Atg8/LC3 system function much like their yeast counterparts (Figure II.6) (Geng and Klionsky, 2008). Unlike yeast, which have only one Atg4 and one Atg8, mammals have four isoforms of ATG4 and several Atg8-like proteins, the latter of which are divided into the LC3 and GABARAP subfamilies (Hemelaar *et al.*, 2003; Mariño *et al.*, 2003; Weidberg *et al.*, 2010). Whereas both subfamilies can localize with autophagosomes (Kabeya *et al.*, 2004), it has been proposed that they function at different steps in phagophore elongation and completion, with the LC3 subfamily acting prior to the GABARAP subfamily (Weidberg *et al.*, 2010). Among the Atg8-like proteins in mammals, LC3 has been the best characterized. The ATG4-processed form of LC3 is referred to as LC3-I and the PE-conjugated form is called LC3-II (Geng and Klionsky, 2008). Lipidation of LC3 in mammalian cells is accelerated under conditions of nutrient starvation or other types of stress (Kabeya *et al.*, 2000). While the mechanism of the conjugation system of Atg8/LC3 is well understood, the precise role of Atg8/LC3 in macroautophagy is still unclear. Atg8, and to some extent LC3 (Tanida *et al.*, 2005; Martinet *et al.*, 2006), shows a substantial increase in synthesis during macroautophagy

induction (Kirisako *et al.*, 1999), and in yeast this is a determining factor in autophagosome size (Xie *et al.*, 2008).

Atg9 and membrane recruitment

Another protein thought to function in elongation of the phagophore is the transmembrane protein ATG9. In yeast, Atg9 may cycle between the PAS and peripheral sites (Reggiori *et al.*, 2004). These peripheral sites are referred to as Atg9 reservoirs or tubulovesicular clusters (TVCs). The TVCs may be direct membrane precursors to the PAS, and thus to phagophores (Mari *et al.*, 2010; Nair *et al.*, 2011). The movement of Atg9 is dependent on the Atg1-kinase complex as well as multimerization of Atg9 (Reggiori *et al.*, 2004; He *et al.*, 2008). The abilities of Atg9 to traffic and multimerize are necessary for autophagosome formation, suggesting that these properties of Atg9 contribute to a role for this protein in recruiting membrane to the expanding phagophore (Reggiori *et al.*, 2004; He *et al.*, 2008).

The mammalian homolog of Atg9 (ATG9) is also seen to shift localization within the cell and is proposed to have a similar role in membrane recruitment (Young *et al.*, 2006). Under nutrient-rich conditions, ATG9 localizes to the *trans*-Golgi network and late endosomes (Young *et al.*, 2006). When cells are starved for nutrients, however, ATG9 colocalizes with autophagosomal markers (Young *et al.*, 2006). This cycling to autophagosomes is dependent on both ULK1 and PtdIns3K activity and is negatively regulated by MAPK14/p38α (Young *et al.*, 2006; Webber and Tooze, 2010). The exact function of ATG9 in the cell, and how the ULK1 complex regulates ATG9 movement, are poorly understood.

III.B.4 Autophagosome completion and fusion

In what is perhaps the least understood step of macroautophagy, the expanding phagophore must eventually mature and close to form a completed autophagosome, which

traffics to and fuses with an endosome and/or lysosome, becoming an autolysosome. Movement of autophagosomes to lysosomes is dependent on microtubules (Monastyrska *et al.*, 2009). Fusion of autophagosomes with endosomes involves the protein VTIlB (Atlashkin *et al.*, 2003). UVRAG, which can associate with the PtdIns3K complex, can activate the GTPase RAB7, which promotes fusion with lysosomes (Jäger *et al.*, 2004; Liang *et al.*, 2008). It has also been suggested that components of the SNARE machinery, such as VAM7 and VAM9, have a role in fusion (Fader *et al.*, 2009; Furuta *et al.*, 2010). Recent work has identified another SNARE, syntaxin 17 (STX17), which localizes to completed autophagosomes and is required for fusion with the endosome/lysosome through an interaction with SNAP29 and the endosomal/lysosomal SNARE VAMP8 (Itakura *et al.*, 2012).

III.C Regulation of macroautophagy

Macroautophagy helps cells respond to a wide range of extra- and intracellular stresses including nutrient starvation, the presence/absence of insulin and other growth factors, hypoxia, and endoplasmic reticulum stress (Figure II.7) (He and Klionsky, 2009). Two pathways involved in nutrient starvation are regulated by the cAMP-dependent protein kinase A (PKA) and TOR pathways, which sense primarily carbon and nitrogen, respectively (Stephan *et al.*, 2010). In yeast, PKA is an inhibitor of macroautophagy under nutrient-rich conditions (Budovskaya *et al.*, 2004). In mammals, this inhibition occurs at least partially through the phosphorylation of LC3 by PKA (Cherra *et al.*, 2010). For its role in nitrogen sensing, MTORC1 is positively regulated by the presence of amino acids. Amino acids regulate RAG proteins, RAS-related small GTPases that activate MTORC1 (Kim *et al.*, 2008; Sancak *et al.*, 2008). There is thought to be some crosstalk between the carbon- and nitrogen-sensing pathways, based on studies that demonstrated that mammalian PKA can phosphorylate, and thus activate, MTORC1 (Mavrakis *et al.*, 2006;

Blancquaert *et al.*, 2010). PKA can also indirectly activate MTORC1 through inactivation of the AMP-activated protein kinase (AMPK) (Djouder *et al.*, 2010).

AMPK is not simply a substrate of PKA. It is the major energy-sensing kinase in the cell and responds to intracellular AMP/ATP levels to regulate a variety of cellular processes, including macroautophagy (Meley *et al.*, 2006; Alers *et al.*, 2012). AMP and ATP have opposite effects on the activity of AMPK, with AMP binding activating the kinase activity of AMPK (Hardie, 2007). When activated by low energy levels, AMPK can phosphorylate and activate the TSC1/2 complex, which indirectly inhibits the activity of MTORC1 (Inoki *et al.*, 2003). Alternatively, AMPK can directly inhibit MTORC1 (Gwinn *et al.*, 2008; Yang and Klionsky, 2010). Several studies have also reported that AMPK can phosphorylate and activate ULK1 to induce macroautophagy (Lee *et al.*, 2010; Egan *et al.*, 2011; Kim *et al.*, 2011; Shang *et al.*, 2011). The modulation of macroautophagy by energy sensing is conserved in yeast where Snf1, the yeast ortholog of AMPK, serves as a positive regulator (Huang and Snider, 1995; Wang *et al.*, 2001).

It has also been observed that an increase in cytosolic Ca^{2+} concentrations resulting from ER stress causes calcium/calmodulin-dependent kinase kinase 2, beta (CAMKK2/CaMKKβ) to activate AMPK and induce macroautophagy (Høyer-Hansen *et al.*, 2007). Another way in which ER stress can induce macroautophagy is through unfolded protein response (UPR) signaling. Accumulation of unfolded proteins in the ER can be caused by a variety of cellular stressors, and induces macroautophagy in both yeast and mammals. However, the role of macroautophagy in response to ER stress seems to vary, with some studies reporting that it enhances cell survival, while others suggest that it may result in autophagic cell death (Ding *et al.*, 2007; He and Klionsky, 2009).

Additional signals that cause the induction of macroautophagy include hypoxia and the absence of growth factors. Even in the presence of adequate nutrients, the absence of growth factors leads to the induction of macroautophagy (Lum *et al.*, 2005). Both growth factor concentrations and hypoxia regulate macroautophagy at least in part through MTORC1, and hypoxia can inhibit MTORC1 even in the presence of adequate nutrients and growth factors (Arsham *et al.*, 2003; Alers *et al.*, 2012). Given its complex regulation by a variety of cellular signaling pathways, the involvement of MTORC1 in the regulation of macroautophagy is a very intriguing and active area of research, and is discussed in greater detail in another review in this forum series.

III.D Selective macroautophagy and cellular maintenance

While nonspecific macroautophagy can be induced in response to nutrient or energy deprivation to enable cell survival, macroautophagy can also be highly specific, and in this mode functions more in cell maintenance and homeostasis (Chen and Klionsky, 2011; Isakson *et al.*, 2012). Specific autophagic cargoes can include, but are not limited to peroxisomes, mitochondria, and ubiquitinated proteins (Weidberg *et al.*, 2011; Lee *et al.*, 2012; Till *et al.*, 2012).

The selective macroautophagic degradation of peroxisomes, termed pexophagy, is important for a majority of the turnover of peroxisomes under normal growth conditions (Huybrechts *et al.*, 2009). For example, in mouse livers, macroautophagy is responsible for degradation of 70-80% of the peroxisomal mass (Yokota and Dariush Fahimi, 2009). Peroxisomes can also be degraded under starvation conditions, during which they can be specifically recognized by autophagosomes through binding of LC3-II to PEX14, a component

of the peroxisomal translocon complex found on the peroxisomal membrane (Hara-Kuge and Fujiki, 2008). Given the role of peroxisomes in a variety of metabolic functions and the negative effects of peroxisomal dysfunction on human health, pexophagy has an important role in maintaining proper cellular physiology (Till *et al.*, 2012).

Mitophagy is another type of selective macroautophagy that involves the selective degradation of mitochondria, and has been shown to be important in mammals not only for steady-state turnover of these organelles (Tal *et al.*, 2007), but also for the development of certain cell types and the clearance of damaged mitochondria (Kim *et al.*, 2007; Schweers *et al.*, 2007; Kundu *et al.*, 2008). For example, in order for mammalian red blood cells to mature, mitophagy is used to remove mitochondria from the immature cells (Kundu *et al.*, 2008; Zhang *et al.*, 2009; Mortensen *et al.*, 2010). During this process, it is thought that a mitochondrial outer membrane protein called BNIP3L/NIX interacts through a WXXL-like motif (also called the LC3-interacting region) with LC3 and GABARAP on the expanding phagophore, allowing for recognition (Figure II.8) (Youle and Narendra, 2011).

The clearance of damaged mitochondria, however, is thought to proceed in a slightly different way. In this case, the cytosolic E3 ubiquitin ligase PARK2/Parkin is recruited to damaged mitochondria by the mitochondrial outer membrane kinase PINK1, whereupon PARK2 ubiquitinates mitochondrial substrates, leading to mitophagy (Youle and Narendra, 2011). In healthy mitochondria, PINK1 is imported into the mitochondrial inner membrane, and subsequent cleavage by mitochondrial processing peptidase (PMPCB) and presenilin associated, rhomboid-like protease (PARL) leads to its eventual degradation. This prevents the accumulation of PINK1 on the mitochondrial outer membrane, which would otherwise lead to mitophagy of healthy mitochondria (Jin *et al.*, 2010; Meissner *et al.*, 2011). The genes encoding both PINK1

and PARK2 are mutated in autosomal recessive Parkinson disease (Kitada *et al.*, 1998; Valente *et al.*, 2004), emphasizing the importance of mitophagic clearance of damaged mitochondria in maintaining cellular, and thus organismal, health.

Another mechanism used by the cell to identify cargo for selective degradation by macroautophagy involves ubiquitination. The ubiquitin-binding protein SQSTM1/p62 targets intracellular bacteria for degradation by a specific type of macroautophagy called xenophagy (Zheng *et al.*, 2009). SQSTM1 is also important for the clearance of ubiquitinated protein aggregates by acting as an adaptor protein that interacts with LC3-II to target aggregates for macroautophagy-specific degradation in a process termed aggrephagy (Vadlamudi *et al.*, 1996; Bjørkøy *et al.*, 2005; Øverbye *et al.*, 2007). NBR1 and OPTN are other receptors that function in targeting ubiquitinated proteins or pathogens to autophagosomes (Kirkin *et al.*, 2009b; Wild *et al.*, 2011).

Conclusions

Given the wide array of extra- and intracellular signals that can regulate autophagy and the range of possible cargos, it is not surprising to learn that autophagy has been implicated in various aspects of human health and pathophysiology. Several of these topics will be explored in depth in other reviews in this forum series. One area that especially warrants further study is the regulatory network controlling macroautophagy. While several key regulators of macroautophagy have been identified, it is likely that many regulatory factors are not yet defined. Even in the case of relatively well-characterized regulators, such as MTORC1, the relevant downstream targets are not completely known, as is true for most of the kinases that control macroautophagy, and very little information is available with regard to the complementary phosphatases. Similarly, the crosstalk among the different regulatory pathways

has not been well elucidated. The identification and characterization of such factors will be important in the development of therapeutics targeting regulatory proteins; without a deeper understanding of how the cell integrates various extracellular and intracellular signals into a cohesive macroautophagic response, it is difficult to predict how the regulatory network will function when perturbed by therapeutics.

Along these lines, potentially interesting targets for therapeutic applications include ULK1/2, ATG3, ATG4, ATG7, ATG10 and PIK3C3/VPS34. The crystal structures of most of these proteins have been determined from various organisms (Sugawara *et al.*, 2005; Yamada *et al.*, 2007; Satoo *et al.*, 2009; Miller *et al.*, 2010; Hong *et al.*, 2011; Noda *et al.*, 2011; Hong *et al.*, 2012), and, importantly, they have clearly defined functions and functional motifs, making them interesting targets for rational drug design. Further elucidation of the individual steps of macroautophagy, additional structural studies, and a more complete knowledge of the role of this process in different disease conditions will provide a better understanding of this integral cellular process, and can guide the development of improved methods and/or drugs for the treatment of autophagy defects related to human disease.

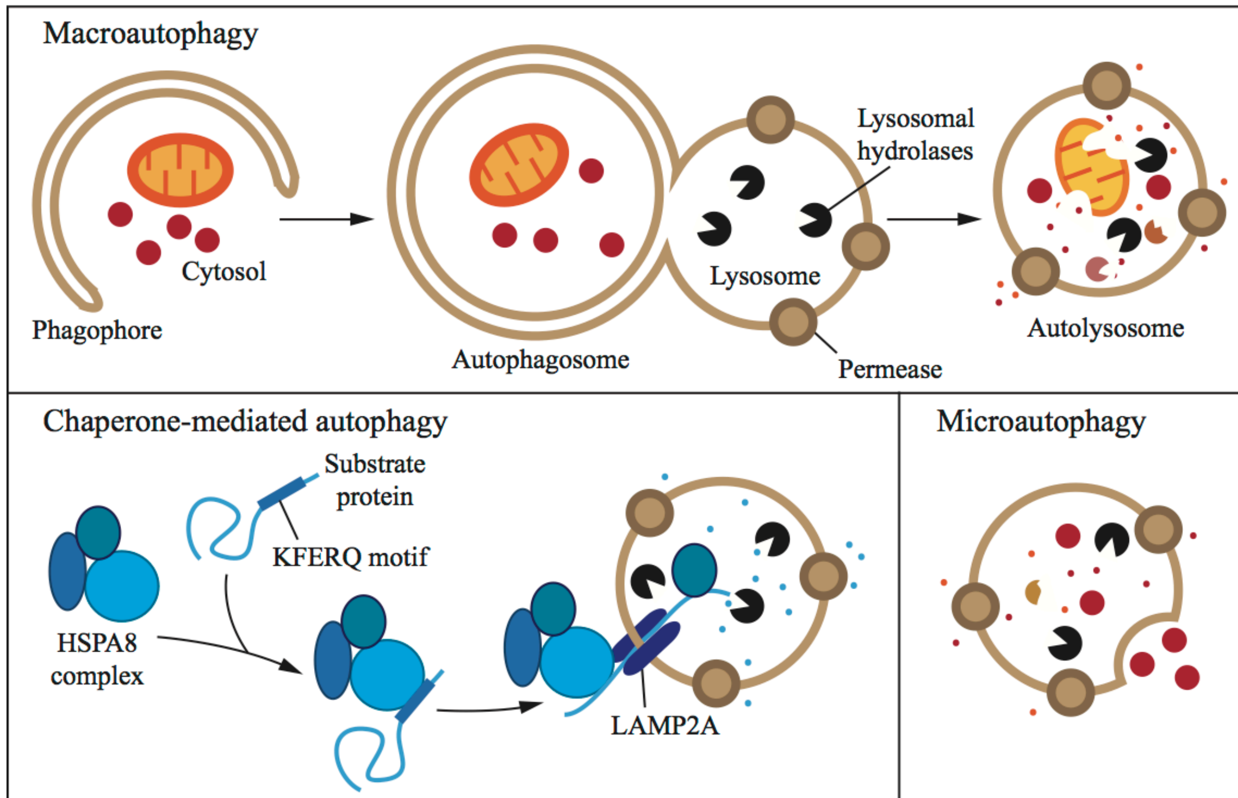

Figure II.1. Three types of autophagy in mammalian cells.
Macroautophagy relies on *de novo* formation of cytosolic double-membrane vesicles, autophagosomes, to sequester and transport cargo to the lysosome. Chaperone-mediated autophagy transports individual unfolded proteins directly across the lysosomal membrane. Microautophagy involves the direct uptake of cargo through invagination of the lysosomal membrane. All three types of autophagy lead to degradation of cargo and release of the breakdown products back into the cytosol for reuse by the cell. See the text for details.

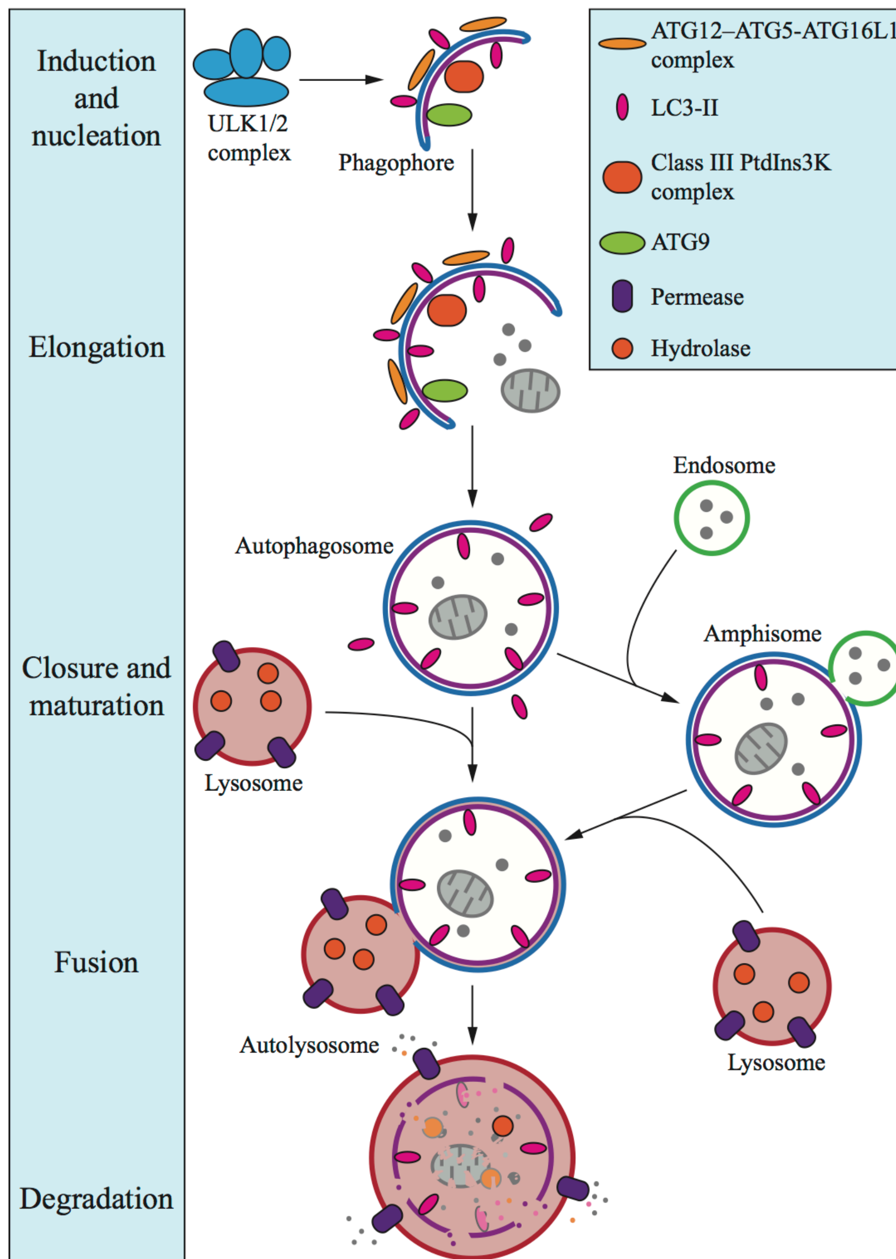

Figure II.2. Morphology of macroautophagy.
Nucleation of the phagophore occurs following induction by the ULK1/2 complex. Elongation of the phagophore is aided by the ATG12−ATG5-ATG16L1 complex, the class III PtdIns3K complex, LC3-II, and ATG9. Eventually, the expanding membrane closes around its cargo to form an autophagosome and LC3-II is cleaved from the outer membrane of this structure. The outer membrane of the autophagosome will then fuse with the lysosomal membrane to form an autolysosome. In some instances, the autophagosome may fuse with an endosome, forming an amphisome, before fusing with the lysosome. The contents of the autolysosome are then degraded and exported back into the cytoplasm for reuse by the cell. See the text for details. This figure was modified from figure 1 in Yang, Z, Klionsky DJ. Eaten alive: a history of macroautophagy. *Nat Cell Biol* 12(9): 814-22, 2010.

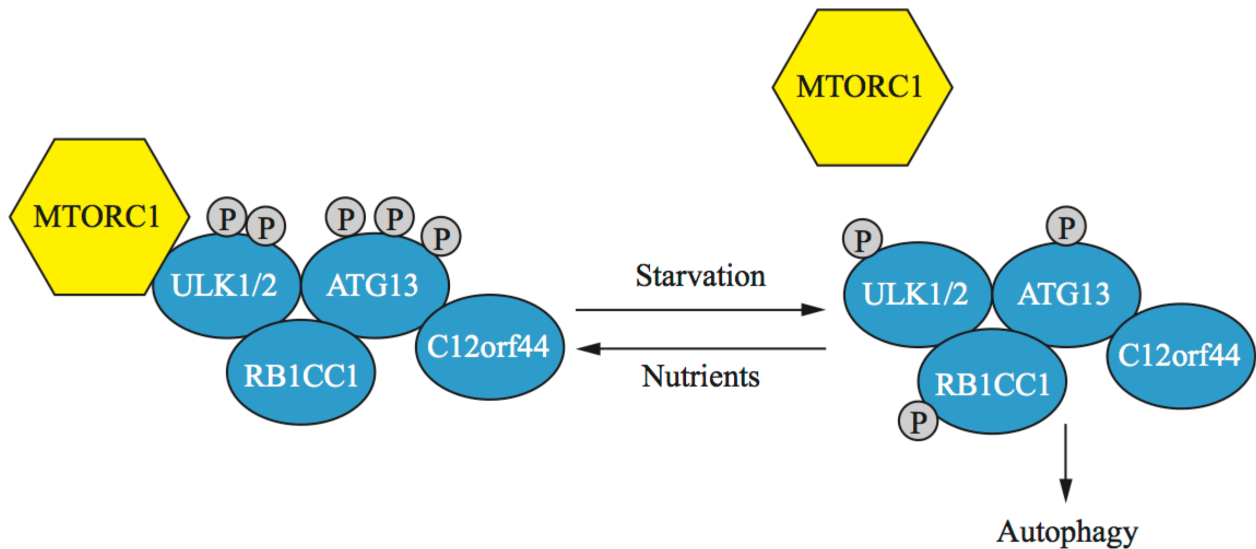

Figure II.3. The induction complex consists of ULK1/2, ATG13, RB1CC1, and C12orf44. Under nutrient-rich conditions, MTORC1 associates with the complex and inactivates ULK1/2 and ATG13 through phosphorylation. During starvation, MTORC1 dissociates from the complex and ATG13 and ULK1/2 become partially dephosphorylated by as yet unidentified phosphatases, allowing the complex to induce macroautophagy. RB1CC1/FIP200 and C12orf44/ATG101 are also associated with the induction complex and are essential for macroautophagy. RB1CC1/FIP200 may be the ortholog of yeast Atg17, whereas the function of C12orf44/ATG101 is not known. This figure was modified from figure 1 in Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 22: 124-31, 2010.

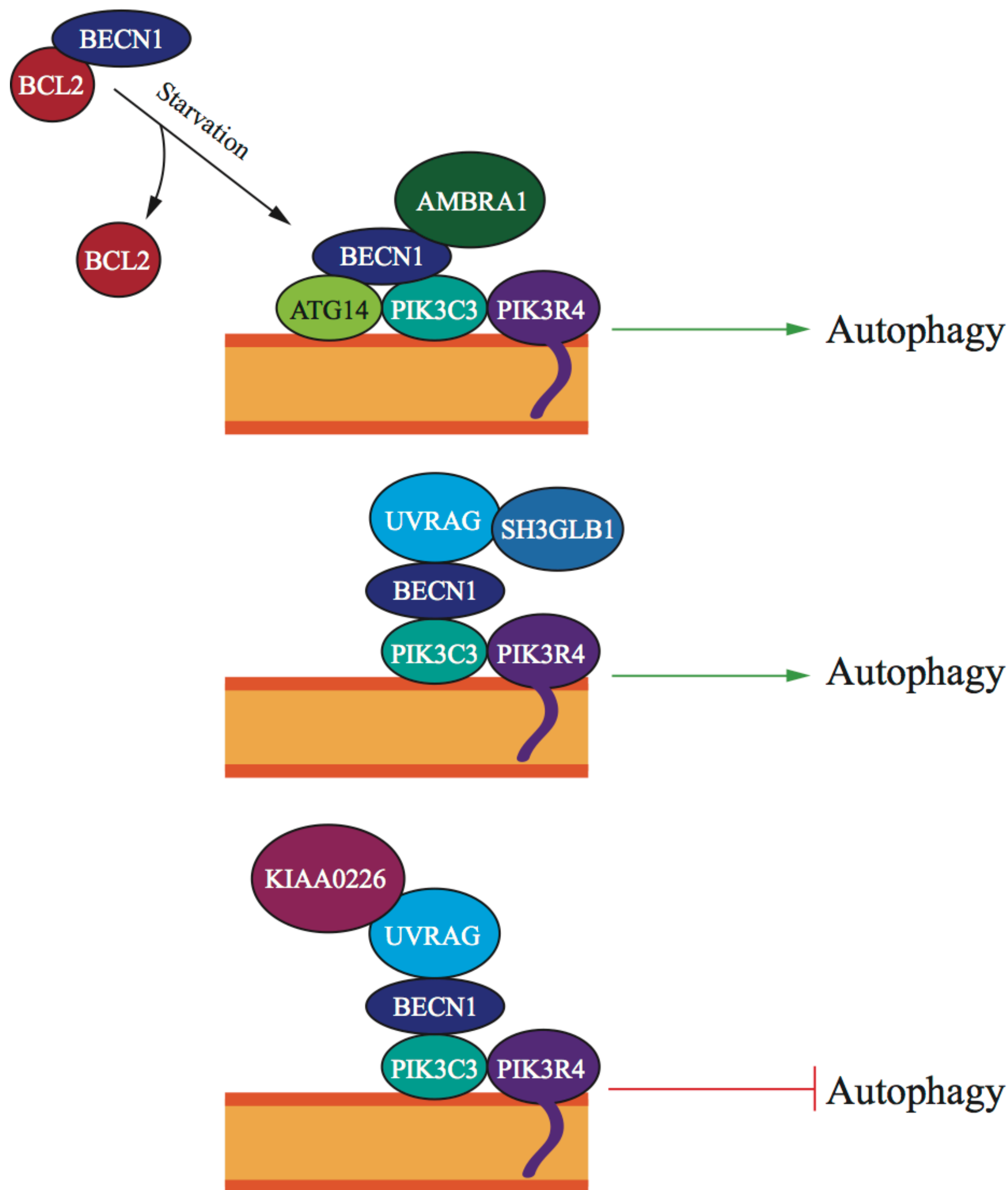

Figure II.4. The activity of the class III PtdIns3K complex is regulated by subunit composition.
The ATG14 complex (ATG14-BECN1-PIK3C3-PIK3R4) is required for macroautophagy. It can be positively regulated by AMBRA1 and negatively regulated by BCL2 binding to BECN1 and preventing association with the complex. The UVRAG (UVRAG-BECN1-PIK3C3-PIK3R4) complex is involved in the endocytic pathway and also participates in macroautophagy. SH3GLB1/Bif-1 positively regulates this complex by binding UVRAG. The KIAA0226/Rubicon complex (KIAA0226-UVRAG-BECN1-PIK3C3-PIK3R4) negatively regulates macroautophagy. This figure was modified from figure 1 in Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 22: 124-31, 2010.

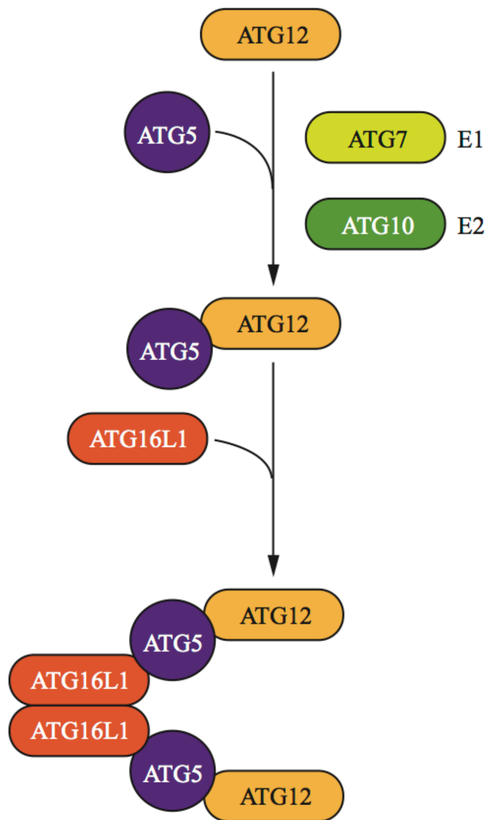

Figure II.5. ATG12–ATG5-ATG16L1 conjugation complex.
The ubiquitin-like protein ATG12 is irreversibly conjugated to ATG5 in an ATG7- and ATG10-dependent manner. ATG7 and ATG10 function as E1 activating and E2 conjugating enzymes, respectively. The ATG12–ATG5 conjugate binds ATG16L1 through ATG5. ATG16L1 dimerizes and allows association with the phagophore, promoting membrane expansion.

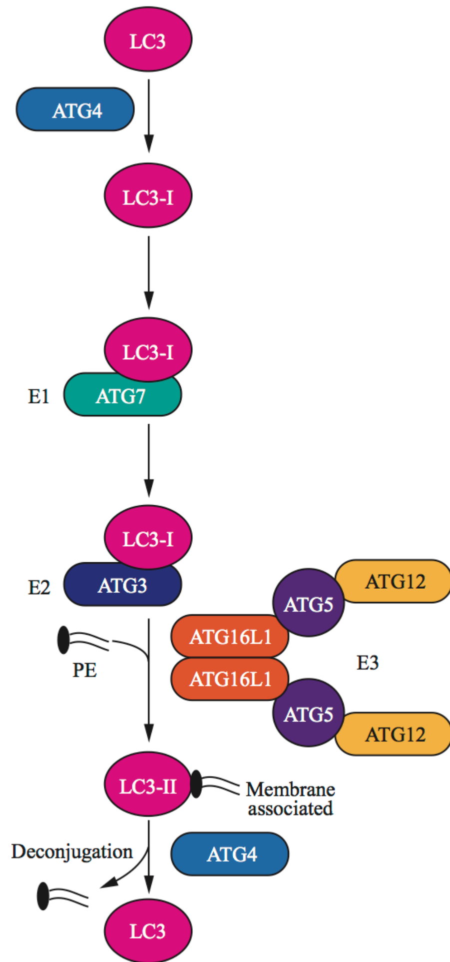

Figure II.6. The LC3 conjugation system.
LC3 is processed by ATG4 to reveal a C-terminal glycine (LC3-I). ATG7, an E1-like enzyme, activates LC3-I and transfers it to the E2-like enzyme ATG3. The ATG12–ATG5-ATG16L1 complex may participate as an E3 ligase in the conjugation of phosphatidylethanolamine (PE) to LC3-I to create LC3-II, which can associate with the phagophore. LC3-II can subsequently be cleaved by ATG4 to release LC3 (deconjugation).

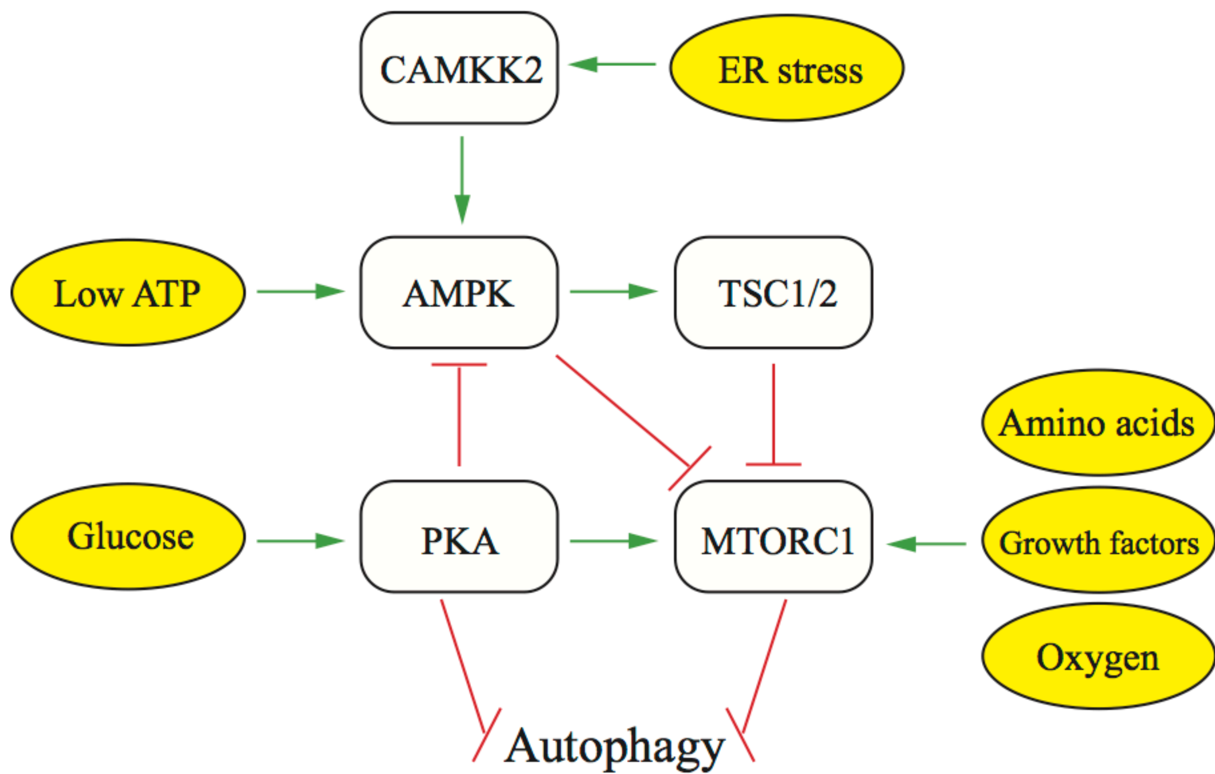

Figure II.7. Regulation of macroautophagy.
Three of the major kinases that regulate macroautophagy are cAMP-dependent protein kinase A (PKA), AMP-activated protein kinase (AMPK), and mechanistic target of rapamycin complex 1 (MTORC1). These kinases, along with proteins such as TSC1/2 and calcium/calmodulin-dependent kinase kinase 2, beta (CAMKK2), respond to a variety of intracellular and extracellular signals to regulate macroautophagy. Green arrows indicate activation of a target and red bars indicate inhibition of a target. See the text for details. This figure was modified from figure 4 of Chen Y, Klionsky, DJ. The regulation of autophagy – unanswered questions. *J Cell Sci* 124: 161-70, 2011.

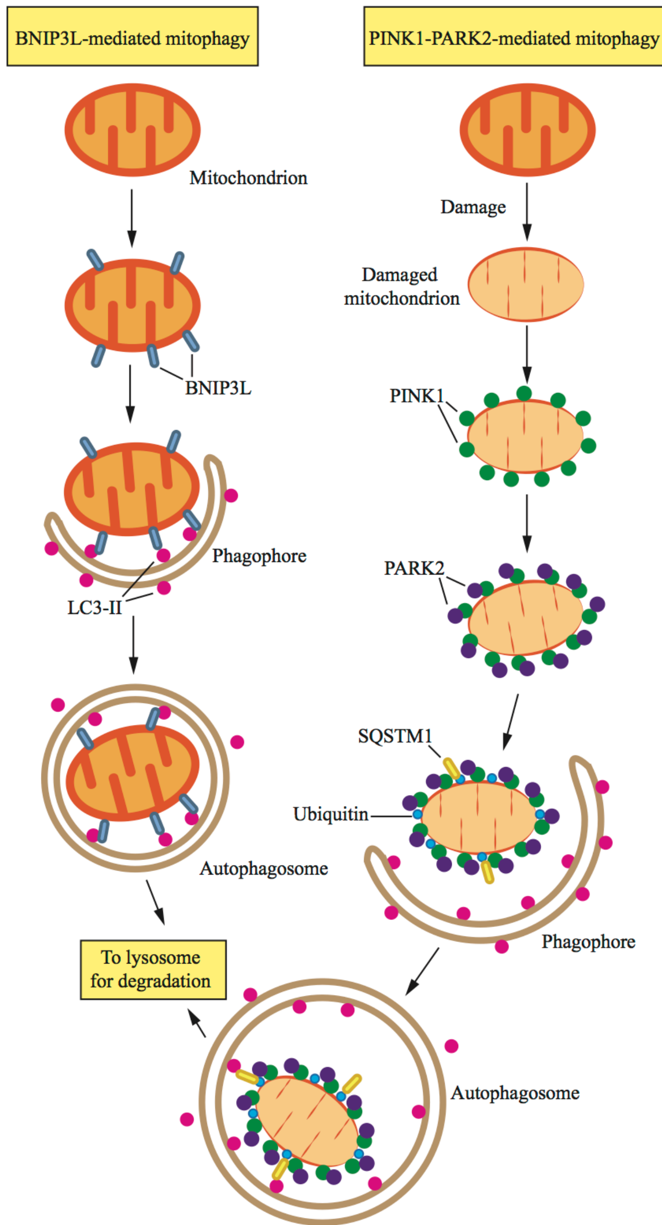

Figure II.8. Two mechanisms of mitophagy.
Mitochondria are cleared from maturing red blood cells through a mechanism involving autophagic recognition of mitochondria through a BNIP3L-LC3 interaction. During removal of damaged mitochondria, PARK2 binds to PINK1 on the mitochondrial surface and ubiquitinates mitochondrial outer membrane proteins, which may then bind SQSTM1, a receptor that interacts with LC3. In either case, the interaction with LC3 leads to sequestration by the phagophore and eventual degradation. This figure was modified from figure 2 of Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol* 12: 9-14, 2011.

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Chapter III

A newly characterized vacuolar serine carboxypeptidase, Atg42/Ybr139w, is required for normal vacuole function and the terminal steps of autophagy in the yeast *Saccharomyces cerevisiae*[3]

Abstract

Macroautophagy (hereafter autophagy) is a cellular recycling pathway essential for cell survival during nutrient deprivation that culminates in the degradation of cargo within the vacuole in yeast and the lysosome in mammals, followed by efflux of the resultant macromolecules back into the cytosol. The yeast vacuole is home to many different hydrolytic proteins and while few have established roles in autophagy, the involvement of others remains unclear. The vacuolar serine carboxypeptidase Prc1 (carboxypeptidase Y) has not been previously shown to have a role in vacuolar zymogen activation and has not been directly implicated in the terminal degradation steps of autophagy. Through a combination of molecular genetic, cell biological, and biochemical approaches, we have shown that Prc1 has a functional homolog, Ybr139w, and that cells deficient in both Prc1 and Ybr139w have defects in autophagy-dependent protein synthesis, vacuolar zymogen activation, and autophagic body breakdown. Thus, we have demonstrated that Ybr139w and Prc1 have important roles in proteolytic processing in the vacuole and the terminal steps of autophagy.

[3] This chapter has been submitted for publication in *Molecular Biology of the Cell*. All experiments were completed by Katherine Parzych.

Introduction

The vacuole in the yeast *Saccharomyces cerevisiae* is analogous to the mammalian lysosome and performs a variety of functions including metabolite storage and maintenance of pH and ion homeostasis, but it is perhaps best known as the major degradative organelle of the cell (Klionsky *et al.*, 1990; Thumm, 2000). Autophagy is an intracellular recycling pathway that depends on the vacuole for degradation of various substrates (Reggiori and Klionsky, 2013). Upon induction of autophagy by nutrient stress conditions such as nitrogen starvation, transient double-membrane compartments, called phagophores, form *de novo* to envelop cellular contents. The phagophore expands, and upon completion forms an autophagosome. Autophagosomes traffic to the vacuole, where the outer membrane of the autophagosome fuses with the vacuolar membrane, releasing the inner membrane-bound compartment, now termed the autophagic body, into the vacuolar lumen. The autophagic body and its contents are broken down and released back into the cytosol for reuse by the cell (Reggiori and Klionsky, 2013).

Although autophagy has attracted substantial attention over the past two decades, and defects in this process are associated with a wide array of diseases, relatively little attention has been focused on the final steps of this process—breakdown of the autophagic cargo, and efflux of the resulting macromolecules. As a degradative organelle, the vacuole is home to many hydrolases, responsible for degrading a wide array of substrates, including proteins, carbohydrates, lipids, and nucleic acids (Klionsky *et al.*, 1990; Epple *et al.*, 2001; Teter *et al.*, 2001). As with the final breakdown process in general, the biosynthesis and function of vacuolar/lysosomal hydrolases has been largely ignored in recent years, yet there are clearly many unanswered questions about hydrolase function. For example, several of these enzymes appear to have redundant activities: the yeast vacuole contains at least two carboxypeptidases

and two aminopeptidases (Klionsky *et al.*, 1990; Van Den Hazel *et al.*, 1996; Hecht *et al.*, 2014); however, it is likely that each of these enzymes has at least some unique substrates and specificities. In fact, the absence of a single lysosomal hydrolase often results in a disease phenotype (Kaminskyy and Zhivotovsky, 2012). As one example, patients with the disease galactosialidosis exhibit a deficiency of the multifunctional lysosomal hydrolase CTSA (cathepsin A) (Hiraiwa, 1999). CTSA functions as a carboxypeptidase and has structural homology to, and similar substrate specificity as, the yeast vacuolar serine carboxypeptidase Prc1 (carboxypeptidase Y) (Hiraiwa, 1999).

In yeast, two resident vacuolar proteases in particular, Pep4 (proteinase A) and Prb1 (proteinase B), are critical for the final steps of autophagy, in part because they play a role in the activation of many of the other zymogens present in the vacuole lumen (Van Den Hazel *et al.*, 1996). Cells deficient in these proteases show an accumulation of autophagic bodies in the vacuole (Takeshige *et al.*, 1992). Additionally, cells lacking Pep4 display decreased survival in nitrogen starvation conditions (Teichert *et al.*, 1989; Tsukada and Ohsumi, 1993). During times of nutrient stress, cells will increase expression of Pep4, Prb1, and Prc1 to cope with the increased demand for autophagic recycling (Klionsky *et al.*, 1990; Van Den Hazel *et al.*, 1996). Thus far, Prc1 has not been shown to have a role in autophagy, as there is no accumulation of autophagic bodies in the vacuoles of Prc1-deficient cells during nitrogen starvation (Takeshige *et al.*, 1992). However, this may be due to the presence of a functionally-redundant homolog; the vacuole contains one other putative serine carboxypeptidase, Ybr139w, which shows a high degree of similarity to Prc1 at the amino acid level; the other known vacuolar carboxypeptidase, Cps1, is a zinc metallopeptidase (Nasr *et al.*, 1994; Huh *et al.*, 2003; Baxter *et al.*, 2004; Hecht *et al.*, 2014). Microarray and northern blotting analysis show that *YBR139W* expression is induced

in nitrogen-poor conditions or following rapamycin treatment (Scherens *et al.*, 2006). In one study examining the synthesis of phytochelatins, peptides that bind excess heavy metal ions, deletion of *YBR139W* had little-to-no effect on synthesis, whereas deletion of *PRC1* resulted in moderate inhibition (Wünschmann *et al.*, 2007). However, deletion of both genes abolished phytochelatin synthesis altogether (Wünschmann *et al.*, 2007). This finding suggests that there may indeed be some functional redundancy between these two proteins. Thus, it is possible that no autophagy phenotype has yet been seen in Prc1-deficient cells due to a compensatory effect by Ybr139w.

We set out to determine whether Ybr139w is a functional homolog of Prc1 and whether either, or both, of these proteins participate in the terminal steps of autophagy. We demonstrate that the absence of both of these proteins results in defects in the maturation of several vacuolar hydrolases, lysis of autophagic bodies in the vacuole, and maintenance of the amino acid pool during nitrogen starvation conditions. Additionally, there is functional redundancy between Prc1 and Ybr139w as regards these phenotypes.

Results

Ybr139w is a resident vacuolar glycoprotein

As can be inferred from the absence of a standard name, Ybr139w has been essentially uncharacterized. A previous large-scale study of protein localization indicated that Ybr139w localized to the vacuole, similar to Prc1 (Huh *et al.*, 2003). To verify this localization, we tagged the carboxy terminus of Ybr139w with GFP and examined its intracellular distribution using fluorescence microscopy. In both growing and starvation conditions, Ybr139w-GFP localized to the vacuole and displayed a diffuse signal throughout the lumen, similar to Prc1-GFP (Figure III.1A). Similarly, in the *pep4*Δ strain, where most proteolytic processing is blocked, localization

was diffuse throughout the vacuole lumen, suggesting that Ybr139w is a soluble, rather than membrane-associated, protein (Figure III.1A).

Western blot analysis of protein extracts from cells expressing Ybr139w-GFP showed that the GFP tag was cleaved from Ybr139w in both growing and starvation conditions in a Pep4-dependent manner (Figure III.1B). Many chimeric GFP-tagged proteins that are delivered to the vacuole display a similar phenotype, that is, cleavage of intact GFP from the remainder of the protein (Shintani and Klionsky, 2004; Kanki and Klionsky, 2008); the GFP moiety is relatively resistant to degradation, and the appearance of the free GFP band serves as an indication of vacuolar delivery. Thus, the liberation of GFP from Ybr139w-GFP is another line of evidence suggesting that Ybr139w is exposed to the proteolytic environment of the vacuole. Together, these results suggest that, similar to Prc1, Ybr139w is a resident vacuolar protein.

As with many of the vacuolar proteases, Prc1 is a glycoprotein (Klionsky *et al.*, 1990; Van Den Hazel *et al.*, 1996). Prc1 is N-glycosylated at Asn124, Asn198, Asn279, and Asn479 (Hasilik and Tanner, 1978a, b; Winther *et al.*, 1991) (Figure III.1C). Based on BLAST alignment, two of these sites, Asn198 and Asn279, are conserved in Ybr139w as Asn163 and Asn242. To determine whether Ybr139w is glycosylated at these sites, we mutated them to glutamine and looked for a change in gel mobility using western blotting. Mutation of the predicted glycosylated residues resulted in a reduction in apparent molecular weight of approximately 5 kDa, which would correspond to the average mass of two glycosylation sites (Figure III.1D). This observation suggests that Ybr139w is glycosylated at these two conserved sites.

***prc1Δ ybr139wΔ* cells exhibit defects in vacuolar function**

One important function of the yeast vacuole during autophagy is to generate a pool of free amino acids to be used in the synthesis of proteins. During nitrogen starvation, cellular amino acid levels decrease drastically but are largely recovered after 3-4 h (Onodera and Ohsumi, 2005; Müller *et al.*, 2015); this recovery is dependent on autophagy, and is required to support the increased synthesis of various proteins (Onodera and Ohsumi, 2005; Müller *et al.*, 2015). One such protein that displays a substantial increase in synthesis under autophagy-inducing conditions is Ape1 (aminopeptidase I), a resident vacuolar hydrolase that is delivered to the vacuole through the cytoplasm-to-vacuole targeting (Cvt) pathway (Harding *et al.*, 1995; Gasch *et al.*, 2000). Under conditions of nitrogen starvation, Ape1 is dependent on the release of amino acids from the vacuolar pool for its increased synthesis (Onodera and Ohsumi, 2005; Yang *et al.*, 2006). Thus, the level of Ape1 during starvation serves as a useful marker for vacuolar function and recycling of amino acids. Accordingly, we monitored the synthesis of this protein when cells were shifted from growing to starvation conditions. Whereas a robust increase in Ape1 occurred in wild-type cells upon nitrogen starvation, this was markedly reduced in *prc1*Δ *ybr139w*Δ double-knockout cells (Figure III.2A), suggesting a defect in the generation or efflux of the vacuolar amino acid pool in these cells; considering the soluble nature of Ybr139w and its similarity to Prc1, the former seems most likely. We also noted that the proteolytic processing of the precursor form of Ape1 (prApe1) was substantially delayed in *prc1*Δ *ybr139w*Δ cells (Figure III.2A) (Hecht *et al.*, 2014). The *prc1*Δ *ybr139w*Δ double-knockout cells accumulated prApe1, similar to proteolytically-deficient *pep4*Δ cells (Figure III.2, B and C). In contrast, neither the *prc1*Δ nor the *ybr139w*Δ single null strain displayed a defect in the synthesis or processing of prApe1 (Figure III.2B), suggesting that there is at least some degree of functional redundancy between Prc1 and Ybr139w.

Another marker for vacuolar recycling of amino acids is Prb1, which, like Ape1, is upregulated during nitrogen starvation and is synthesized as a zymogen (Klionsky *et al.*, 1990; Van Den Hazel *et al.*, 1996; Hecht *et al.*, 2014). Prb1 undergoes a self-catalyzed N-terminal cleavage event in the ER followed by glycosylation, resulting in a 40-kDa species (proPrb1) being delivered to the vacuole (Nebes and Jones, 1991; Hirsch *et al.*, 1992; Van Den Hazel *et al.*, 1996). Once in the vacuole, it undergoes two more cleavage events, this time at the C terminus. The first cleavage is Pep4-mediated and results in a 37-kDa intermediate species (Moehle *et al.*, 1989), which we have termed intPrb1 (Figure III.2D). The second cleavage event results in the 31-kDa mature form of Prb1 (Moehle *et al.*, 1989). Similar to the Ape1 biosynthesis defects seen in the *prc1*Δ *ybr139w*Δ strain, Prb1 levels were lower and proteolytic processing was reduced compared to the wild type (Figure III.2, E and F). The migration pattern of Prb1 in the *prc1*Δ *ybr139w*Δ double-knockout strain, however, was not identical to that seen in the *pep4*Δ strain (Figure III.2E); the *pep4*Δ mutant showed a mix of the proPrb1 and intPrb1 precursors, whereas *prc1*Δ *ybr139w*Δ cells accumulated intPrb1 and the mature Prb1, suggesting that the initial cleavage event in the vacuole depends on Pep4, but that subsequent maturation requires the activity of these carboxypeptidases, at least for maximal efficiency; neither of these steps appeared to be completely blocked in *pep4*Δ or *prc1*Δ *ybr139w*Δ cells, respectively, suggesting the possibility of less efficient compensatory processing mechanisms. The defect in protein synthesis and processing of prApe1 and intPrb1 were complemented by addition of either *YBR139W* or *PRC1* genes to the *prc1*Δ *ybr139w*Δ strain (Supplementary figure III.S1). Based on these data, we conclude that Ybr139w and Prc1 share some functional redundancy and in cells lacking both of these proteins, vacuolar function is impaired, as demonstrated by effects on

protein synthesis under starvation conditions and proteolytic processing of certain zymogens.

Ybr139w is a serine carboxypeptidase

To determine whether Ybr139w exhibited serine carboxypeptidase activity similar to Prc1, we sought to assess potential serine carboxypeptidase activity through mutagenesis of the predicted Ybr139w active site. Serine proteases have a catalytic triad consisting of a serine, histidine, and aspartate (Kraut, 1977). In Prc1, these residues are at positions Ser257, Asp449, and His508 (Stennicke *et al.*, 1996). Mutation of either Ser257 or His508 to alanine drastically reduces the activity of Prc1 (Bech and Breddam, 1989; Stennicke *et al.*, 1996), whereas mutating Asp449 has only a minor effect (Stennicke *et al.*, 1996). The analogous residues in Ybr139w are Ser219, Asp425, and His474 (Nasr *et al.*, 1994). Mutation of all three predicted active site residues to alanine abolished enzymatic activity, as evidenced by the inability of the mutated Ybr139w to complement the prApe1- and intPrb1-processing defects in *prc1*Δ *ybr139w*Δ cells (Figure III.3, A and B); although we detected partial processing of intPrb1, a similar result was seen with the non-transformed *prc1*Δ *ybr139w*Δ strain, or the double-knockout strain transformed with an empty vector. Mutation of individual residues showed only a partial block in enzymatic activity (Figure III.3, C and D). These results suggest that Ybr139w functions as a serine carboxypeptidase, similar to Prc1.

We next used a complementary in vitro biochemical assay to measure the carboxypeptidase Y activity of various mutants. Hydrolysis of the Prc1 peptide substrate *N*-(3-[2-furyl]acryloyl)-Phe-Phe-OH (FA-Phe-Phe-OH) added to cell lysates results in a decrease in absorbance at 337 nm (Caesar and Blomberg, 2004; Gombault *et al.*, 2009). As expected, wild-type cells showed a decrease in absorbance over time, indicative of carboxypeptidase Y activity in the cell lysates (Supplementary figure III.S2). Deletion of *PRC1* or both *PRC1* and *YBR139W*

almost completely abolished carboxypeptidase Y activity, whereas deletion of *YBR139W* alone had little-to-no effect. We propose that the observed results are due to a difference in substrate specificity between Prc1 and Ybr139w.

***prc1*Δ *ybr139w*Δ cells are defective in the terminal steps of autophagy**

Because *prc1*Δ *ybr139w*Δ cells showed a clear defect in vacuolar function, we next wanted to determine whether autophagy was affected in these cells. Atg8 is an autophagic protein that becomes conjugated to a phosphatidylethanolamine (PE) lipid moiety in the cytoplasm (Ichimura *et al.*, 2000). Atg8–PE is present on both sides of the phagophore, and the protein that is localized to the concave side becomes trapped within the completed autophagosome (Kirisako *et al.*, 1999). This population of Atg8–PE is delivered into the vacuole within the autophagic body and is degraded during autophagy, but accumulates in the vacuoles of *pep4*Δ cells (Klionsky *et al.*, 2007). We analyzed the potential role of Prc1 and Ybr139w in the vacuolar turnover of Atg8 by western blot. In wild-type cells, relatively little Atg8 or Atg8–PE is detected because the protein is degraded in the vacuole (Figure III.4A). In contrast, *pep4*Δ cells displayed the expected accumulation of this protein. In fact, *pep4*Δ cells accumulated both non-lipidated Atg8 and Atg8–PE. Atg8 synthesis increases during starvation (Kirisako *et al.*, 1999); it is possible that the ineffective generation of amino acids from vacuolar hydrolysis in the absence of Pep4 results in a continued starvation signal, causing further upregulation of Atg8 synthesis, and the small size of the protein may leave it relatively insensitive to the limited pool of free amino acids. Deletion of *PRC1* caused no change in Atg8/Atg8–PE accumulation as compared to wild-type, whereas the *ybr139w*Δ strain showed a slight reduction in total Atg8/Atg8-PE (Figure III.4A). In contrast to the single mutants, the *prc1*Δ *ybr139w*Δ double mutant showed a substantial accumulation of Atg8/Atg8–PE, comparable to that of the *pep4*Δ

strain. Reintroduction of the *PRC1* gene into the *prc1*Δ *ybr139w*Δ strain fully complemented this phenotype, whereas reintroduction of the *YBR139W* gene could only partially complement (Supplementary figure III.S3A); there was still a substantial accumulation of Atg8–PE suggesting a continued partial starvation response. This finding demonstrates that the vacuolar serine carboxypeptidases participate in terminal steps of autophagy and further supports functional overlap between these two proteins.

Given that Prb1 cleaves the propeptide from prApe1 in the vacuole in a Pep4-dependent manner (i.e., Prb1 is the direct processing enzyme, but its activation requires Pep4) (Klionsky *et al.*, 1992; Van Den Hazel *et al.*, 1996), it is possible that the observed defects in prApe1 maturation in *pep4*Δ and *prc1*Δ *ybr139w*Δ cells (Figure III.2, A-C) are a result of the defects in Prb1 processing in these strains (Figure III.2, E and F). However, a previous observation that cells deficient in Pep4 or Prb1 accumulate autophagic bodies in the vacuole upon nitrogen starvation suggests another possible explanation (Takeshige *et al.*, 1992). In addition to delivery via the Cvt pathway (Harding *et al.*, 1995), prApe1 can be delivered to the vacuole through nonspecific autophagy (Scott *et al.*, 1996). We hypothesized that inefficient maturation of prApe1 in *pep4*Δ and *prc1*Δ *ybr139w*Δ cells (Figure III.2, A-C) resulted from impaired lysis of autophagic bodies in the vacuole, preventing exposure of this zymogen to the proteolytic environment of the vacuolar lumen. We investigated whether autophagic bodies accumulate in the vacuole in *prc1*Δ *ybr139w*Δ cells by examining the localization of GFP-Atg8. In growing conditions, GFP-Atg8 appears primarily as a single perivacuolar punctum that corresponds to the phagophore assembly site (Kim *et al.*, 2002). During nitrogen starvation, GFP-Atg8 is delivered to the vacuole via autophagy (Suzuki *et al.*, 2001). In wild-type cells that undergo normal breakdown of autophagic bodies within the vacuole, GFP from GFP-Atg8 appears as a diffuse

signal throughout the vacuolar lumen. However, if breakdown of autophagic bodies is impeded, such as in a *pep4*Δ strain, the GFP signal appears punctate within the vacuole, which corresponds to the presence of intact autophagic bodies (Kim *et al.*, 2001; Klionsky *et al.*, 2007). The deletion of *PRC1* or *YBR139W* alone resulted in the presence of diffuse vacuolar GFP-Atg8 fluorescence upon nitrogen starvation, similar to wild-type cells (Figure III.4, B and C). In contrast, deletion of both genes showed an accumulation of GFP-Atg8 puncta in the vacuole, similar to, but not as severe as the *pep4*Δ strain (Figure III.4, B and C). This result suggests that at least one of the serine carboxypeptidases, Ybr139w or Prc1, must be present for efficient lysis of autophagic bodies in the vacuole lumen.

Due to its roles in vacuolar function and the terminal steps of autophagy, we propose to rename *YBR139W* as *ATG42*.

Discussion

In this work, we set out to characterize the putative Prc1 homolog Atg42/Ybr139w and to determine whether either, or both, of these proteins are involved in the terminal steps of autophagy. Through fluorescence microscopy and western blotting, we demonstrated that, similar to Prc1, Atg42/Ybr139w is a resident soluble vacuolar glycoprotein (Figure III.1). Moreover, Atg42/Ybr139w was shown to be a serine carboxypeptidase (Figure III.3), based on mutation of predicted active site residues that were identified through alignment with Prc1. However, we suggest that Atg42/Ybr139w may have a slightly different substrate specificity than Prc1, as *prc1*Δ cells showed an inability to break down the Prc1 substrate FA-Phe-Phe-OH, despite the presence of Atg42/Ybr139w (Supplemental figure III.S2).

We also found that at least one of these proteins is required for regeneration of the vacuolar amino acid pool during starvation, as demonstrated by the reduced synthesis of Ape1 in

*atg42*Δ*/ybr139w*Δ *prc1*Δ mutant cells (Figure III.2A). Loss of both Atg42/Ybr139w and Prc1 also resulted in decreased maturation of the vacuolar zymogens prApe1 and intPrb1 (Figure III.2, B-C and E-F). Our results regarding the maturation defects of Prb1 in the *atg42*Δ*/ybr139w*Δ *prc1*Δ strain in particular provide further information regarding the proteolytic processing of this protein. The second cleavage of the Prb1 zymogen, which occurs in the vacuole (conversion of intPrb1 to Prb1; Figure III.2D), was previously reported to be Prb1-dependent (i.e., autocatalytic), because the Prb1 inhibitor chymostatin inhibits processing (Mechler *et al.*, 1988). Also, the intPrb1 species accumulates in cells with the *prb1-628* allele, in which Ala171 is changed to Thr; this mutation is thought to possibly interfere with the Prb1 active site (Moehle *et al.*, 1989; Nebes and Jones, 1991). However, our data suggest that the second cleavage event is at least partially dependent on Atg42/Ybr139w and/or Prc1.

The vacuolar breakdown and efflux steps of autophagy are mediated by a host of hydrolases and permeases, including Pep4 and Prb1. Evidence of this exists in the accumulation of autophagic bodies in the vacuoles of Pep4- and Prb1-deficient cells (Takeshige *et al.*, 1992). It was previously thought that Prc1 had no involvement in autophagy because deletion of the *PRC1* gene had no effect on autophagic body formation in the vacuole (Takeshige *et al.*, 1992). However, our work suggests that the role of Prc1 in autophagy was previously obscured due to compensatory activity by the homolog Atg42/Ybr139w in Prc1-deficient cells, and that both Prc1 and Atg42/Ybr139w do in fact participate in the terminal steps of autophagy. Analysis of *prc1*Δ or *atg42*Δ*/ybr139w*Δ single mutant strains would seem to support the previous notion that neither of these genes are required for autophagy; Atg8 protein is turned over as in wild-type cells (Figure III.4A), and GFP-Atg8 fluorescence is diffuse within vacuoles during nitrogen starvation (Figure III.4B), suggesting efficient lysis of autophagic bodies within the vacuole.

However, the *atg42Δ/ybr139wΔ prc1Δ* double mutant was strikingly similar to the autophagy-deficient *pep4Δ* strain; there was a marked accumulation of Atg8 protein (Figure III.4A), suggesting a defect in protein turnover, and GFP-Atg8 appeared primarily as punctate clusters within the vacuole, indicating an accumulation of autophagic bodies and a defect in autophagic body lysis (Figure III.4, B and C).

It is unclear from our results how Atg42/Ybr139w and Prc1 function in the breakdown of autophagic bodies in the vacuole. As previously mentioned, autophagic bodies accumulate in Prb1- and Pep4-deficient cells (Takeshige *et al.*, 1992), so one possibility is that the defects in Prb1 maturation seen in the *atg42Δ/ybr139wΔ prc1Δ* strain are responsible for this block. Accumulation of autophagic bodies also occurs in cells lacking the vacuolar lipase Atg15 (Epple *et al.*, 2001; Teter *et al.*, 2001). How Atg15 activity is regulated in the vacuole remains unknown, but it has been previously speculated that, similar to many other vacuolar proteins, it may be activated through proteolytic processing (Teter *et al.*, 2001). Further study is required to understand this activation and whether Atg42/Ybr139w, Prc1, and Prb1 are involved. The cascade of events that combines vacuolar acidification, zymogen activation, and the lipase Atg15 to result in autophagic body breakdown remains poorly understood; however, its importance cannot be overlooked—without these critical terminal events, autophagy cannot complete its recycling of macromolecules to support protein synthesis and survival during starvation.

Materials and Methods

Strains and Media

Yeast strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. C-terminal tagging with GFP (Longtine *et al.*, 1998) and gene disruption (Gueldener *et al.*, 2002) were performed using a PCR-based method. Due to slight overlap

between the *YBR139W* gene and the chromosomal autonomously replicating sequence, we did not delete the entire gene, but instead deleted nucleotides coding for the first 491 of 508 amino acids. We refer to this truncation as *ybr139w*Δ for simplicity. Site-directed mutagenesis of plasmid-borne *YBR139W* was done using a standard method (Zheng *et al.*, 2004).

Cells were cultured in rich medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal medium (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed) as appropriate. Autophagy was induced by shifting cells in mid-log phase from growth medium to nitrogen starvation medium (SD-N; 0.17% yeast nitrogen base without ammonium sulfate or amino acids, 2% glucose) for the indicated times. All cells were grown at 30˚C.

Protein Extraction and Immunoblot Analysis

Protein extraction and immunoblotting were performed as previously described (Yorimitsu *et al.*, 2007). PVDF membranes were stained with Ponceau S to monitor protein transfer prior to immunoblotting.

Antisera to Ape1 and Atg8 were used as described previously (Klionsky *et al.*, 1992; Huang *et al.*, 2000). The anti-Pgk1 antiserum was a generous gift from Dr. Jeremy Thorner, University of California, Berkeley. The anti-Prb1 antiserum was a generous gift from Dr. Elizabeth Jones (Moehle *et al.*, 1989). Additional antisera used were anti-PA (Jackson Immunoresearch), anti-YFP (Clontech, JL-8), rabbit anti-mouse (Jackson Immunoresearch), and goat anti-rabbit (Fisher Scientific).

Fluorescence microscopy

For FM 4-64 (Life Technologies) vacuole membrane staining, cells were grown to mid-log phase in SMD complete medium or SMD medium lacking selective nutrients at 30˚C. Cells

(0.75 OD_{600} units) were collected by centrifugation at 855 x g for 1 min; pellets were resuspended in 100 µl growth medium and stained with 30 µM FM 4-64 for 30 min at 30ºC, agitating every 10 min. Cells were then washed 2 times with 1 ml growth medium or starvation medium (SD-N), resuspended in 1 ml growth medium or SD-N, and incubated at 30ºC for either 1 h (growth medium) or 2 h (starvation medium) before imaging.

Carboxypeptidase Y activity assay

Samples were prepared and carboxypeptidase Y activity was determined similar to the method described in Caesar and Blomberg (Caesar and Blomberg, 2004). Briefly, cells were lysed by glass bead disruption in MES buffer (50 mM MES, 1 mM EDTA, pH 6.5). Cell debris was pelleted and the supernatant (lysate) was collected. The BCA assay was used to determine the protein concentration of the lysates.

Hydrolysis of the carboxypeptidase Y substrate *N*-(3-[2-furyl]acryloyl)-Phe-Phe (FA-Phe-Phe-OH; Bachem) was measured over time in MES buffer. Reactions contained 200 µg/ml lysate and 1 mM FA-Phe-Phe-OH (dissolved in methanol) and were incubated at room temperature. Hydrolysis of FA-Phe-Phe-OH was measured by reading the absorbance at 337 nm.

Statistical analysis

Where appropriate, a one-sample *t*-test was used to determine statistical significance.

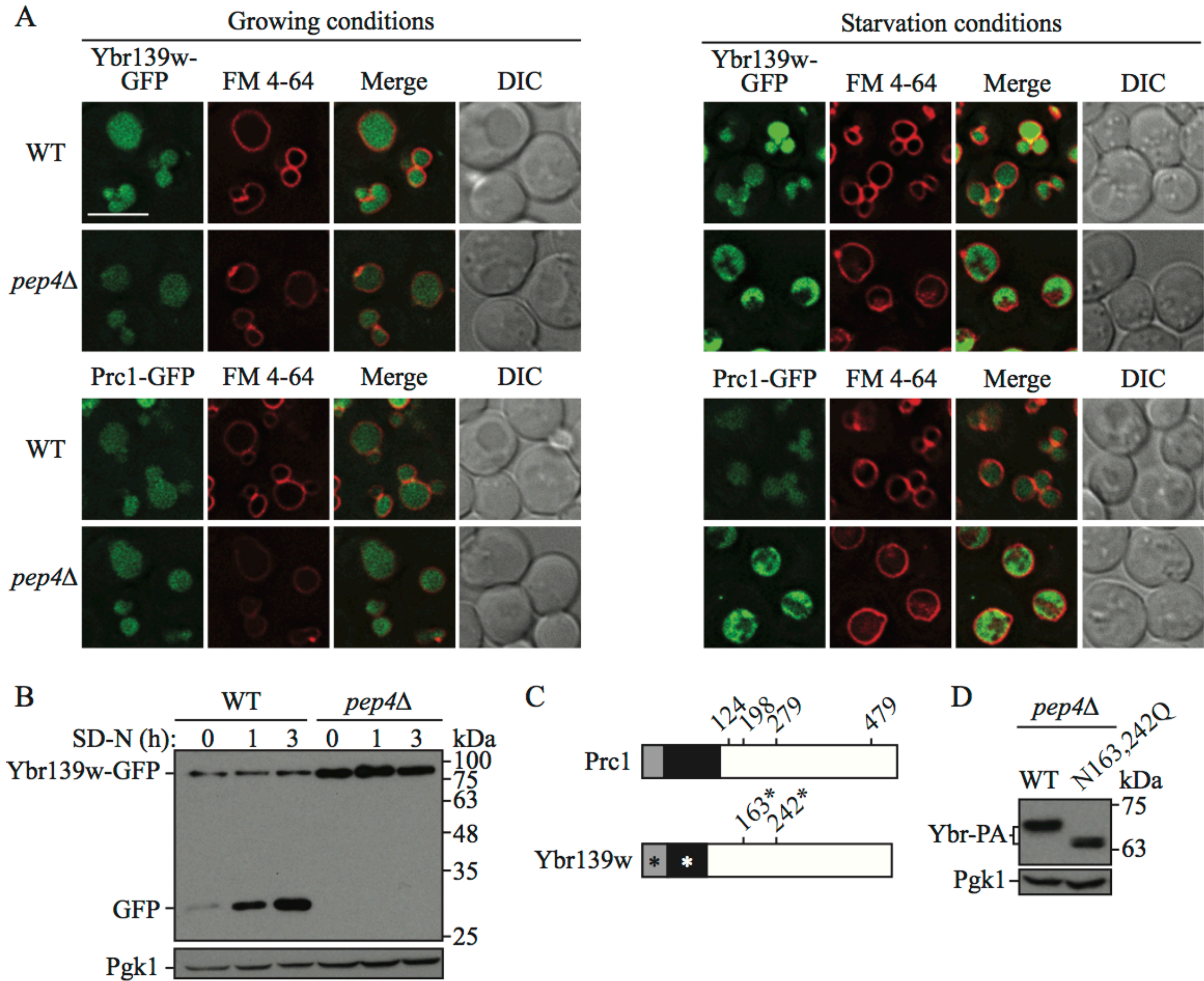


Figure III.1. Ybr139w is a soluble vacuolar glycoprotein.
(A) The localization of Ybr139w-GFP and Prc1-GFP was examined in wild-type (KPY382 and KPY384) and *pep4*Δ (KPY383 and KPY385) cells in growing and starvation conditions. FM 4-64 was used to label the vacuole limiting membrane. DIC, differential interference contrast. Scale bar: 5 µm. (B) GFP is cleaved from Ybr139w-GFP in a *PEP4*-dependent manner. Wild-type (KPY382) and *pep4*Δ (KPY383) cells expressing chromosomally-tagged Ybr139w-GFP were grown to mid-log phase in YPD and then shifted to starvation conditions for the indicated times. Protein extracts were resolved by SDS-PAGE and blotted with anti-YFP antibody as described in Materials and Methods. Pgk1 is used as a loading control. (C) Schematic representation of Prc1 and Ybr139w. Gray box, signal peptide; black box, propeptide; numbers, glycosylated residues; *, predicted. (D) *pep4*Δ (TVY1) cells expressing wild-type Ybr139w-PA (pKP105) or Ybr139w^{N163,242Q}-PA (pKP110) were grown to mid-log phase in SMD-URA, cells were harvested and protein extracts were analyzed as in (B) using antibodies to protein A.

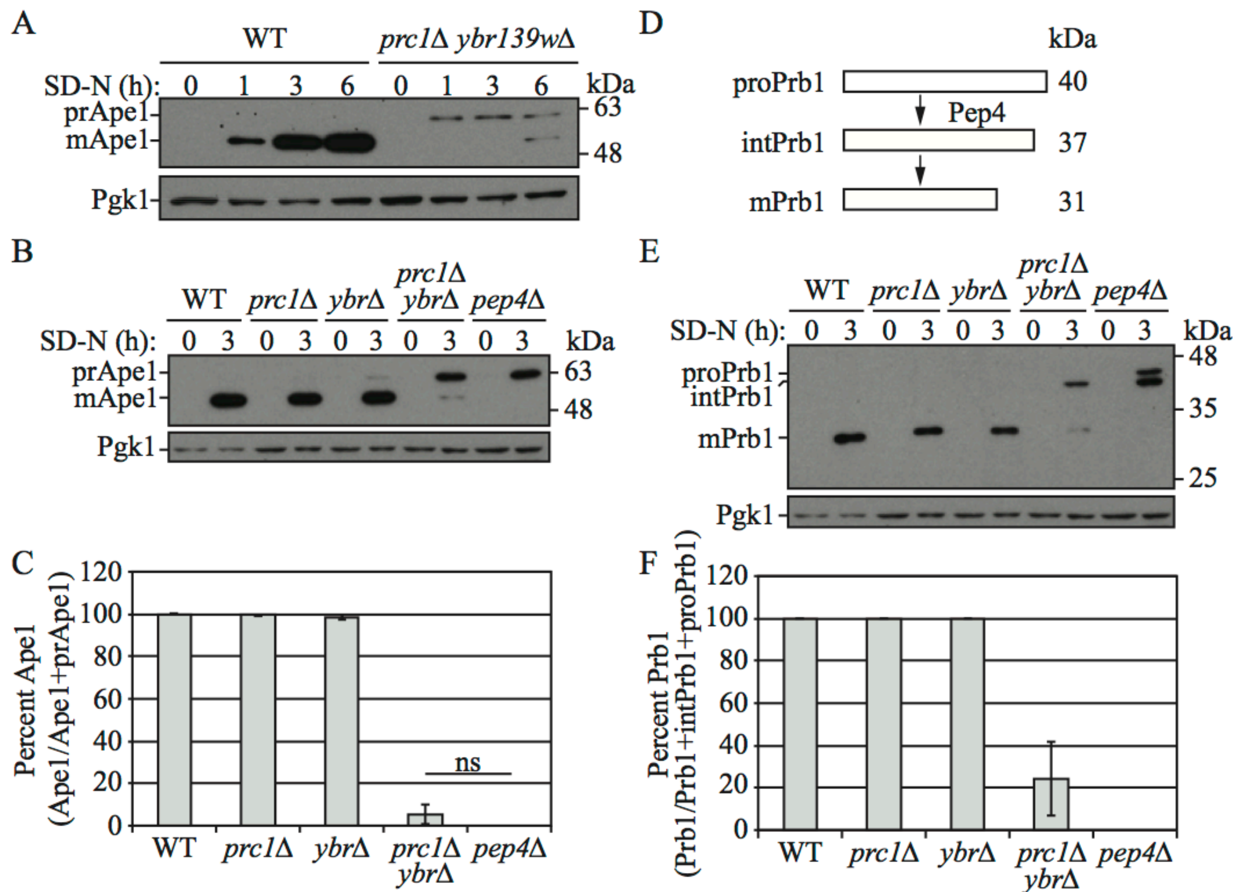


Figure III.2. Vacuolar function is impaired in cells lacking *PRC1* and *YBR139W*.
(A) Wild-type (SEY6210) and *prc1*Δ *ybr139w*Δ (KPY325) cells were grown to mid-log phase in YPD medium and then shifted to starvation conditions for the indicated times. Protein extracts were analyzed by western blot using antiserum to Ape1. The positions of precursor (pr) and mature Ape1 are indicated. (B and E) Wild-type (SEY6210), *prc1*Δ (KPY301), *ybr139w*Δ (KPY323), *prc1*Δ *ybr139w*Δ (KPY325), and *pep4*Δ (TVY1) cells were grown to mid-log phase in YPD and then shifted to starvation conditions for 3 h. Cells were harvested and protein extracts were analyzed by western blot using antiserum to Ape1 (B) or Prb1 (E). The positions of the precursor (pro), intermediate (int), and mature forms of Prb1 are indicated. (C) Quantification of results in (B). Percent Ape1 was calculated as amount of Ape1/total Ape1 (Ape1 + prApe1). Average of three experiments. Error bars, standard deviation; ns, not significant. (D) Schematic representation of Prb1 processing in the vacuole. See text for details. (F) Quantification of results in (E). Average of three experiments. Percent Prb1 was calculated as amount of Prb1/total Prb1 (Prb1 + intPrb1 + proPrb1). Average of three experiments. Error bars, standard deviation.

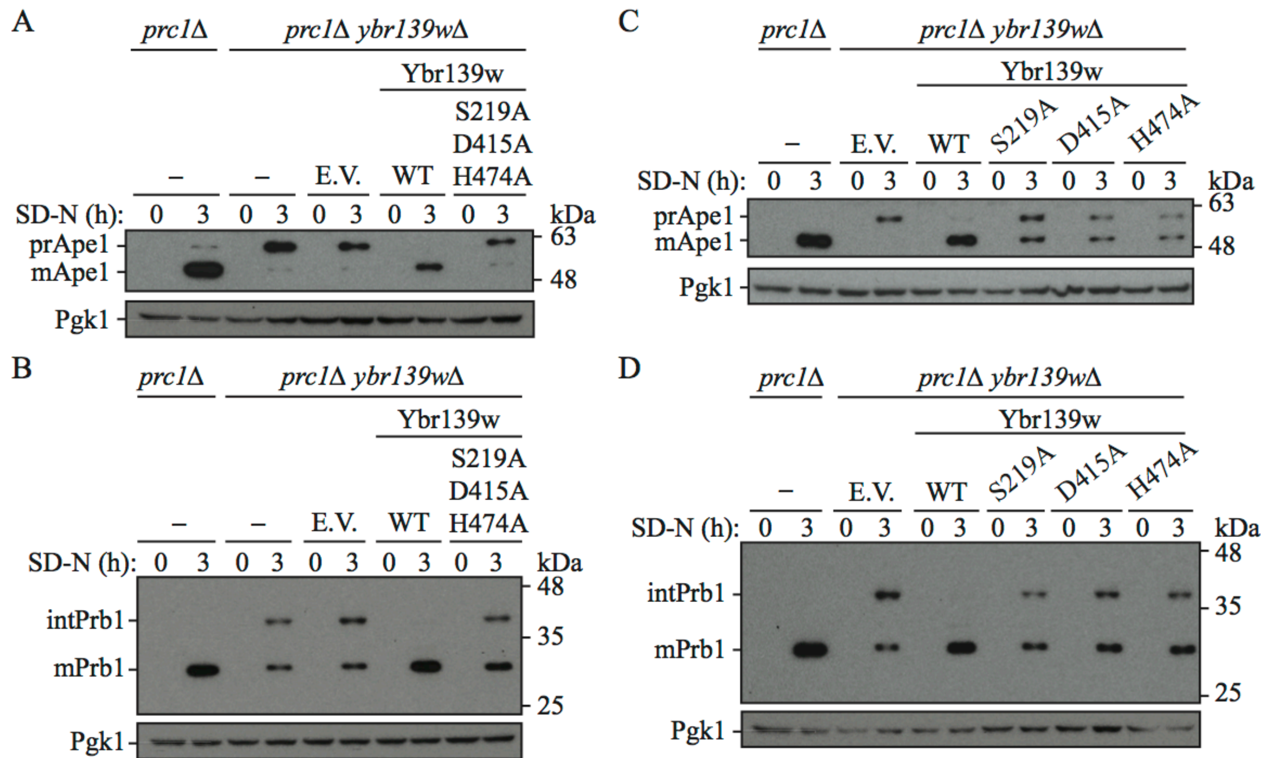


Figure III.3. Ybr139w is a serine carboxypeptidase.
(A and B) *prc1Δ* (KPY301), *prc1Δ ybr139wΔ* (KPY325), and *prc1Δ ybr139wΔ* cells with integrated empty vector (KPY332), *YBR139W* (KPY336), or *YBR139W*^{S219,D415,H474A} (KPY418) genes were grown to mid-log phase in YPD medium and then shifted to starvation conditions for 3 h. Cells were harvested and protein extracts were analyzed by western blot using antiserum to Ape1 (A) or Prb1 (B). (C and D) *prc1Δ* (KPY301), and *prc1Δ ybr139wΔ* cells with integrated empty vector (KPY332), *YBR139W* (KPY336), *YBR139W*^{S219A} (KPY404), *YBR139W*^{D415A} (KPY416), or *YBR139W*^{H474A} (KPY406) genes were grown to mid-log phase in YPD and then shifted to starvation conditions for 3 h. Cells were harvested and protein extracts were analyzed by western blot using antiserum to Ape1 (C) or Prb1 (D).

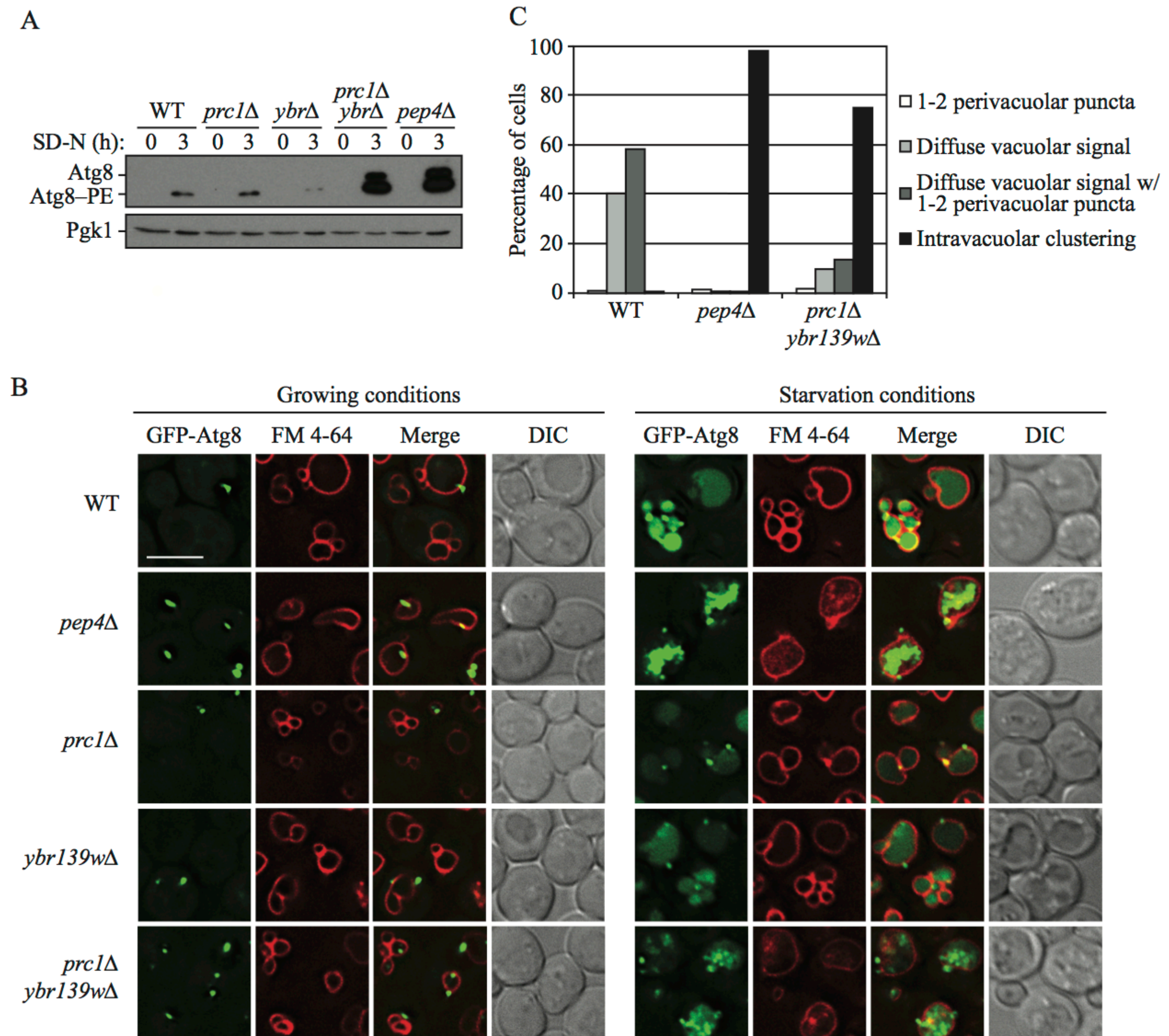


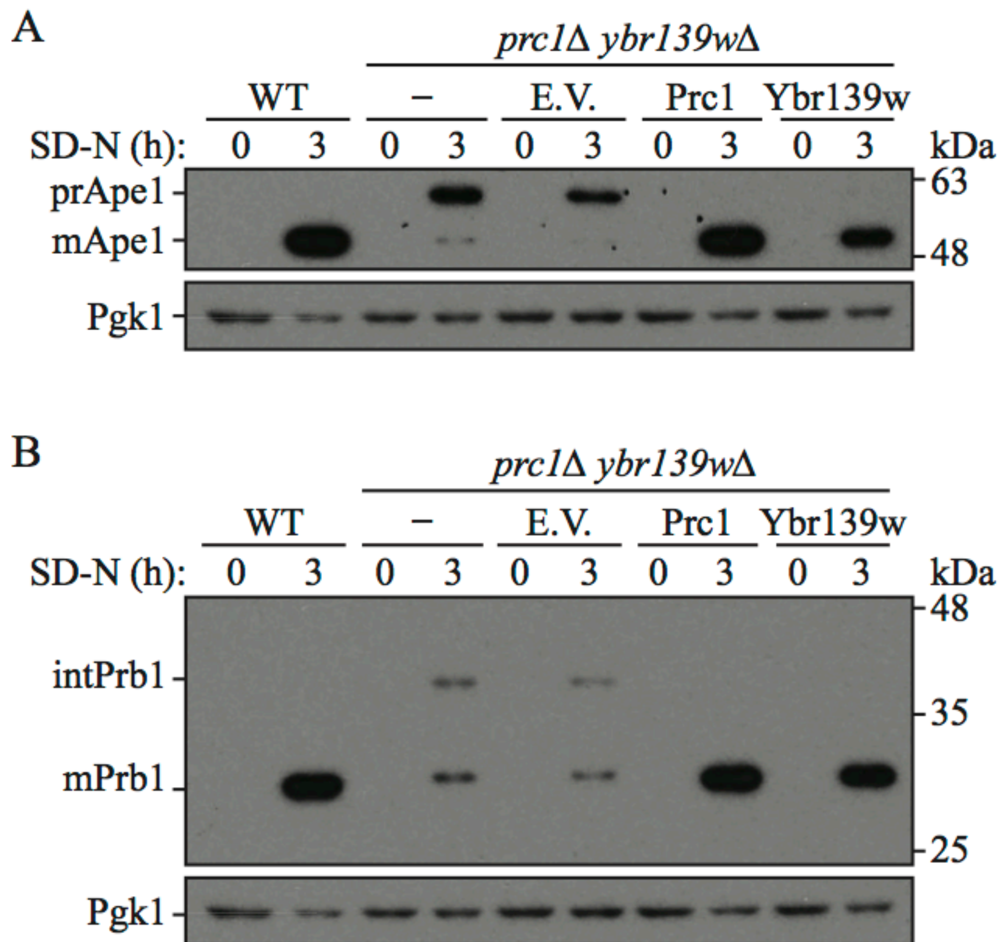
Figure III.4. Cells lacking *PRC1* and *YBR139W* are defective in the terminal steps of autophagy.
(A) Wild-type (SEY6210), *prc1*Δ (KPY301), *ybr139w*Δ (KPY323), *prc1*Δ *ybr139w*Δ (KPY325), and *pep4*Δ (TVY1) cells were grown to mid-log phase in YPD medium and then shifted to starvation conditions for 3 h. Cells were harvested and protein extracts were analyzed by western blot using antiserum to Atg8. (B) Wild-type (SEY6210), *pep4*Δ (TVY1), *prc1*Δ (KPY301), *ybr139w*Δ (KPY323), and *prc1*Δ *ybr139w*Δ (KPY325) cells expressing GFP-Atg8 from a plasmid were grown in SMD-TRP medium to mid-log phase. Cells were stained with FM 4-64 for 30 min to label the vacuole and chased in either SDM-TRP for 1 h (growing) or SD-N for 2 h (starvation) before imaging. DIC, differential interference contrast. Scale bar: 5 µm. (C) Quantification of results in (B). Cells with GFP-Atg8-positive vacuoles were divided into four categories based on the appearance of the GFP signal as indicated. Wild-type, n = 183 cells; *pep4*Δ, n = 475 cells; *prc1*Δ *ybr139w*Δ, n = 309 cells.

Table III.1. Strains used in this study.

Strain	Genotype	Source
KPY301	SEY6210 *prc1Δ::his5*	this study
KPY323	SEY6210 *ybr139wΔ::LEU2*	this study
KPY325	SEY6210 *prc1Δ::his5 ybr139wΔ::LEU2*	this study
KPY332	KPY325 + pKP112	this study
KPY334	KPY325 + pKP113	this study
KPY336	KPY325 + pKP115	this study
KPY350	SEY6210 + pRS406	this study
KPY351	KPY325 + pRS406	this study
KPY382	SEY6210 *YBR139W-GFP(S65T)-His3MX6*	this study
KPY383	TVY1 *YBR139W-GFP(S65T)-His3MX6*	this study
KPY384	SEY6210 *PRC1-GFP(S65T)-His3MX6*	this study
KPY385	TVY1 *PRC1-GFP(S65T)-His3MX6*	this study
KPY404	KPY325 + pKP129	this study
KPY406	KPY325 + pKP131	this study
KPY416	KPY325 + pKP133	this study
KPY418	KPY325 + pKP134	this study
KPY420	KPY325 + pKP135 (isolate #1)	this study
KPY421	KPY325 + pKP135 (isolate #4)	this study
KPY422	KPY325 + pKP136 (isolate #1)	this study
KPY423	KPY325 + pKP136 (isolate #4)	this study
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801; GAL*	(Robinson *et al.*, 1988)
TVY1	SEY6210 *pep4Δ::LEU2*	(Gerhardt *et al.*, 1998)

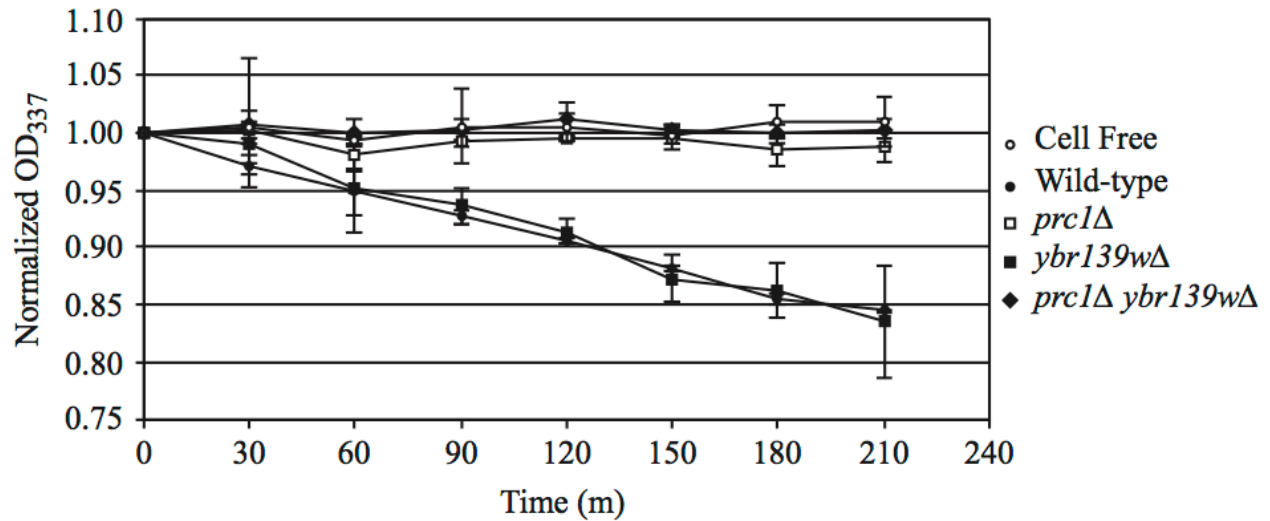
Table III.2. Plasmids used in this study.

Plasmid	Genotype	Source
pGFP-Atg8 (414)		(Abeliovich *et al.*, 2003)
pKP105	pRS416-*YBR139Wp-YBR139W-PA-ADH1t*	this study
pKP110	pRS416-*YBR139Wp-YBR139W*^{N163,242Q}-*PA-ADH1t*	this study
pKP112	pRS406-*GFP-ADH1t*	this study
pKP113	pRS406-*PRC1p-PRC1-GFP-ADH1t*	this study
pKP115	pRS406-*YBR139Wp-YBR139W-GFP-ADH1t*	this study
pKP129	pRS406-*YBR139Wp-YBR139W*^{S219A}-*GFP-ADH1t*	this study
pKP131	pRS406-*YBR139Wp-YBR139W*^{H474A}-*GFP-ADH1t*	this study
pKP133	pRS406-*YBR139Wp-YBR139W*^{D415A}-*GFP-ADH1t*	this study
pKP134	pRS406-*YBR139Wp-YBR139W*^{S219,D415,H474A}-*GFP-ADH1t*	this study
pKP135	pRS406-*YBR139Wp-YBR139W-PA-ADH1t*	this study
pKP136	pRS406-*PRC1p-PRC1-PA-ADH1t*	this study
pRS406		(Sikorski and Hieter, 1989)



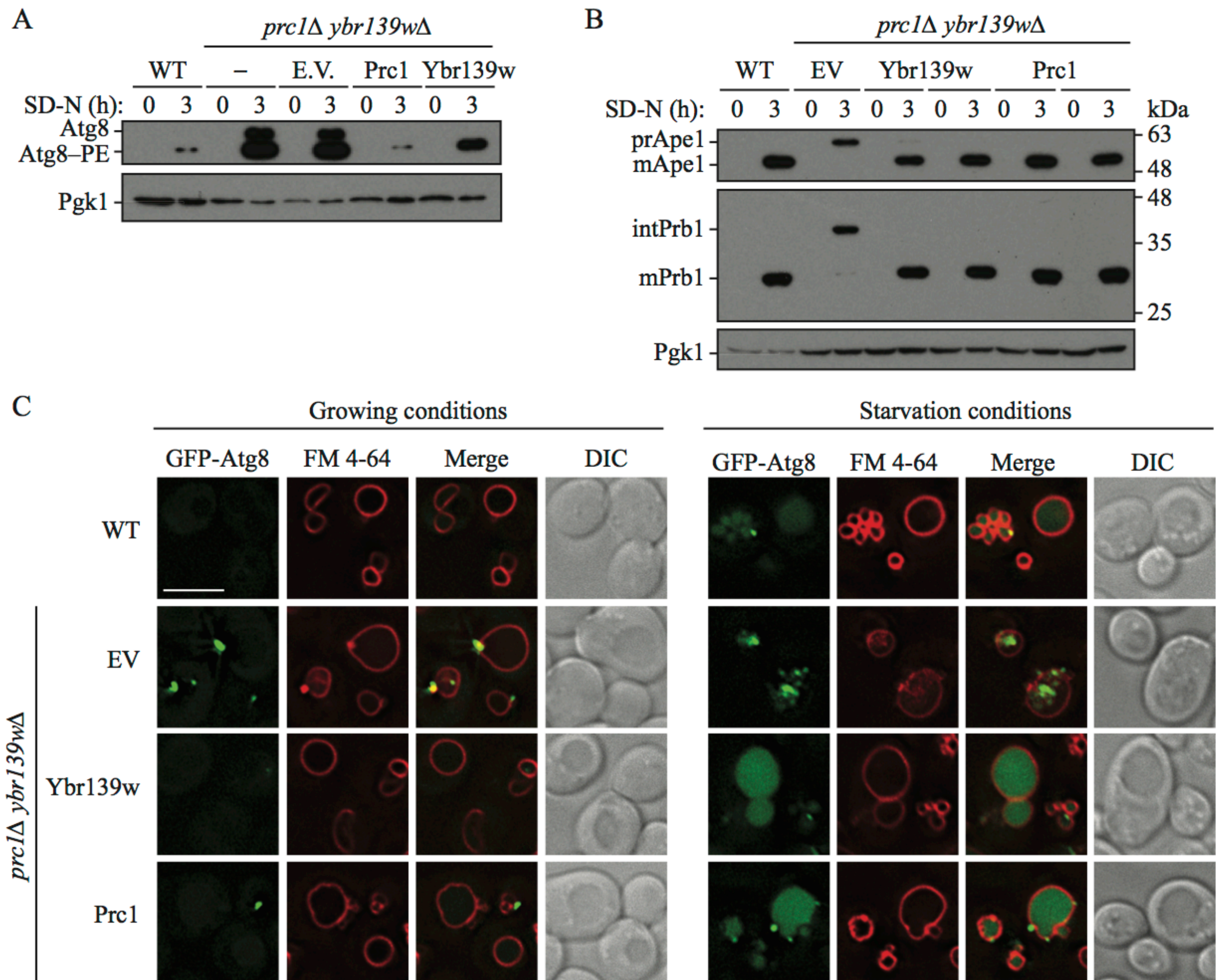
Supplemental Figure III.S1. Reintroduction of *PRC1* or *YBR139W* complements protein processing defects in *prc1*Δ *ybr139w*Δ mutants.

(A and B) Wild-type (SEY6210), *prc1*Δ *ybr139w*Δ (KPY325), and *prc1*Δ *ybr139w*Δ cells with integrated empty vector (KPY332), *PRC1* (KPY334), or *YBR139W* (KPY336) genes were grown to mid-log phase in YPD medium and then shifted to starvation conditions for 3 h. Protein extracts were resolved by SDS-PAGE and blotted with antiserum to Ape1 (A) or Prb1 (B). Pgk1 is used as a loading control.



Supplemental Figure III.S2. Prc1, but not Ybr139w, is required for Carboxypeptidase Y activity.

Wild type (SEY6210), *prc1*Δ (KPY301), *ybr139w*Δ (KPY323), and *prc1*Δ *ybr139w*Δ (KPY325) cells were grown to mid-log phase in YPD medium. Cell lysates were collected and protein concentration was determined using the BCA assay. Lysate (200 µg) or cell-free buffer (control) was combined in a reaction with 1 mM FA-Phe-Phe-OH substrate. Absorbance at 337 nm was measured every 30 min. The value for the 0 h time point was set to 1.0 and subsequent absorbance measurements were normalized to the initial measurement for each sample. N = 3.



Supplemental Figure III.S3. Reintroduction of *PRC1* or *YBR139W* complements autophagy defects in *prc1Δ ybr139wΔ* mutants.
(A) Wild-type (SEY6210), *prc1Δ ybr139wΔ* (KPY325), and *prc1Δ ybr139wΔ* cells with integrated empty vector (KPY332), *PRC1* (KPY334), or *YBR139W* (KPY336) genes were grown to mid-log phase in YPD medium and then shifted to starvation conditions for 3 h. Protein extracts were resolved by SDS-PAGE and blotted with antiserum to Atg8. Pgk1 is used as a loading control. (B) Wild-type (KPY350), *prc1Δ ybr139wΔ* (KPY351), and *prc1Δ ybr139wΔ* cells with integrated *YBR139W* (KPY420 and KPY421) or *PRC1* (KPY422 and KPY423) genes were grown to mid-log phase in YPD medium and then shifted to starvation conditions for 3 h. Cells were harvested and protein extracts were analyzed as in (A) using antiserum to Ape1 (upper panel) or Prb1 (middle panel). (C) Wild-type (KPY350) and *prc1Δ ybr139wΔ* cells with an integrated empty vector (KPY351), *YBR139W* (KPY421), or *PRC1* (KPY423) genes and all expressing GFP-Atg8 from a plasmid were grown in SMD-TRP medium to mid-log phase. Cells were stained with FM 4-64 to label the vacuole limiting membrane for 30 min and chased in either SDM-TRP medium for 1 h (growing) or SD-N for 2 h (starvation) before imaging. DIC, differential interference contrast. Scale bar: 5 µm.

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Chapter IV

Conclusions and Future Directions

The work presented in this dissertation identifies Atg42/Ybr139w as a functional homolog of the vacuolar serine carboxypeptidase Prc1 and sheds new light on the overlapping functions of these proteases in the activation of vacuolar zymogens and breakdown of autophagic bodies. These new findings also raise interesting questions and provide grounds for future studies.

Comparative studies of Atg42/Ybr139w and Prc1

There is clear functional redundancy between Prc1 and Atg42/Ybr139w. As described in chapter III, only when cells are lacking both of these proteins are defects in Prb1 processing, Ape1 synthesis, and autophagic body breakdown observed. Additionally, whereas deletion of either gene reduces synthesis of phytochelatin peptides and the breakdown of glutathione (GSH), only when both are deleted is there complete abrogation of these two functions (Wünschmann *et al.*, 2007; Wünschmann *et al.*, 2010). It is as yet unclear why there are two vacuolar serine carboxypeptidases, Prc1 and Atg42/Ybr139w. Continued comparative studies will help to further define both their redundant and distinct intracellular functions.

Prc1 has broad substrate specificity, but preferentially cleaves between hydrophobic amino acids (Hayashi, 1976; Jung *et al.*, 1999). The substrate specificity of Atg42/Ybr139w is currently unknown. In chapter III, I described the results of an *in vitro* carboxypeptidase Y (CPY) activity assay using the synthetic Prc1 substrate *N*-(3-[2-furyl]acryloyl)-Phe-Phe-OH (FA-Phe-Phe-OH) (Caesar and Blomberg, 2004; Gombault *et al.*, 2009). Whereas cells lacking

PRC1 failed to degrade the synthetic substrate, deletion of *ATG42/YBR139W* had no effect. This may indicate that Atg42/Ybr139w has different substrate specificity than Prc1. *S. cerevisiae* has one additional serine carboxypeptidase, Kex1, a Golgi-localized integral membrane protein (Cooper and Bussey, 1989, 1992), that may provide a clue as to the substrate specificity of Atg42/Ybr139w. While Kex1 has two tracts of homology to Prc1, including the active site serine, Kex1 has different substrate specificity, preferentially cleaving between basic Arg and Lys residues (Dmochowska *et al.*, 1987; Cooper and Bussey, 1989; Latchinian-Sadek and Thomas, 1993).

Additional clues as to divergent functions may lie in the differences and similarities in transcriptional regulation of the *PRC1* and *ATG42/YBR139W* genes. Comparative analysis of the 800-base pair promoter regions directly upstream of both genes using the YEASTRACT database reveals the presence of predicted binding sites for 32 different predicted transcription factors; 7 are unique to the *PRC1* promoter, 12 are unique to the *ATG42/YBR139W* promoter, and 13 are found in both promoter regions (Teixeira *et al.*, 2014). Here I will discuss several differences of note that may prompt future studies.

Unique to the *PRC1* promoter region are predicted DNA binding sites for the transcription factors Abf1, Stp1, and Stp2. Abf1 is considered a general regulatory factor due to its involvement in a diverse array of regulatory functions, including control of ribosome protein gene expression in response to nutrient starvation or TORC1 inactivation (Fermi *et al.*, 2016, 2017). Additionally, Abf1, Stp1, and Stp2 are involved in induction of Bap3, a branched-chain amino acid permease, in response to extracellular amino acids (de Boer *et al.*, 1998; de Boer *et al.*, 2000).

There are several predicted transcription factor binding sites in the *ATG42/YBR139W* promoter that are of interest. There are 4 sites predicted to be bound by Gcn4, which is responsible for induction of a variety of genes during amino acid starvation, including genes involved in amino acid transport and biosynthesis, peroxisome biogenesis, glycogen synthesis, and autophagy (Natarajan *et al.*, 2001). There is one predicted binding site for Swi5 and its paralog Ace2; activity of Swi5, and likely Ace2, is regulated by Pho85 and Cdc28 (Moll *et al.*, 1991; O'Conallain *et al.*, 1999; Measday *et al.*, 2000), both of which also regulate glycogen synthesis and autophagy (François and Parrou, 2001; Wang *et al.*, 2001). Of particular interest is a Yap1-binding site in the *ATG42/YBR139W* promoter region, which further supports a role for Atg42/Ybr139w in GSH catabolism and phytochelatin synthesis, which occurs in response to exposure to heavy metals (Wünschmann *et al.*, 2007; Wünschmann *et al.*, 2010). Yap1-deficient cells are hypersensitive to cadmium (Wu *et al.*, 1993). Transcriptional regulation of genes encoding both Gsh1 (γ-glutamylcysteine synthetase), which is involved in the first step of GSH biosynthesis (Kistler *et al.*, 1990), and Ycf1, which imports glutathione-*S*-conjugates into the vacuolar lumen (Li *et al.*, 1996), is dependent on Yap1 and confers cadmium tolerance (Wemmie *et al.*, 1994; Wu and Moye-Rowley, 1994). Yap1 also regulates *GSH1* transcription in response to depletion of the GSH pool (Wheeler *et al.*, 2003), H_2O_2-induced oxidative stress (Stephen and Jamieson, 1997), and to some degree in response to arsenic stress (Menezes *et al.*, 2008). *YCF1* induction in response to arsenic stress is also Yap1-dependent (Menezes *et al.*, 2004). Interestingly, *ATG42/YBR139W* expression is also upregulated in response to arsenate treatment (Menezes *et al.*, 2008).

Whether the transcription factors discussed here do in fact regulate transcription of *PRC1* or *ATG42/YBR139W* has yet to be determined.

Zymogen activation cascade

While previously thought to be an autocatalytic event (Mechler *et al.*, 1988; Nebes and Jones, 1991), the work presented in chapter III demonstrates that the presence of either Prc1 or Atg42/Ybr139w is required for efficient processing of Prb1 from an intermediate form to the mature enzyme. However, it is also known that Prc1 maturation depends on Prb1 function, as an intermediate form of Prc1 accumulates in Prb1-deficient cells (Mechler *et al.*, 1987). More work must be done to fully dissect the sequence of events leading to processing and maturation of both Prb1 and Prc1. Prb1 processing is not fully blocked in the *atg42/ybr139w*Δ *prc1*Δ strain. What accounts for this residual processing? Is it Prb1-mediated, or is another protease involved? Is it this residual activity that allows for Prc1 processing? Does this Prc1 processing lead to more processing of Prb1, beginning a processing amplification loop between Prb1 and Prc1? Is Ybr139w also proteolytically activated and if so, how does this factor in to the Prb1-Prc1 processing relationship?

Overall, these findings and questions encourage further characterization of the entire zymogen processing and activation cascade within the vacuole. As Prb1 processing is impaired in the *atg42/ybr139w*Δ *prc1*Δ strain, what effects will be seen on Prb1-dependent processing targets such as Pho8 and Ape3 in this strain (Yasuhara *et al.*, 1994; Merz and Wickner, 2004)? Ppn1 activity is also dependent on proteolytic activation, as there is no Ppn1 activity in cells lacking Pep4, Prb1, and Prc1 (Sethuraman *et al.*, 2001), but the protease(s) directly responsible for this activation have yet to be determined. Also, for many vacuolar zymogens, it is not clear whether processing by Pep4 and/or Prb1 is direct or indirect and whether additional proteases may be involved.

Lysis of autophagic bodies and effects on substrate degradation

Lysis of autophagic bodies within the vacuole lumen allows autophagic cargo to be broken down by the vast array of hydrolases present in the vacuole. Cells lacking Pep4 or Prb1 show an accumulation of autophagic bodies in the vacuolar lumen upon nitrogen starvation (Takeshige *et al.*, 1992). Both mitochondria and peroxisomes, cargoes of selective macroautophagy, can be observed within intact autophagic bodies in cells defective for autophagic body breakdown (Epple *et al.*, 2003; Okamoto *et al.*, 2009). RNA degradation can occur in the vacuole in an autophagy-dependent manner (Frankel *et al.*, 2017). In *pep4*Δ *prb1*Δ cells, the breakdown to nucleosides is impaired (Huang *et al.*, 2015). It is unclear whether this is due to failure of autophagic body breakdown, failure to process the vacuolar phosphatase Pho8 to its active form (Klionsky and Emr, 1989), or a combination of both.

The results presented in chapter III indicate that autophagic body breakdown is also defective in cells lacking Prc1 and Atg42/Ybr139w, and that this has an effect on amino acid recycling and protein synthesis. It is therefore likely that deletion of both *PRC1* and *ATG42/YBR139W* will also affect breakdown of other autophagic substrates, both selective and non-selective. Alongside investigating Prc1- and Atg42/Ybr139w-dependent breakdown of additional autophagic cargoes, it may be worthwhile to investigate whether other vacuolar proteases have effects on autophagic body breakdown in conjunction with the further characterization of the zymogen activation cascade.

Whereas Pep4, Prb1, Prc1, and Atg42/Ybr139w all have a role in autophagic body breakdown, it is unlikely that they are acting on the autophagic bodies themselves. It is more likely that they are acting through the lipase Atg15, which is also required for breakdown of Cvt bodies and autophagic bodies (Epple *et al.*, 2001; Teter *et al.*, 2001). It is currently unknown

how Atg15 is regulated within the vacuole. However, as Pep4, Prb1, Prc1, and Atg42/Ybr139w have been shown to have roles in vacuolar zymogen activation, it may be that Atg15 is also synthesized as a zymogen that depends on these proteases (and possibly others) for proper activation. Further study of the regulation and potential proteolytic activation of Atg15 will be important for understanding the terminal events of autophagy.

In studies of lipophagy, it was demonstrated that breakdown of lipid droplets is largely dependent on Atg15, but that lipase activity is not completely abrogated in Atg15-deficient cells (van Zutphen *et al.*, 2014). It will therefore be of interest to determine the identity of other vacuolar lipases and characterize their roles in turnover of lipid droplets as well as other autophagic cargoes.

Characterization of additional proteases

The work presented in this dissertation indicates that many important biological functions of vacuolar proteases may have been obscured in the past due to the presence of uncharacterized or poorly characterized homologs. Using BLAST (Basic Local Alignment Search Tool) to search for possible homologs of Cps1 identified Yol153c, a predicted integral membrane metallocarboxypeptidase, which has approximately 56.9% amino acid sequence identity with Cps1 (The UniProt Consortium, 2017). A BLAST search for Prb1 homologs identified Ysp3, which has 58.4% identity with Prb1 and is a predicted serine endopeptidase (Finn *et al.*, 2017; The UniProt Consortium, 2017). Localization of Ysp3 in large-scale studies has been reported in both the endoplasmic reticulum and vacuolar lumen (Sarry *et al.*, 2007; Yofe *et al.*, 2016); however, the GFP tag used to visualize the protein at the ER was fused to the N terminus (Yofe *et al.*, 2016), which is predicted to contain a signal sequence, perhaps preventing Ysp3 from reaching its intended location; thus, further study is required. While it does not arise via BLAST

search, Dap2 has homology to Ste13, a protease which cycles between the *trans*-Golgi network and endosomes, is also annotated as being a vacuolar membrane protein, and is involved in proteolytic activation of α-factor (Fuller *et al.*, 1988; Johnston *et al.*, 2005; The UniProt Consortium, 2017). Further study will be required to understand the relationship between Dap2 and Ste13.

As mentioned in chapter I, there are several predicted vacuolar proteases that have yet to be characterized. Pff1 is a vacuolar membrane protein with a predicted metalloprotease domain facing the vacuolar lumen (Hecht *et al.*, 2013). Ecm14 also localizes to the vacuole and is a predicted zinc-dependent carboxypeptidase (Huh *et al.*, 2003; The UniProt Consortium, 2017). For both of these proteins, activity, substrate specificity, proteolytic processing, and biological function have yet to be determined. Ynl115c localizes to the vacuolar membrane and has a predicted α/β-hydrolase fold (Huh *et al.*, 2003; Finn *et al.*, 2017). This fold is found in a large family of structurally related enzymes with wide-ranging functions including lipases and a variety of peptidases (Holmquist, 2000). Further study will be required to determine the function of Ynl115c. There are also several large-scale studies that identify other potential proteins of the vacuolar membrane and lumen that may warrant investigation (Sarry *et al.*, 2007; Wiederhold *et al.*, 2009).

Final Perspectives

The actions of vacuolar/lysosomal hydrolases and transporters are critically important in maintaining cellular health and survival, yet there are many existing gaps in our knowledge of these enzymes and their intracellular functions, especially concerning vacuolar proteolysis. It is my hope that the work presented herein will increase understanding of vacuolar proteases and reinvigorate efforts to reexamine and characterize these proteins, as well as the mechanisms of

vacuolar substrate degradation and efflux, in order to further our knowledge of vacuolar function.

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