CHARACTERIZATION OF YBR074 (PFF1), A CONSERVED VACUOLAR MEMBRANE METALLOPROTEASE FAMILY MEMBER

by

Karen Alice Hecht

H.B.Sc. Biochemistry, University of Toronto, Toronto Canada, 2006

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This dissertation was presented

by

Karen Alice Hecht

It was defended on

July 27, 2012

and approved by

Meir Aridor, Associate Professor, Cell Biology and Physiology

Karen M. Arndt, Professor, Biological Sciences

Michael Grabe, Assistant Professor, Biological Sciences

Joseph A. Martens, Assistant Professor, Biological Sciences

Committee chair: Jeffrey L. Brodsky, Professor, Biological Sciences
In yeast, the vacuole is the principal intracellular compartment associated with protein degradation. The vacuole acts as a buffering organelle that accumulates nutrients in times of plenty, and releases them into the cytosol during periods of nutrient starvation. This important biological function is mediated by vacuolar proteases, which exhibit a variety of conserved catalytic mechanisms. Metalloproteases represent one of the most diverse classes of proteases, and they are defined by a characteristic dependence on coordinated metal ions for their catalytic activity.

In higher eukaryotes, metalloproteases are associated with both intracellular homeostasis and remodeling of the extracellular environment. In humans, remodeling of the extracellular matrix is mediated by secreted matrix metalloproteases regulating cell motility during development and wound healing, and also serving as markers of cancer metastasis. Within the cell, metalloproteases play a major role in the maturation and trafficking of proteins, as well as in the turnover of long-lived, superfluous, or damaged proteins and organelles.

This dissertation represents the first characterization of the putative yeast metalloprotease Ybr074, which is named herein, protein in FXNA-related family (Pff1). Pff1 is a member of the conserved family of M28 metalloproteases, which includes the mammalian ortholog, FXNA. FXNA has been reported to be localized in the endoplasmic reticulum (ER), and is expressed in
multiple tissues in rats, where it has been implicated in ovarian development. This dissertation shows that, unlike the ER-localized FXNA, Pff1 is a vacuolar protein. This finding is in agreement with extensive data, presented herein, demonstrating that Pff1 is not involved in protein quality control in the ER. However, genetic and chemical-genetic analyses suggest that Pff1 may have a role in yeast cell wall maintenance. Finally, this dissertation describes proteomic approaches employed in an attempt to identify endogenous substrates of Pff1, and outlines additional strategies aimed at defining the biological function of this novel vacuolar protease family member.
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PREFACE

What’s in a name? One would hope to find a lot of meaning in a name. Pffl is a name that is drawn from the comparison of related protein sequences. Although none of the members of the Brodsky lab are related (as far as I know), they have certainly come to feel like family over the course of my six years as a graduate student, and as lab mates go, they are beyond compare. So, I’ll take this space to thank them in writing, so that they know I really mean it.

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1.0 INTRODUCTION

The characterization of Ybr074 was initiated when YBR074 was found to be induced in yeast cells exogenously expressing the mammalian cystic fibrosis transmembrane conductance regulator (CFTR). Since CFTR is a multi-domain protein that folds inefficiently, it was hypothesized that novel protein quality factors could be identified among genes upregulated in response to general stress conditions caused by CFTR expression in yeast. Preliminary evidence suggested that a yeast strain in which YBR074 was partially disrupted, exhibited stabilization of CFTR (Ahner & University of Pittsburgh. School of Arts and Sciences, 2005). Since Ybr074 was computationally predicted to be an ER-localized protein with homology to a family of metalloproteases, it was hypothesized that it may play a role in the degradation of CFTR, and perhaps other quality control substrates.

The results presented in this dissertation show that the putative protease Ybr074 is not involved in CFTR quality control. Furthermore, this work demonstrates that Ybr074 is localized to the vacuole. In order to understand what role Ybr074 may be fulfilling in the vacuole, this chapter will focus on the various biological functions associated with the vacuole and the proteases found therein. The chapter concludes with an overview, in which I describe the significance and goals of my research, and outline the content of each chapter in this dissertation.
1.1 STRUCTURE AND FUNCTION OF THE YEAST VACUOLE

The yeast vacuole is a prominent organelle that can occupy ~20% of the cell volume. It is functionally similar to the plant vacuole and the lysosome of higher eukaryotes, with an important role in degradation, storage, homeostasis, and detoxification. (Broach et al, 1991; Li & Kane, 2009)

The vacuole was first described in the 1930’s from light microscopy images of plant cells showing large “empty” (vacuus, in Latin) structures within the cytosol (Marty, 1999). However, the vacuole in yeast is host to numerous proteases, lipases, nucleases, and transporters (Broach et al, 1991; Klionsky et al, 1990; Veses et al, 2008).

The vacuole is a dynamic organelle that undergoes fusion and fission events in response to the cell cycle, and to environmental cues. Logarithmically growing cells have a multi-lobed vacuole, while in stationary phase cells, or cells starved of a carbon source, these lobes fuse to produce a single round vacuolar structure (Wiemken et al, 1970).

The vacuole maintains a slightly acidic environment (pH 6.2), compared to the cytosolic pH 7.2 in logarithmically growing yeast (Orij et al, 2009; Preston et al, 1989). This proton gradient is generated by the vacuolar ATPase, V-type ATPase, which pumps protons into the vacuolar lumen. In addition to decreasing the pH of the vacuole, the V-ATPase also generates a membrane potential of 75 mV, vacuole interior positive (Bowman & Bowman, 1986; Kakinuma et al, 1981). This potential difference is maintained by cation channels and chloride transport (Klionsky et al, 1990). These transporters include the Na⁺/H⁺ and K⁺/H⁺ cation exchanger Nhx1, and the chloride transporter Gef1 (Braun et al, 2010; Qiu, 2012).

The vacuole also has a characteristic lipid composition. Ergosterol and sphingolipid levels increase along the secretory pathway, with the plasma membrane having the highest
proportion of both these lipids and the ER having the lowest levels (Zinser et al, 1993). However, the vacuole has a relatively low ergosterol to phospholipid ratio and a reduced concentration of sphingolipids (Zinser et al, 1991). Regulating the lipid composition of the vacuolar membrane is essential to its function. In particular, phosphoinositides, ergosterol, and diacylglycerol regulate proteins that mediate vacuolar fusion events. For example, misregulation of vacuolar phosphoinositides disrupts vacuolar fusion and results in fragmented vacuoles (Fratti et al, 2004; Li & Kane, 2009).

A specific host of vacuolar proteins also define the function of this organelle. Approximately 200 of the 6000 yeast genes are annotated as encoding proteins having vacuolar localization, and 27% of those are described as having transporter function (Li & Kane, 2009). The remaining proteins comprising the vacuole possess functions in macromolecule hydrolysis, membrane fusion, protein sorting and targeting to the vacuole, acidification, and cell wall biogenesis (Wiederhold et al, 2009). A proteomic study found five cell wall biogenesis proteins enriched in the vacuole, which are thought to be targeted to the vacuole for degradation. These are Chs2, involved in chitin synthesis; Gas1 and Fks1, involved in β-glucan synthesis; Bgl2, a β-glucanase; and Yjl171, a glycosylphosphatidylinositol- (GPI) anchored cell wall protein of unknown function (Wiederhold et al, 2009).

Most proteins are targeted to the vacuole via the secretory pathway. The secretory pathway relays proteins and lipids to the subcellular compartment where their function is required. Secretory proteins are translated in the cytosol by ribosomes and are post-translationally or co-translationally inserted into the endoplasmic reticulum (ER) via the Sec61 protein channel known as the translocon (Marie et al, 2008; Mellman & Warren, 2000). Secretory proteins that are translocated co-translationally bear a signal sequence peptide with a
characteristic stretch of 8-20 hydrophobic residues that are recognized by the Signal Recognition Particle (SRP) associated with the ribosome (Janda et al., 2010). The SRP-ribosome nascent chain complex docks at the translocon, at which point soluble proteins are inserted into the ER lumen, while transmembrane proteins are inserted into the membrane through a lateral gating mechanism of the translocon (Alder & Johnson, 2004; Egea & Stroud, 2010). Once in the ER, nascent proteins fold with the assistance of chaperones, including those in the heat shock protein (Hsp) 70 family and Hsp40 family, and undergo post-translational modifications, including proteolytic cleavage of the signal peptide, disulphide bond formation, and addition of carbohydrate residues (Braakman & Bulleid, 2011; Hebert & Molinari, 2007).

Once secretory proteins have folded correctly in the ER, they are packaged into coat protein complex-II (COPII) vesicles for delivery to the Golgi. The COPII complex is made up of the Sar1 GTP-binding protein, the Sec23-Sec24 complex, and the Sec13-Sec31 complex (Hughes & Stephens, 2008). Transmembrane cargo can bind directly to COPII-coat proteins via a cytosolic ER exit signal located at the C-terminus of the cargo protein. The ER exit signal can be a di-hydrophobic sequence such as Phe-Phe, Tyr-Try, Ile-Ile, or Leu-Leu. The ER exit signal might also be comprised of the di-acidic motif Asp/Glu-Xaa-Asp/Glu (where Xaa is any amino acid). Other types of ER exit signals have also been characterized, including Phe-Tyr, Leu-Val, Ile-Leu, Phe-Xaa, Xaa-Val, and Tyr-Xaa-Xaa-Phi (where Phi is a bulky hydrophobic amino acid) (Nufer et al., 2002; Watanabe & Riezman, 2004). It is important to note these C-terminal ER exit signal are not always sufficient for vesicle budding, and that other signals within the cargo protein may be required in combination with a C-terminal signal to direct ER exit (Otte & Barlowe, 2002). Furthermore, soluble protein cargos require membrane-spanning adaptor
proteins to interact with the cytosolic COPII coat. These adaptor proteins, or cargo receptors, include Erv29 and Erv14 (Sato & Nakano, 2007).

Vesicle fusion to the Golgi is mediated by soluble n-ethylmaleimide sensitive factor adaptor protein receptor (SNARE) proteins that form a complex in trans between the vesicle-specific SNAREs (v-SNAREs) and the target membrane-specific SNAREs (t-SNAREs) of the Golgi (Brittle & Waters, 2000). Once cargo has been successfully delivered to the Golgi, the SNARE complex is disassembled by the dissociation chaperones Sec17 and Sec18, which in mammalian cells are SNAP (Soluble NSF-Attachment Protein) and NSF (N-ethylmaleimide-Sensitive Factor), respectively (Brittle & Waters, 2000). COPI vesicles direct retrograde transport of v-SNAREs back to the ER. The COPI coat also retrieves other factors from the Golgi, such as the ER chaperones Kar2 and Pdi1, by recognition of an ER-retention signal: KDEL for soluble ER-resident proteins, and KKXX for transmembrane ER-resident proteins (Duden, 2003).

In the Golgi, carbohydrate residues of secretory proteins are modified and extended, particularly on secretory proteins destined for the cell wall (Goto, 2007; Yan & Lennarz, 2005). It is at this point, in the Golgi, that cargo proteins reach a cross-road in the secretory pathway, and can be retained in the Golgi, targeted to the plasma membrane, or targeted to the vacuole. The adaptor protein (AP) 1 complex is involved in targeting cargo to endosomes (Valdivia et al, 2002). The AP-3 complex is more specific for delivery of cargo from the Golgi to the vacuole (Cowles et al, 1997).

The principle routes described for vacuolar sorting of secretory proteins are known as the carboxypeptidase Y (CPY) pathway and the alkaline phosphatase (ALP) pathway (Bowers & Stevens, 2005). The CPY pathway targets proteins to the pre-vacuolar compartment, also known
as the multivesicular body (MVB), which subsequently fuses with the vacuole. The CPY pathway involves sorting into vesicles associated with clathrin and adaptor proteins Gga1 and Gga2 (Bonifacino, 2004; Bowers & Stevens, 2005). The ubiquitin ligase, Rsp5, appends a K63-linked polyubiquitin signal on cargo, which ensures targeting to the MVB instead of the plasma membrane (Lauwers et al, 2010). This is achieved by association with the ubiquitin-binding endosomal sorting complex required for transport (ESCRT) complexes (Van Den Hazel et al, 1996). These vesicles are subsequently tethered to the MVB by a multi-subunit tethering complex (MTC) known as the homotypic fusion and vacuole protein sorting (HOPS) complex (Brocker et al, 2012). The HOPS complex, together with the Rab family GTPase, Ypt7, and SNARE proteins direct vesicle fusion to the MVB, and fusion of the MVB to the vacuole (Lachmann et al, 2011). In contrast, the ALP pathway bypasses the MVB completely, such that vesicles budding from the Golgi are targeted directly to the vacuole by AP-3 complex in a clathrin-independent manner (Bowers & Stevens, 2005). These pathways are summarized in Figure 1.
Figure 1: Protein trafficking pathway and vacuolar sorting pathways

Cellular compartments are labeled in black and secretory pathways discussed in this chapter are highlighted and color coded. Of particular note are the vacuolar sorting pathways: the CPY pathway, shown in light blue; the ALP pathway, shown in green; and the Cvt pathway, shown in brown. This image was adapted from Bowers et al. 2005.
1.1.1 Proteolysis

The vacuole is host to numerous exopeptidases and endopeptidases that contribute to what is often considered the vacuole’s principal function, protein degradation (Van Den Hazel et al, 1996). The vacuole maintains protein homeostasis under physiological conditions by degrading senescent, superfluous, and damaged proteins and organelles. It is also important under conditions of nutrient stress, when cell growth and proliferation are down-regulated, and macromolecules are broken down in the vacuole so that their constituent amino acids can be recycled (Thumm, 2000). Degradation of up to ~85% of the cell’s intracellular protein content has been observed during nutrient starvation (Teichert et al, 1989). Such nutrient stress is encountered when cells reach stationary phase, or when they are undergoing sporulation. Some of the cues known to trigger nutrient stress include carbon and nitrogen starvation, and to a lesser extent a lack of essential amino acids, nucleotides, and sulphates (Mizushima, 2007; Takeshige et al, 1992). This section discusses the various degradation functions attributed to the vacuole.

1.1.1.1 Autophagy

Autophagy, also known as macroautophagy, is a process whereby bulk cytoplasmic material and organelles are isolated in a double-membrane vesicle known as an autophagosome (Mari et al, 2011). The autophagosome fuses with the vacuole, where its contents are degraded and their constituent parts recycled. This mechanism is conserved throughout eukaryotes, and is used to maintain cellular homeostasis under physiological conditions by eliminating long-lived proteins and damaged organelles. Autophagy can also be induced under conditions of nutrient stress, and
during specific stages in the cell cycle such as in stationary phase or during sporulation (Hopper et al., 1974; Reggiori & Klionsky, 2002). Autophagy is mediated by over 30 autophagy-related proteins, known as Atg proteins encoded by ATG genes (Xie & Klionsky, 2007).

Autophagy is described in a series of sequential steps: induction, cargo recognition, vesicle nucleation, expansion and completion of the autophagosome, Atg protein recycling, fusion of the autophagosome with the vacuole, vesicle breakdown, and macromolecule recycling (Figure 2).

![Figure 2: Steps mediating autophagy.](image)

A) The phagophore assembly site (PAS) recruits cargo and Atg proteins required to extend the vesicle membrane to form a B) phagophore, which closes to form a double membrane vesicle called the C) autophagosome, which D) fuses to the vacuole, resulting in an internalized single membrane vesicle called the E) autophagic body. Ultimately, the autophagic membrane is broken down, releasing its contents into the vacuolar lumen for degradation.

In yeast, autophagy is regulated by the protein kinase known as Target of rapamycin (Tor). Under nutrient-rich conditions, a Tor-containing complex (TORC1) is active and inhibits autophagy. During nutrient limitation, TORC1 is inactive, resulting in induction of autophagy (Mizushima, 2007). Inactivation of TORC1 allows the Atg1 kinase to associate with
hypophosphorylated Atg13 and Atg17. Formation of the Atg1-Atg13-Atg17 kinase complex increases the activity of Atg1. Although the substrate of Atg1 has not been identified, formation of this complex is associated with increased levels of autophagy (He & Klionsky, 2009).

Once autophagy has been induced, Atg proteins begin to accumulate at the Phagophore Assembly Site (PAS). In yeast, the PAS is found adjacent to the vacuole, and acts as a nucleation site for recruitment of Atg proteins, cargo, and lipids needed to form the phagophore. This event is marked by accumulation of the transmembrane protein, Atg9, which is a core member of the autophagy machinery and is conserved amongst eukaryotes (Suzuki et al, 2001). The scaffold proteins Atg11 and Atg17 also localize to the PAS and facilitate its expansion by recruiting other Atg proteins (Kabeya et al, 2009; Xie & Klionsky, 2007).

Another protein with a putative role in PAS nucleation is the lipid kinase Vps34. This phosphatidylinositol 3-kinase forms at least two distinct complexes in yeast, denoted Complex I and II. It is thought that Vps34 produces phosphatidylinositol-3-phosphate at the PAS, which serves as a binding site for Atg proteins containing phox homology (PX) domains, such as Atg20 and Atg24 (Nice et al, 2002). However, the role of these PX domain-containing proteins is not understood.

Expansion of the phagosome is dependent on two ubiquitin-like protein conjugating systems involving the ubiquitin-like proteins Atg12 and Atg8. Based on structural data derived from studies on plant Atg12 and mammalian Atg8, these proteins display a ubiquitin fold at the C-terminus, even though they are not considered homologs based on sequence similarity (Paz et al, 2000; Suzuki et al, 2005). During autophagy, Atg12 is activated by the E1-like protein Atg7, which mediates the transfer of Atg12 to the E2-like protein Atg10. Atg12 is then covalently linked to a lysine residue on Atg5. The Atg12-Atg5 conjugate then associates with Atg16 to form
an Atg12-Atg5-Atg16 ternary complex that coats the outer membrane of the expanding phagophore and is thought to drive membrane curvature and to prevent premature fusion with the vacuole (Yang & Klionsky, 2009).

The second ubiquitin-like conjugating system required for phagosome formation involves the cleavage of the ubiquitin-like protein Atg8 by the cysteine protease Atg4, followed by conjugation of the cleaved Atg8 to phosphatidylethanolamine (PE) by the E2-like enzymes Atg7 and Atg3. Atg8-PE is associated with both the inner and outer membranes of the phagophore and serves to link cargo to the phagophore membrane (Pyo et al, 2012). Intriguingly, Atg8-PE has been shown to interact with Shp1, a co-factor of the AAA+ ATPase Cdc48, which will be discussed in detail in Chapter 2. It is thought that Cdc48 together with Shp1 play an important, though uncharacterized role in phagosome maturation (Dargemont & Ossareh-Nazari, 2012; Krick et al, 2010).

Once expansion of the phagophore is complete the resulting double membrane vesicle enclosing its cargo is referred to as the autophagosome. Atg8-PE on the outer membrane is cleaved by Atg4 and recycled. Other Atg proteins are also recycled at this stage by an uncharacterized mechanism. Fusion of the outer membrane of the autophagosome with the vacuole is mediated by SNAREs, Vam3 and Vam7; the ATPase Sec18; and the Rab-family GTP-binding protein, Ypt7 (Yang & Klionsky, 2009). Once internalized by the vacuole, the cytoplasmic cargo encapsulated by the inner membrane of the autophagosome is referred to as an autophagic body. The membrane of the autophagic body is degraded by the lipase Atg15, and its contents are hydrolyzed by vacuolar proteases (Epple et al, 2001). It is interesting to note that Atg15 is currently the only known vacuolar lipase.
Although autophagy is generally considered to be non-selective, a few forms of selective autophagy have been described. These involve degradation of superfluous or damaged organelles. Unlike non-selective autophagy, phagophores associated with selective autophagy engulf very little cytoplasmic material besides the target organelle. These selective forms of autophagy are known to target the ER, lipid droplets, mitochondria, the nucleus, peroxisomes, and ribosomes, and are referred to as ERphagy, lipophagy, mitophagy, Piecemeal Microautophagy of the Nucleus (PMN), pexophagy, and ribophagy, respectively (Beau et al, 2008; Bernales et al, 2007; Dong & Czaja, 2011; Dunn et al, 2005; Kisko et al, 2007; Kraft et al, 2009; Krick et al, 2008).

Another notable type of selective autophagy is known as the cytoplasm-to-vacuole targeting (Cvt) pathway. Unlike the forms of autophagy discussed previously, the Cvt pathway occurs during nutrient-rich conditions and is a constitutive process (Khalfan & Klionsky, 2002). The Cvt pathway mediates the selective transport of homooligomeric vacuolar proteins. Only three substrates are known to be targeted to the vacuole by this method: aminopeptidase I (Ape1), α-mannosidase I (Ams1), and aminopeptidase 4 (Ape4). Notably, Ape1 and Ams1 are resident vacuolar proteins, while Ape4 is cytosolic under physiological conditions, with Cvt-mediated vacuolar targeting occurring during nutrient starvation conditions (Yuga et al, 2011).

Cvt substrates are synthesized in the cytoplasm as precursors, where they homooligomerize and bind to Atg19. Atg19 acts as a receptor for Cvt substrates and recruits them to the PAS in an Atg11-dependent manner (Baba et al, 1997; Hutchins & Klionsky, 2001; Scott et al, 1997; Yuga et al, 2011). The autophagosomes associated with the Cvt pathway are smaller, and take approximately 10 times longer to form than those associated with autophagy (Kraft et al, 2009).
The Cvt pathway may be an important alternative trafficking mechanism to target vacuolar resident proteins that may otherwise be damaging to secretory pathway residents. Alternatively, the Cvt pathway may facilitate transport of stable oligomeric proteins which are too large to traverse the vacuolar membrane via transporters. The Cvt pathway may also facilitate vacuolar localization of membrane-associated proteins, such as Ams1, which lack a signal sequence to direct their translocation into the secretory pathway (Figure 1) (Hutchins & Klionsky, 2001).

1.1.1.2 Microautophagy

Microautophagy differs from macroautophagy because cargo is engulfed directly by vacuolar membrane extensions and not by de novo synthesis of a phagophore membrane. Microautophagy is constitutively active but can also be induced to higher levels under starvation conditions. Microautophagy targets both cytosolic proteins and organelles. Internalization of cargo is achieved by invagination of the vacuolar membrane, or by protruding vacuolar membrane extensions known as autophagic tubes. The cytosolic material is engulfed and the resulting vacuolar vesicle pinches off and becomes completely internalized by the vacuole. The vesicle and its contents are then degraded, and constituent parts may be recycled. It is thought that the uptake of vacuolar boundary membrane by microautophagy is required to balance the influx of autophagosomal membranes resulting from macroautophagy (Li et al, 2012; Uttenweiler & Mayer, 2008).

1.1.1.3 Endocytosis

Endocytosis represents another pathway targeting proteins to the vacuole for degradation. Endocytosis is a conserved mechanism that mediates protein turnover at the cell surface, nutrient
uptake, and adaptation to the extracellular environment (Boettner et al, 2012). Yeast must both internalize plasma membrane proteins that have become superfluous and deliver new ones to replace them. The most well characterized endocytic pathway is clathrin-mediated endocytosis (CME), which is mediated by ~60 proteins (Weinberg & Drubin, 2012). Until recently, the CME was the only demonstrated mechanism for endocytosis in yeast. However clathrin-independent pathways are beginning to be described as well (Prosser et al, 2011).

The process of endocytosis can be divided into a stationary phase, in which early proteins, early coat proteins, and cargo are recruited, and a mobile phase, in which intermediate and late coat proteins mediate membrane deformation and actin polymerization that drives invagination of the endocytic vesicle. The process is completed by vesicle scission and uncoating. This allows recycling of coat proteins and vesicle fusion with the early endosome (Figure 3).

The mechanism that leads to endocytic site selection is not understood, but is marked by recruitment of early proteins Ede1 and Syp1 to sites of the plasma membrane inner leaflet referred to as eisosomes (Moreira et al, 2009). Syp1 contains an F-BAR domain, which binds lipid and deforms membranes. Ede1 is a ubiquitin-binding protein that plays an important role in the recruitment of proteins involved in later stages of endocytosis. Recruitment of these early proteins is followed by enrichment of endocytic cargo proteins to these sites, and association of early coat proteins including clathrin, and cargo-specific adaptors such as the AP-2 complex and Yap1801/Yap1802. Syp1 also functions as a cargo-specific adaptor for the cell wall stress sensor Mid2 (Boettner et al, 2012).

The subsequent recruitment of intermediate coat proteins brings together machinery that mediates coupling of actin assembly and vesicle formation. For example, the intermediate coat
protein, Sla2, serves as an adaptor linking the plasma membrane, clathrin coat, and actin at the site of endocytosis. The Sla2 N-terminal domain binds phosphoinositol-4,5-bisphosphate (PIP2) at the endocytic membrane, its central coiled-coil region interacts with clathrin light chain, and its C-terminal domain interacts with actin filaments (Sun et al, 2005). Furthermore, a number of late coat protein are recruited, which act as nucleation promoting factors (NPFs) that activate the Arp2/3 complex mediating actin assembly. These NPFs include Pan1, Las17 (mammalian Wiskott-Aldrich syndrome protein; WASP), Myo3, Myo5, and Abp1. Actin polymerization provides a directional pulling force and the tensile strength required to extend the membrane deformation inward, forming an elongated tubule with a clathrin-coated tip (Girao et al, 2008; Weinberg & Drubin, 2012). Although dynamin is essential for scission in mammalian cells, its yeast counterpart, Vps1, it thought to plays a lesser role in this process (Weinberg & Drubin, 2012). It is thought that the combination of actin assembly, membrane deformation by BAR and F-BAR proteins, such as Syp1 and the heterodimeric Rvs161/167 complex, and lipid phase separation between the plasma membrane and endocytic vesicle are the key factors facilitating vesicle scission (Liu et al, 2006; Weinberg & Drubin, 2012). Finally, vesicle uncoating is facilitated by the PIP2 phosphatase Sjl2 and other late coat proteins, such as Lsb5 and Gts1 (Weinberg & Drubin, 2012). The uncoated endocytic vesicle undergoes a series of fusion events with the early endosome, the late endosome, and finally the vacuole. Vesicle fusion is facilitated by Rab GTPases, multisubunit tethering complexes (MTC) and SNAREs (Boettner et al, 2012; Munn et al, 1999).
Figure 3: Schematic of endocytosis

Early, early coat protein and cargo are recruited to endocytic sites. Intermediate and late coat proteins, as well as actin remodeling proteins assemble. Invagination of the endocytic vesicle is mediated by actin assembly, as well as BAR and F-BAR proteins. The endocytic vesicle undergoes scission and uncoating. This diagram was adapted from Weinberg J. et al. 2012.
1.1.2 Trehalose metabolism

Trehalose is a disaccharide that accumulates in yeast during stationary phase, and in response to various stress conditions, including salt stress, heat or cold stress, oxidative stress, and dehydration (Garre & Matallana, 2009; Nwaka & Holzer, 1998). Trehalose is thought to act as a protectant of membranes under conditions of stress by forming hydrogen bonds with lipid polar head groups, and maintaining appropriate spacing between lipid acyl chains. This mechanism of trehalose-mediated membrane protection is known as the water replacement hypothesis (Golovina et al, 2009). Under conditions of heat stress, trehalose associates with and stabilizes proteins, and prevents misfolded proteins from aggregating. Furthermore, recovery of cells after heat stress is associated with rapid degradation of trehalose, which is thought to be required in order to allow Hsps access to misfolded proteins in order to facilitate their re-folding (Singer & Lindquist, 1998).

Degradation of trehalose into two glucose molecules is mediated by the cytosolic trehalases, Nth1 and Nth2, as well as the vacuolar trehalase, Ath1 (Garre & Matallana, 2009). Although Ath1 is considered a vacuolar enzyme, whose activity is optimal at acidic pH, some evidence suggests Ath1 is also found in the periplasmic space (Jules et al, 2004). Although a role for Ath1 in trehalose degradation after stress has not been ruled out, it is thought that the principal role of Ath1 in trehalose hydrolysis is its utilization as a carbon source. In fact, an ath1Δ strain was unable to grow on media in which trehalose was the sole carbon source (Nwaka et al, 1996). However, ATH1 was also induced by the high osmolarity glycerol (HOG) pathway.
under conditions of salt stress, suggesting that a role for Ath1 in stress tolerance may yet be discovered (Garre & Matallana, 2009).

1.1.3 Other functions of the vacuole

In addition to proteolytic functions, the vacuole has other important roles in the maintenance of cellular homeostasis. The vacuole acts as a storage compartment for amino acids, phosphates, Ca\textsuperscript{2+}, and metal ions (Li et al, 2012). It stores basic and neutral amino acids at particularly high levels in order to buffer a decrease in cytosolic levels of amino acids arising during nutrient starvation (Broach et al, 1991; Li et al, 2012). The vacuole stores inorganic phosphate and polyphosphate, which are used in the synthesis of nucleotides, and phospholipids (Shirahama et al, 1996). Polyphosphates may also help regulate vacuolar pH by releasing protons when hydrolyzed. They also play an important role as counterions, facilitating the retention of basic amino acids and positively charged ions, such as Ca\textsuperscript{2+}. Calcium ATPase and Ca\textsuperscript{2+}/H\textsuperscript{+} transporters facilitate calcium storage in the vacuole, and regulate cytosolic levels of this important signaling molecule. Ca\textsuperscript{2+} mediates the cell wall stress response, release from pheromone-induced cell cycle arrest, and the salt stress response (Pittman, 2011; Rusnak & Mertz, 2000). The vacuole also stores other physiologically relevant ions such as Mg\textsuperscript{2+} and Zn\textsuperscript{2+}, which may have deleterious effects on the cell if allowed to accumulate in the cytosol (Klionsky et al, 1990). The vacuole sequesters toxic compounds, such as Cd\textsuperscript{2+}, Co\textsuperscript{2+}, and Pb\textsuperscript{2+}, in the form of glutathione S-conjugates (Broach et al, 1991). Interestingly, a screen for genes required for transition metal tolerance revealed 18 genes required for tolerance on all six metals tested in this screen: Zn\textsuperscript{2+}, Ni\textsuperscript{2+}, Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, Cu\textsuperscript{2+}, and Co\textsuperscript{2+}. These included subunits of the V-ATPase, an ER-resident ATPase assembly factor, Vma21, and members of the Regulator of the H\textsuperscript{+}-ATPase of the
Vacuolar and Endosomal membranes (RAVE) complex, which is involved in assembly of the V-ATPase. Factors involved in vacuolar protein sorting, such as the Rho family GTPase Ypt7, and the phosphatidylinositol kinases Vps34 and Fab1, were also isolated (Bleackley et al, 2011). These findings highlight the importance of vacuolar sequestration in mediating tolerance to metals. Finally, cross-talk between the yeast vacuole and mitochondria has been shown to regulate apoptosis in response to external pro-apoptotic signals such as acetic acid (Sousa et al, 2011). This process is thought to be mediated by translocation of Pep4 from the vacuole into the cytosol as a result of vacuolar membrane permeabilization (Mason et al, 2005; Schauer et al, 2009). Pep4 release from the vacuole appears to protect cells from apoptosis by facilitating the removal of damaged mitochondria by an uncharacterized, and autophagy-independent mechanism (Pereira et al, 2010).
1.2 VACUOLAR PROTEASES

The yeast vacuole contains seven known vacuolar proteases, only one of which is a transmembrane protease. Of these, three are metalloproteases, three are serine proteases, and one is an aspartyl protease. The vacuolar proteases include three aminopeptidases, two carboxypeptidases, and two endopeptidases. This section describes the known characteristics and function of these proteases, which are summarized in Table 1.

Table 1: *Saccharomyces cerevisiae* vacuolar proteases

<table>
<thead>
<tr>
<th>Protease</th>
<th>Gene</th>
<th>Activity</th>
<th>Proteolytically activated by</th>
<th>Trafficking pathway</th>
<th>Function</th>
<th>Known P1 site amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase A</td>
<td>PEP4</td>
<td>aspartyl</td>
<td>Pep4 and Prb1</td>
<td>secretory</td>
<td>initiation of protease activation cascade;</td>
<td>Phe, Leu, Tyr, Trp, Thr, Asn, Gln, Glu, Lys, Ala, Ile</td>
</tr>
<tr>
<td></td>
<td></td>
<td>endopeptidase</td>
<td></td>
<td></td>
<td>protein degradation</td>
<td></td>
</tr>
<tr>
<td>Proteinase B</td>
<td>PRB1</td>
<td>serine</td>
<td>Pep4 and Prb1</td>
<td>secretory</td>
<td>protease activation; protein degradation</td>
<td>Leu, Arg, Phe, Tyr, Gln, Lys</td>
</tr>
<tr>
<td></td>
<td></td>
<td>endopeptidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase Y</td>
<td>PRC1</td>
<td>serine</td>
<td>Pep4 and Prb1</td>
<td>CPY pathway</td>
<td>peptide degradation</td>
<td>Ala, Gly, Val, Leu, Ile, Met, Phe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carboxypeptidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Carboxypeptidase S</td>
<td>CPS1</td>
<td>zinc</td>
<td>Prb1</td>
<td>CPY pathway</td>
<td>peptide degradation</td>
<td>Gly, Leu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>metalloprotease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase I</td>
<td>APE1</td>
<td>zinc</td>
<td>Prb1</td>
<td>Cvt pathway</td>
<td>glutathione degradation</td>
<td>Leu, Cys/Gly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>metalloprotease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase Y</td>
<td>APE3</td>
<td>metalloprotease</td>
<td></td>
<td>secretory</td>
<td>unknown</td>
<td>Pro, Ala, Leu, Met, Phe, Tyr, Ser, Lys, Arg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipeptidylaminopeptidase B</td>
<td>DAP2</td>
<td>serine</td>
<td>none</td>
<td>CPY pathway</td>
<td>unknown</td>
<td>Xaa-Ala, Xaa-Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dipeptidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The MEROPS peptidase database (http://merops.sanger.ac.uk) has classified known proteases into evolutionarily related groups, which will be referenced in the following discussion of vacuolar proteases. A protease clan refers to proteases derived from a single common
ancestor. Clans are subdivided into families. A protease family refers to a sub-group of proteases that share sequence similarity, either throughout the entire protein sequence or only within the catalytic domain (Rawlings & Barrett, 1993). Protease families are assigned a letter referring to the catalytic mechanism of the protease: M for metalloprotease, S for serine protease, and A for aspartyl protease. The catalytic designation is followed by a numeric identifier for the family (Rawlings et al, 2012). Another important nomenclature refers to the site of cleavage of the substrate. The amino acid on the N-terminal side of the hydrolyzed peptide bond is referred to as P1, while the amino acid on the C-terminal side is referred to as P1’. Similarly, the site on the proteolytic enzyme known to bind to the P1 residues is referred to as S1, while the site recognizing the P1’ residue is referred to as S1’.

Briefly, serine proteases comprise a large family whose enzymatic activity is mediated by a characteristic “catalytic triad” consisting of Asp, His, and Ser. The members, substrates and catalytic mechanism of the serine type proteases is reviewed in Hedstrom et al. (Hedstrom, 2002). Metalloproteases depend on metal ions, especially Zn$^{2+}$, for their catalytic function, and are discussed in detail in Section 1.3 (Rawlings & Barrett, 1995). The aspartyl proteases are characterized by two catalytic Asp residues mediating an acid-base hydrolysis reaction (Tang & Wong, 1987). The structure and function of aspartyl proteases was reviewed by Dunn (Dunn, 2002).

1.2.1 Soluble vacuolar proteases.

Proteinase A (PrA), encoded by the PEP4 gene, is a monomeric 42 kDa aspartyl endoprotease of the A1 family of proteases. PrA is targeted to the vacuole via the secretory pathway (Broach et al, 1991; Van Den Hazel et al, 1996; Westphal et al, 1996). It is a key vacuolar enzyme in the
vacuolar protease activation cascade. Many vacuolar hydrolases, including PrA, are initially produced as inactive precursor forms, known as zymogens (van den Hazel et al, 1992). Zymogens are not activated until they have been delivered to the vacuole where an inhibitory propeptide is removed by proteolysis. This allows hydrolase activity to be sequestered within the vacuole, and protects substrates outside the vacuole from proteolytic damage. The vacuolar proteases, proteinase B (PrB), carboxypeptidase Y (CPY), and aminopeptidase I (API), which are discussed in detail below, all depend on PrA for proteolytic activation, and PrA mutant strains are deficient in the activity of these proteases (Van Den Hazel et al, 1996).

PrA is initially synthesized as an inactive precursor referred to as preproPrA. PreproPrA is 405 amino acids in length and has a molecular mass of 52 kDa. PreproPrA has a hydrophobic 22 amino acid signal sequence that directs its translocation into the ER, where it is modified by N-linked glycans at Asn67 and Asn266 (Mechler et al, 1982; Parr et al, 2007). The signal peptide is cleaved by the signal peptidase to produce ProPrA, which is then transported to the Golgi. The carbohydrate residues of ProPrA are mannosylated in the Golgi. ProPrA is then recognized by the vacuolar protein sorting receptor, Vps10, which targets it to the vacuole (Jorgensen et al, 1999). Either within the transport vesicle or the vacuole, proPrA undergoes autocatalytic activation, whereby a 54 amino acid propeptide is removed to produce active PrA. Active PrA preferentially cleaves substrates between two hydrophobic amino acids, such as Phe, Ile, Leu, Glu, and Ala (Kondo et al, 1998).

Proteinase B (PrB) is a serine endoprotease of the S8 family, encoded by the PRB1 in yeast. The zymogen preproPrB is initially synthesized in a 76 kDa form that is translocated into the ER where it is modified by a single N-linked glycan, and its 20 amino acid signal peptide is removed (Moehle et al, 1989). PreproPrB undergoes autocatalytic cleavage in the ER, removing
an N-terminal 260 amino acid propeptide (Nebes & Jones, 1991). The ER-resident protein, Pbn1, acts as a chaperone in this process to facilitate proper folding of proPrB, which is a pre-requisite for autocatalytic cleavage of the proPrB propeptide (Naik & Jones, 1998; Subramanian et al, 2006). ProPrB is further modified by O-linked glycans in the Golgi resulting in a 42 kDa species (Moehle et al, 1989). The N-terminal propeptide is thought to remain non-covalently associated with proPrB, partially inhibiting its enzymatic activity, until proPrB is targeted to the vacuole where the N-terminal propeptide is degraded by PrA (Naik & Jones, 1998). In the vacuole a C-terminal propeptide (~30 amino acids) of proPrB is cleaved by PrA to produce a 37 kDa PrB species. The final cleavage event is mediated autocatalytically by PrB, removing a C-terminal peptide modified by a single N-linked glycan, and yielding the mature 31 kDa PrB (Moehle et al, 1989). Intriguingly, while disrupting PrA activity leads to accumulation of proPrB and the N-terminal propeptide, proPrB retains residual catalytic activity (Van Den Hazel et al, 1996).

Information on the substrate specificity of PrB comes from studies of PrB isolated from both Saccharomyces cerevisiae and Candida albicans. Both artificial peptides and non-yeast proteins, such as insulin, were used to test PrB substrate specificity in these studies (Farley et al, 1986; Kominami et al, 1981). Based on these studies, PrB has a broad substrate specificity that may depend on amino acid context. The protease cleaves substrates at Arg, Leu, Tyr, Phe, Lys, or Asp in the P site, and Phe, Tyr, Val, Gly, Leu, Glu, or His in the P’ site (Farley et al, 1986; Kominami et al, 1981).

Carboxypeptidase Y (CPY) is a serine carboxypeptidase of the S10 family, which is encoded by PRC1 in yeast. CPY is initially synthesized as ~60 kDa precursor 532 amino acids in length. It is translocated into the ER where a 20 amino acid signal peptide is removed by signal peptidase and four N-linked glycan residues are appended to produce a 67 kDa from called p1-
CPY (Jung et al, 1999). CPY protein folding in the ER involves the formation of five disulphide bonds and is facilitated by a 91 amino acid propeptide, which acts as an intramolecular chaperone (Tang et al, 2002). The p1-CPY protein is delivered to the Golgi where glycan residues are extended to produce a 69 kDa form referred to as p2-CPY. In the Golgi, p2-CPY is recognized by Vps10 via a QRPL recognition sequence in the propeptide region for delivery to the MVB, which subsequently fuses with the vacuole (Jung et al, 1999; Marcusson et al, 1994; Van Den Hazel et al, 1996). This vacuolar targeting pathway, named the CPY pathway, was described in Section 1.1 and is used by other substrates targeted to the vacuole, two of which are discussed below. In the vacuole, the propeptide of CPY is removed by the sequential action of PrA and PrB to produce active CPY (Broach et al, 1991; Jung et al, 1999).

CPY contains a catalytic triad characteristic of serine proteases, which is comprised of Ser146, His397, and Asp338 (Jung et al, 1999). The enzyme is active at low pH and high salt concentrations, which are characteristic of the vacuolar environment. Substrates of CPY are recognized via their C-terminal carboxyl group, which associates with CPY by hydrogen bonding near the S1’ binding pocket. The S1’ subsite in the substrate binding pocket is large and can recognize both hydrophobic and hydrophilic residues. However, the hydrophobic S1 subsite exhibits greater specificity towards hydrophobic amino acids by virtue of being lined with bulky Tyr residues and having a Leu at the bottom of the binding pocket (Jung et al, 1999).

Carboxypeptidase S (CpS) is a zinc-dependent metallo-carboxypeptidase of the M20 family, which is encoded by CPS1 in yeast. CpS is synthesized as a ~64 kDa precursor containing a signal anchor sequence spanning amino acids 20 through 40 that is inserted into the ER membrane such that CpS is oriented with its C-terminus facing the lumen (Spormann et al, 1991). The CpS membrane-bound precursor is glycosylated at 2 or 3 N-glycan acceptor sites and
passes through the Golgi before being targeted to the vacuole via the CPY pathway (Lauwers et al, 2009).

Once in the vacuole, CpS is processed by PrB, and is released in its soluble form into the vacuolar lumen. Both 74 kDa and 77 kDa mature forms of CpS are observed, representing CpS modified by 2 or 3 N-linked glycans, respectively. It is interesting to note that the membrane-bound form of CpS also shows proteolytic activity (Spormann et al 1992).

CpS has partially overlapping substrate specificity with CPY, contributing ~60% of the enzymatic activity required to hydrolyze the synthetic dipeptide Cbz-Gly-Leu, where Cbz is the amine protecting group benzyloxy carbonyl (Wolf & Ehmann, 1981). In a prc1Δ strain, CpS is required for growth on media where Cbz-Gly-Leu is the sole nitrogen and leucine source (Spormann et al, 1991). Finally, CpS has also been found to play a role in sporulation efficiency. Disrupting PrB activity produces a partial defect in sporulation. However, when PrB activity is disrupted together with CPY and CpS activity, cells lose nearly all capacity for sporulation (Wolf & Ehmann, 1981).

Aminopeptidase I (Ape1) is a zinc-dependent metallo-aminopeptidase of the M18 family, encoded by the APE1 gene in yeast. Ape1 is synthesized as 61 kDa precursor known as preApe1, which contains a 45 amino acid N-terminal helix-loop-helix domain that is required for vacuolar localization. PreApe1 homooligomerizes in the cytoplasm, forming a dodecamer complex with a molecular mass of 372 kDa (Kim et al, 1997). Unlike other protease precursors, the preApe1 complex is catalytically active. It is thought that preApe1 dodecamers aggregate into large complexes in order to inhibit protease activity (Adachi et al, 2007). This Ape1 complex is recognized by its receptor Atg19, which interacts with Atg11 to tether the preApe1 complex to the PAS (Morales Quinones et al, 2012). The preApe1 complex is encapsulated in a Cvt vesicle
that fuses with the vacuole. Exposure to the acidic pH of the vacuolar lumen results in disassembly of the preApe1 complex to preApe1 dodecamers (Adachi et al, 2007). Finally, PrB cleaves the N-terminal propeptide of the preApe1 subunits in the vacuole to produce 50 kDa mApe1 subunits (Morales Quinones et al, 2012; Schu, 2008).

Ape1, is also known as Lap4 because it was initially characterized as a leucine aminopeptidase in a screen for yeast mutants defective in their ability to hydrolyze the synthetic substrate leucine beta-naphthylamide (Trumbly & Bradley, 1983). Ape1 was also shown to mediate resistance to Cd$^{2+}$, which is a toxic metal ion that induces oxidative stress in yeast (Adamis et al, 2009). Yeast sequesters Cd$^{2+}$ in the vacuole as a glutathione (GSH) S-conjugate. GSH is a tripeptide made up of Glu, Cys, and Gly residues. The Glu residue is linked to the Cys residue by a gamma peptide bond, where the carboxyl group of the Glu side-chain is bound to the amine group of the Cys peptide backbone. The GSH is recycled from the vacuole by the action of gamma-glutamyltranspeptidase (Ecm38), which hydrolyzes Glu, leaving a Cys-Gly dipeptide which is thought to be further degraded by Ape1 (Adamis et al, 2009). However, more recent work has identified an alternative GSH degradation pathway involving the cytosolic dipeptidase, Dug1, of the M20 family of metalloproteases. This study demonstrated that Ape1 was not required for growth of a met15Δ strain on media in which GSH was the only source of sulphur (Ganguli et al, 2007).

There are three other leucine aminopeptidases in yeast: Lap1 is found in the cytosol and periplasm, Lap2 is found in the cytosol and nucleus, and Lap3 is found in the cytosol. Cells in which all four leucine aminopeptidase-encoding genes have been deleted are viable, suggesting these enzymes do not play an essential role or that enzymes such as Dug1 have overlapping activity with the leucine aminopeptidases (Schu, 2008).
Aminopeptidase Y (Ape3), encoded by the gene APE3, is a metallo- aminopeptidase of the same M28 family of proteases as Ybr074 (see Chapter 2). Like YBR074, the APE3 locus is found on chromosome II. Global pairwise sequence alignment of Ape3 and Ybr074, shown in Figure 4, reveals 5.7% amino acid sequence identity, while comparison of the M28 protease domains share 21.9% sequence identity (Myers & Miller, 1988).
Ape3  | MHFSLKQLAVAAFYATNLGSAYVIPQFFQEFQEEPIENYLPLQLNNDDSSAIVAANIPKP
Ybr074 | -------------------------------------------------------------
Ape3  | 70    80    90    100    110    120
Ybr074 | -------------------------------------------------------------
Ape3  | 130    140    150    160    170    180
Ybr074 | -------------------------------------------------------------
Ape3  | 190   200   210   220   230   240
Ybr074 | -------------------------------------------------------------
Ape3  | 310   320   330   340   350
Ybr074 | -------------------------------------------------------------
Ape3  | 360   370   380   390   400   410
Ybr074 | -------------------------------------------------------------
Ape3  | 420   430   440   450   460   470
Ybr074 | -------------------------------------------------------------
Ape3  | 480   490   500   510   520   530
Ybr074 | -------------------------------------------------------------
Ape3  | 540   550   560   570   580   590
Ybr074 | -------------------------------------------------------------
Ape3  | 600   610   620   630   640   650
Ybr074 | -------------------------------------------------------------
Ape3  | 660   670   680   690   700   710
Ybr074 | -------------------------------------------------------------
Ape3  | 720   730   740   750   760   770
Ybr074 | -------------------------------------------------------------
Ape3  | 780   790   800   810   820   830
Ybr074 | -------------------------------------------------------------
Ape3  | 840   850   860   870   880   890
Ybr074 | -------------------------------------------------------------
Ape3  | 900   910   920   930   940   950
Ybr074 | -------------------------------------------------------------
Ape3  | 960   970   980   990  1000  1010
Ybr074 | -------------------------------------------------------------
Ape3  | 1020  1030  1040  1050  1060  1070
Ybr074 | -------------------------------------------------------------
Ape3  | 1080  1090  1100  1110  1120  1130
Ybr074 | -------------------------------------------------------------
Ape3  | 1140  1150  1160  1170  1180  1190
Ybr074 | -------------------------------------------------------------
Ape3  | 1200  1210  1220  1230  1240  1250
Ybr074 | -------------------------------------------------------------
Ape3  | 1260  1270  1280  1290  1300  1310
Ybr074 | -------------------------------------------------------------
Ape3  | 1320  1330  1340  1350  1360  1370
Ybr074 | -------------------------------------------------------------
Ape3  | 1380  1390  1400  1410  1420  1430
Ybr074 | -------------------------------------------------------------
Ape3  | 1440  1450  1460  1470  1480  1490
Ybr074 | -------------------------------------------------------------
**Figure 4:** Global sequence alignment of Ape3 and Ybr074.

Global sequence alignment without end-gap penalty was performed using Lalign version 2.2 and default parameters (Huang & Miller, 1991). The M28 domains of Ape3 and Ybr074 are highlighted in yellow according to the Pfam domain boundaries.
Ape3 is synthesized as 60 kDa precursor bearing a 21 amino acid signal sequence directing its translocation into the ER (Nishizawa et al, 1994). The signal sequence is cleaved in the ER, and the Ape3 precursor is targeted to the Golgi, where it is recognized by Vps10 and targets Ape3 to the vacuole (Jorgensen et al, 1999). In the vacuole a 35 amino acid N-terminal propeptide is cleaved by PrB, producing mature Ape3 (Yasuhara et al, 1994). The mature Ape3 protein exists in both a 70 kDa and 75 kDa form that differ in the extent of glycan modification, as Ape3 has eight acceptor sites for N-linked glycans but only 5-7 sites are used (Yasuhara et al, 1994).

Ape3 enzymatic activity was tested by Yasuhara et al. using synthetic peptides with a C-terminal 4-methylcoumaryl-7-amide (MCA) protecting group (Yasuhara et al, 1994). Ape3 exhibits a preference for cleaving the basic residue Lys. However, it also cleaves N-terminal Pro, Ala, Leu, Met, Ser, Phe, and Tyr residues (Yasuhara et al, 1994). The catalytic activity of Ape3 is affected by treatment with Co^{2+}. Specifically, the hydrolysis of amino acid-MCAs and dipeptides was enhanced in the presence of Co^{2+}, while that of dipeptidyl-MCAs and larger unmodified peptides was inhibited. Surprisingly, although M28 family metalloproteases commonly bind two catalytic zinc ions, the authors suggested zinc is inhibitory to Ape3 proteolytic activity (Yasuhara et al, 1994).

1.2.2 Membrane-bound vacuolar protease

Dipeptidylaminopeptidase B (Dap2) is serine protease of the S9 family and is encoded by DAP2 in yeast. Dap2 has a hydrophobic transmembrane segment, positioned 30 amino acids from the N-terminus, that serves both as an ER-targeting sequence and membrane anchor. Dap2 is a type II membrane protein, having a cytosolic N-terminus and lumenal C-terminus, and is initially
synthesized as a 93 kDa protein 818 amino acids in length. Dap2 has eight N-glycans and is modified in the ER with anywhere between 5 and 8 N-linked glycan residues, which undergo minimal extension in the Golgi. This results in a final ~120 kDa product. Dap2 is targeted to the vacuole via the secretory pathway and, unlike other vacuolar proteases, is not modified by proteolytic processing in the vacuole (Roberts et al, 1989; Van Den Hazel et al, 1996).

Although the substrate specificity of Dap2 is unknown, its Golgi-resident homolog, dipeptidylaminopeptidase A (Ste13) is known to process repeating -X-Ala- dipeptides from the yeast α factor mating pheromone (Julius et al, 1983). Disrupting Ste13 function prevents maturation of α factor and results in sterile MATα cells. This defect in mating was partially restored when Dap2 was overexpressed 10 fold in a MATα ste13 mutant strain (Julius et al, 1983).

1.3 METALLOPROTEASE FAMILY MEMBERS IN YEAST

Metalloproteases comprise 24% of all proteases with assigned MEROPS identifiers, and 27% of proteases with solved structures listed in the Protein Data Bank (PDB) (Rawlings et al, 2012). The metalloproteases are divided in the MEROPS database into 16 clans, each representing a group of metalloproteases derived from a common ancestor. Each clan is sub-divided into families of protease sharing sequence similarity. The largest clan, MA, is comprised of 37 families. The MH clan, to which Ybr074 belongs, is comparatively small, consisting of only 4 families: M18, M20, M28, and M42 (Rawlings et al, 2012).

The mechanism of catalysis of metalloproteases involves a water molecule that is polarized by interaction with a divalent metal ion. This “activation” of the water molecule
enables it to hydrolyze the substrate peptide bond via nucleophilic attack. Most metalloproteases function using Zn\(^{2+}\) as the catalytic ion, but a few proteases make use of Mn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), or Cu\(^{2+}\) instead. While 11 of the 16 clans bind a single metal ion, clans MF, MG, MH, MN, and MQ bind two co-catalytic metal ions (Mansfeld, 2007).

The known metalloproteases in *Saccharomyces cerevisiae* are listed in Table 2. A gene ontology (GO) term analysis of these metalloproteases reveals that 36% of them are mitochondrial proteases involved in protein targeting, maturation, and quality control, as well as mitochondrial organization (Gakh et al, 2002). For information on these mitochondrial proteases, the reader is referred to an excellent review by Koppen and Langer (Koppen & Langer, 2007).

Notably, all four proteases involved in maturation of the α-factor mating pheromone are classified as metalloproteases (Boyartchuk & Rine, 1998). These are Ste24, an ER-localized transmembrane protein; Ste23, a peripherally associated membrane protein; Rce1, an ER-localized transmembrane protein; and Axl1, a peripherally associated membrane protein that is found at the bud neck, the tip of mating projections, and the cell periphery (Alper et al, 2009; Huh et al, 2003). Some additional substrates have been identified for these proteases. For example, Ste24 is also involved in chitin biosynthesis, although the substrate linking it to this process is not known (Meissner et al, 2010). In addition, mammalian Ste24 plays an important role in the maturation of lamin A, and disruption of this activity results in progeroid disorders in humans (Barrowman & Michaelis, 2009). Ste23 shares sequence similarity with the mammalian insulin-degrading enzyme, and was found to hydrolyze this substrate *in vitro*, as well as the amyloid-beta peptide associated with Alzheimer’s disease (Alper et al, 2009). Rce1 is also known to process the precursor form of the signaling molecule Ras GTPase in yeast (Manandhar
et al, 2010). Therefore, the physiological functions of this group of metalloproteases may be more diverse than is currently known.

A group of four metalloproteases in yeast were associated with the vacuolar compartment by GO term analysis. Of these, Ape1, Ape3, and Cps1 have been discussed in Section 1.2, while Ape4 was discussed in Section 1.1.1. It is interesting to note that Ecm14 and Tre1, which are non-catalytic metalloprotease homologs, are also associated with the vacuolar compartment by GO term analysis. Ecm14 has been found to associate with the vacuolar membrane in a proteomic analysis and in a high-throughput screen of GFP-tagged proteins (Huh et al, 2003; Wiederhold et al, 2009). The function of Ecm14 is poorly understood, but it has been found to affect adhesion in *Saccharomyces cerevisiae* by contributing to the shedding of flocculin, Flo11, from the cell surface (Karunanithi et al, 2010). Tre1 and Tre2 together with Bsd2 function as an adaptor complex linking the ubiquitin ligase, Rsp5, to its substrate, Smf1. Rsp5-mediated ubiquitination of the metal transporter, Smf1, which facilitates Smf1 endocytosis and targeting to the vacuole for degradation via the vacuolar protein sorting pathway (Leon & Haguenauer-Tsapis, 2009).

Other metalloproteases include Rpn11 and Rpn8, which are notable members of the 26S proteasome. The proteasome is a 2.5 MDa cytosolic protein complex mediating the degradation of ubiquitinated proteins via the ubiquitin-proteasome system (Finley, 2009). Rpn11 is a subunit of the 19S regulatory subunit of the proteasome that functions as a proteasome substrate deubiquitinating enzyme (Lee et al, 2011). Rpn8 is also a subunit of the 19S regulatory particle and shares sequence similarity with Rpn11 (Glickman et al, 1999).
<table>
<thead>
<tr>
<th>Clan</th>
<th>Family</th>
<th>MEROPS ID</th>
<th>Gene</th>
<th>SGD Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>M1</td>
<td>M01.006</td>
<td>APE2</td>
<td>Role in obtaining leucine from dipeptide substrates: cytosolic and mitochondrial</td>
</tr>
<tr>
<td>MA</td>
<td>M1</td>
<td>M01.007</td>
<td>AAP1</td>
<td>Arg/Ala aminopeptidase, overproduction stimulates glycogen accumulation, cytoplasm and nucleus</td>
</tr>
<tr>
<td>MA</td>
<td>M1</td>
<td>M01.017</td>
<td>TMA108</td>
<td>Cytoplasmic protein involved in ribosome biogenesis</td>
</tr>
<tr>
<td>MA</td>
<td>M1</td>
<td>M01.034</td>
<td>LAP2</td>
<td>Leucyl aminopeptidase yscV with epoxide hydrolase activity; cytoplasmic and nuclear</td>
</tr>
<tr>
<td>MA</td>
<td>non-peptidase homologue</td>
<td>TAF2</td>
<td>TFID subunit (150 kDa), involved in RNA polymerase II transcription initiation, nuclear</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>M3</td>
<td>M03.003</td>
<td>PRD1</td>
<td>Degradation of mitochondrial proteins and of presequence peptides</td>
</tr>
<tr>
<td>MA</td>
<td>M3</td>
<td>M03.006</td>
<td>MP1</td>
<td>Mitochondrial DNA polymerase</td>
</tr>
<tr>
<td>MA</td>
<td>M41</td>
<td>M41.002</td>
<td>AFG3</td>
<td>Mediates degradation of misfolded or unassembled mitochondrial proteins</td>
</tr>
<tr>
<td>MA</td>
<td>M41</td>
<td>M41.003</td>
<td>YTA12</td>
<td>Mediates degradation of misfolded or unassembled mitochondrial proteins</td>
</tr>
<tr>
<td>MA</td>
<td>M41</td>
<td>M41.004</td>
<td>YTA11</td>
<td>Degradation of unfolded or misfolded mitochondrial gene products</td>
</tr>
<tr>
<td>MA</td>
<td>M43</td>
<td>M43.001</td>
<td>STE24</td>
<td>Functions in two steps of α-factor maturation: LE</td>
</tr>
<tr>
<td>MA</td>
<td>M43</td>
<td>M43.018</td>
<td>OMA1</td>
<td>Involved in turnover of mitochondrial inner membrane-embedded proteins</td>
</tr>
<tr>
<td>MA</td>
<td>M49</td>
<td>M49.001</td>
<td>YCL057W</td>
<td>Dipeptidyl-peptidase III</td>
</tr>
<tr>
<td>MA</td>
<td>M80</td>
<td>M80.001</td>
<td>WSS1</td>
<td>Sumoylated protein that localizes to a single spot on the nuclear periphery</td>
</tr>
<tr>
<td>MC</td>
<td>non-peptidase homologue</td>
<td>ECM14</td>
<td>Required for normal cell wall assembly</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.003</td>
<td>MAS1</td>
<td>Smaller subunit of the mitochondrial processing protease (MPP)</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.007</td>
<td>AXL1</td>
<td>Performs one of two N-terminal cleavages during maturation of a-factor, cell periphery and bud neck</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.008</td>
<td>STE23</td>
<td>N-terminal processing of pro-a-factor to the mature form</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.013</td>
<td>CYM1</td>
<td>Mitochondrial intermembrane space degrades proteins and presequence peptides</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.971</td>
<td>MAS2</td>
<td>Larger subunit of the mitochondrial processing protease (MPP)</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.973</td>
<td>CORY1</td>
<td>Core subunit of the ubiquinol-cytochrome c reductase complex in mitochondria</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.974</td>
<td>COR2</td>
<td>Subunit 2 of the ubiquinol cytochrome c reductase complex in mitochondria</td>
</tr>
<tr>
<td>ME</td>
<td>M16</td>
<td>M16.994</td>
<td>YCL098C</td>
<td>Putative metalloprotease</td>
</tr>
<tr>
<td>MG</td>
<td>M24</td>
<td>M24.001</td>
<td>MAP4</td>
<td>Removal of N-terminal methionine of nascent polypeptides: cytosolic and nuclear</td>
</tr>
<tr>
<td>MG</td>
<td>M24</td>
<td>M24.002</td>
<td>MAP2</td>
<td>Removal of N-terminal methionine from nascent polypeptides: cytosolic</td>
</tr>
<tr>
<td>MG</td>
<td>M24</td>
<td>M24.026</td>
<td>ICP55</td>
<td>Cleaves the N terminus of imported mitochondrial proteins after MPP</td>
</tr>
<tr>
<td>MG</td>
<td>M24</td>
<td>unassigned</td>
<td>FRA1</td>
<td>Cytosolic negative regulator of transcription of the iron regulon</td>
</tr>
<tr>
<td>MG</td>
<td>M24</td>
<td>unassigned</td>
<td>YFR006W</td>
<td>Putative X-Pro aminopeptidase</td>
</tr>
<tr>
<td>MH</td>
<td>M18</td>
<td>M18.001</td>
<td>APE1</td>
<td>Vacular aminopeptidase I; marker for autophagy and Cyt pathway</td>
</tr>
<tr>
<td>MH</td>
<td>M18</td>
<td>M18.002</td>
<td>APE4</td>
<td>Cytoplasmic aspartyl aminopeptidase that may also function in the vacuole</td>
</tr>
<tr>
<td>MH</td>
<td>M20</td>
<td>M20.002</td>
<td>CPS1</td>
<td>Vacular carboxypeptidase; induced under low-nitrogen conditions</td>
</tr>
<tr>
<td>MH</td>
<td>M20</td>
<td>M20.017</td>
<td>DUG1</td>
<td>Cys-Gly di-peptidase; degrades glutathione (GS) with Dug2 and Dug3</td>
</tr>
<tr>
<td>MH</td>
<td>M20</td>
<td>non-peptidase homologue</td>
<td>DUG2</td>
<td>Component of glutamine amidotransferase (GATase II); degrades GS; cytosolic</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>M28.001</td>
<td>APE3</td>
<td>Vacular aminopeptidase Y, processed to mature form by Prb1p</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>M28.006</td>
<td>YDR145c</td>
<td>Putative protein of unknown function</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>M28.974</td>
<td>YFR018C</td>
<td>Putative protein of unknown function</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>M28.997</td>
<td>YBR074W</td>
<td>Putative metalloprotease</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>non-peptidase homologue</td>
<td>TRE2</td>
<td>Regulates ubiquitylation and vacuolar degradation of the metal transporter Smf1</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>non-peptidase homologue</td>
<td>VPS70</td>
<td>Protein of unknown function involved in vacuolar protein sorting</td>
</tr>
<tr>
<td>MH</td>
<td>M28</td>
<td>non-peptidase homologue</td>
<td>TRE1</td>
<td>Plasma membrane protein, regulates ubiquitylation and vacuolar degradation of the metal transporter Smf1</td>
</tr>
<tr>
<td>MJ</td>
<td>M38</td>
<td>non-peptidase homologue</td>
<td>DAL1</td>
<td>Converts allantoin to allantate in the first step of allantoin degradation</td>
</tr>
<tr>
<td>MK</td>
<td>M22</td>
<td>M22.005</td>
<td>QRI7</td>
<td>Essential for modification of mitochondrial tRNAs that decode ANN codons</td>
</tr>
<tr>
<td>MK</td>
<td>M22</td>
<td>unassigned</td>
<td>RAE1</td>
<td>ATPase of HSP70DnaK family, involved in modification of mitochondrial tRNAs</td>
</tr>
<tr>
<td>MP</td>
<td>M67</td>
<td>M67.001</td>
<td>RPN11</td>
<td>Metalloprotease subunit of the 19S regulatory particle of the 26S proteasome lid</td>
</tr>
<tr>
<td>MP</td>
<td>M67</td>
<td>M67.973</td>
<td>RPN8</td>
<td>Essential, non-ATPase regulatory subunit of the 26S proteasome</td>
</tr>
<tr>
<td>MP</td>
<td>M67</td>
<td>unassigned</td>
<td>RRP1</td>
<td>Catalytic subunit of the COP9 signalosome (CSN) complex; cytosolic</td>
</tr>
<tr>
<td>M-</td>
<td>M79</td>
<td>M79.001</td>
<td>RCF1</td>
<td>CAAX prenyl protease; maturation of Ras and the a-factor mating pheromone</td>
</tr>
</tbody>
</table>
1.4  QUESTIONS FOR FUTURE RESEARCH ON YEAST VACUOLAR FUNCTION

Given the diverse functions associated with the vacuole, issues that need to be addressed in future research into the mechanisms regulating this specialized organelle are very broad. This section highlights only a few of the many important questions requiring attention in this field.

While the substrate specificity of many of the vacuolar proteases has been defined using synthetic substrates, not much information is available in the context of endogenous substrates of vacuolar proteases. The current view is that vacuolar proteases are generally indiscriminant enzymes because the need for substrate specificity is obviated by the presence of the vacuolar membrane, which acts as a physical barrier separating these general proteases from the rest of the cell. However, this does not explain why so many different protease (eight, including Ybr074) are associated with the vacuole. Do certain proteases have dominant roles in response to certain physiological conditions requiring vacuole-dependent degradation? For example, are different vacuolar proteases more active or more abundant during sporulation than under conditions triggering apoptosis? Furthermore, are there functional characteristics that differentiate soluble vacuolar proteases from membrane-bound vacuolar proteases? Perhaps membrane-bound proteases associate more readily with membrane-bound substrates.

In the study of autophagy, the source of autophagosome membranes still represents an outstanding question. Although many sources have been cited, including the ER, mitochondria and plasma membrane, it is not clear whether different lipid donors contribute structural or
functional differences to the autophagosomes they generate (Weidberg et al, 2011). Could the composition of autophagosomes affect cargo selectivity?

1.5 DISSECTATION GOALS AND OVERVIEW

Ybr074 is an uncharacterized, putative metalloprotease of the M28 family in yeast. Ybr074 has a conserved protease domain related to a putative mammalian metalloprotease known as FXNA, or ER Metalloprotease 1 (ERMP1). Both Ybr074 and FXNA are predicted to be multi-pass transmembrane proteins, with Ybr074 having nine predicted transmembrane segments and FXNA having eight predicted transmembrane segments (TopPredII, upper cut-off) (Claros & von Heijne, 1994). Knock-down of FXNA in rat ovaries, achieved using shRNA delivered using a lentiviral vector, resulted in a defect in ovarian development. More specifically, the organization of somatic granulosa cells around the oocyte was impaired upon FXNA knockdown, presumably disrupting intercellular contacts required for the development of the ovarian follicle (Garcia-Rudaz et al, 2007).

FXNA is expressed in other rat tissues, including the kidneys, the adrenal gland, and various areas of the brain (Garcia-Rudaz et al, 2007). Therefore, FXNA may have more extensive, and as yet, undiscovered functions in these tissues. Since the protease domain of Ybr074 shares 32.5% sequence identity with that of FXNA, I hoped that using yeast as a model organism would illuminate the functions of both Ybr074 and FXNA. Nevertheless, the sequence similarity between FXNA and Ybr074 does not extend beyond the protease domain (Figure 5). Therefore, the biological functions and endogenous substrates of Ybr074 and FXNA may well differ.
Even if Ybr074 does not inform directly on the function of FXNA, understanding its biological function has intrinsic value to the field of yeast biology. The role of Ybr074 in the context of the yeast proteome has the potential to inform on pathways connecting vacuolar function and cellular protein homeostasis. These more general biological pathways may be analogous in higher eukaryotes. Furthermore, an understanding of the influence of Ybr074 on the maintenance of the cell wall, described in Chapter 4, may contribute novel targets against pathological yeast associated with human disease (Santamaria et al, 2011). For example, the pathogenic fungus *Candida albicans* is the fourth most common cause of bloodstream infection in cancer patients. A class of antifungal drugs, known as the echinocandins, is used to combat this type of fungal infection. Echinocandins, such as caspofungin, function by inhibiting glucan synthesis in the cell wall of *C. albicans* (Kofla & Ruhnke, 2011). The importance of cell wall glucans in maintaining cell wall integrity will be discussed further in Chapter 4. Furthermore, metalloproteases, such as Ybr074, have important industrial applications. For example, metalloproteases are used to improve beer filtration and reduce calorie content in the brewing industry. Metalloproteases have been used to remove protein coatings to restore old documents and artworks. Metalloproteases are also widely used in the food industry to add flavor-enhancing peptides (Mansfeld, 2007).

Finally, metalloproteases are important pharmaceutical targets. For example, matrix metalloproteases (MMPs) are secreted proteins that normally remodel the extracellular matrix (ECM) to facilitate important physiological functions such as cellular proliferation, cellular migration, wound healing, growth, and development (Klein & Bischoff, 2011). However, MMPs are also linked to a number of pathologies in humans, including cancer, lung disease, vascular disease, inflammation, and neurodegenerative disease (Sbardella et al, 2012). Therefore,
understanding the function of the putative metalloprotease Ybr074, identifying its substrates and its interaction partners is an inherently valuable endeavor.

In the following chapters, I describe the first characterization of Ybr074, such as its topology and sub-cellular localization (Chapter 2). In Chapter 3, I elaborate on genetic techniques used in an attempt to identify the biological function of Ybr074. This chapter also describes proteomic techniques used in an attempt to isolate endogenous substrates of Ybr074. I explore a potential link between Ybr074 and maintenance of the cell wall in Chapter 4. Chapter 5 will conclude with a discussion of the findings presented in this dissertation, and future directions for further characterization of Ybr074.
Figure 5: Global sequence alignment of FXNA and Ybr074.

Global sequence alignment was performed using Lalign version 2.2 and default parameters (Huang & Miller, 1991). The M28 domains of FXNA and Ybr074 are highlighted in yellow according to the Pfam domain boundaries.
2.0 CHARACTERIZATION AND LOCALIZATION OF YBR074

2.1 INTRODUCTION

Saccharomyces cerevisiae was the first eukaryote to have its entire genome sequenced (Goffeau et al, 1996). Since then it has served as a model organism for higher eukaryotes and as a tool in the on-going efforts to surmount the challenge facing the “post-genomic era”: to characterize and annotate the information gleaned from genome sequencing. In 2007, soon after this thesis project began, the biological function of approximately 21% of the Saccharomyces cerevisiae genome (>1000 genes) remained uncharacterized, including that of 11 putative proteases (Pena-Castillo & Hughes, 2007). It is likely that many of these uncharacterized genes do not have strong phenotypes when disrupted, resulting in their under-representation in the research literature. This may be due to functional redundancy, or because of the limited number of conditions testable in a laboratory environment (Pena-Castillo & Hughes, 2007). In order to develop hypotheses about the function of these genes, numerous computational analyses, based on nucleotide or amino acid sequence, are available (Friedberg, 2006). Among these computational methods, sequence pattern searching and structure-based similarity searches were used in this study to predict biological functions of Ybr074. This chapter will focus on the computational predictions that led to testable hypotheses.
Based on the presence of several distinct hydrophobic amino acid motifs, Ybr074 was predicted and demonstrated to be a transmembrane protein. Local sequence similarity comparisons to proteins of known function identified a conserved domain belonging to the M28 family of metalloproteases. Computational predictions of Ybr074’s sub-cellular localization suggested that this protein might be ER-localized. Finally, topological predictions placed the protease domain of Ybr074 on the luminal side of the ER. Although the results detailed below do show that Ybr074 is a transmembrane protein and is modified by N-linked glycans in the ER, it resides in the vacuolar membrane, contrary to what was predicted, with its protease domain facing the vacuolar lumen.

2.2 MATERIALS AND METHODS

2.2.1 Computational Methods

The amino acid sequence of Ybr074 was analyzed using the default parameters of the Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) to identify proteins in the non-redundant protein sequences database, which shared local sequence similarity with Ybr074 (Altschul et al, 1997; Altschul et al, 2005). The European Molecular Biology Library-European Bioinformatics Institute (EMBL-EBI), InterPro Scan sequence search tool, and the PANTHER Classification System version 7.2 were also used to examine proteins related to Ybr074 using default parameters (Mi et al, 2010).

The M28 protease domain of Ybr074, and its putative catalytic and zinc-binding residues, were identified using the National Center for Biotechnology Information (NCBI) Conserved
Domain Database (CDD) (Marchler-Bauer et al, 2011). The M28 protease domain boundaries of Ybr074 were analyzed using Pfam version 26.0 (Punta et al, 2012).

The presence and position of transmembrane segments, as well as the topology of Ybr074 were predicted using ConPredII (Arai et al, 2004), TopPred 2 (von Heijne, 1992), Membrane Protein Explorer, MPEx (Snider et al, 2009), and PSORT II under default settings (Nakai & Horton, 1999). N-linked glycosylation sites were predicted using the NetNGlyc 1.0 server, under default settings, from the Eukaryotic Linear Motif (ELM) resource for functional sites in proteins (Blom et al, 2004). Sub-cellular localization of Ybr074 was predicted using PSORT, PSORT II, and WoLF PSORT under default settings (Horton et al, 2007). Structural homology predictions for the M28 protease domain and C-terminal domain of Ybr074 were analyzed using Protein Homology/analogY Recognition Engine 2 (PHYRE2) under default settings (Kelley & Sternberg, 2009).

2.2.2 Strains, plasmids, yeast growth conditions, and molecular techniques

Strains used in this study are listed in Table 3. Standard growth conditions and molecular techniques were used, unless otherwise indicated (Adams et al, 1998). A chromosomally-integrated $P_{GAL1}$-GFP-YBR074 strain was generated using the integration vector pFA6a-His3MX6-$P_{GAL1}$-GFP, as described (Longtine et al, 1998). Briefly, the His3MX6-$P_{GAL1}$-GFP cassette was amplified using the following primers, forward primer: AAA TTA TCT ACA AGG AAA TAA ATT GAT AGG TAA AGT TAA AGA ATC ACG GCG AAT TCG AGC TCG TTT AAA C; reverse primer: TTT GTT TTC CGG TAC TTT AGA ACT GAT CTG AAT ACA CTT TTT AAT TTC ATT TTG TAT AGT TCA TCC ATG C, and were transformed into the BY4742 strain. Transformed cells were selected on synthetic medium lacking histidine. Integration of the
His3MX6-\text{P}_{\text{GAL1}}\text{-GFP} \text{ cassette was confirmed by amplification of a region upstream of } YBR074 \text{ and internal to the His3MX6-}\text{P}_{\text{GAL1}}\text{-GFP} \text{ cassette (forward primer: CAA TGA CGC CAA ATA TGG ACA CT; reverse primer: TTT GTA TAG TTC ATC CAT ATC GC)} \text{ from purified genomic DNA (Adams et al, 1998).}

Plasmids used in this study are listed in Table 4. To generate an N-terminally HA-tagged \textit{YBR074} construct, \textit{YBR074} was PCR amplified using vector pGP564_1_b11 from the yeast overexpression plasmid library (Jones et al, 2008) (forward primer: AAT CAC GGA TCC AAA TTA AAA AGT GTA TTC AGA TCA GTT CTA AAG; reverse primer: AGA ACT CGA GTT ATA AAA TTA TAG CAT CCT TGA CAA TAA CTA ATC CTT TCT G), and ligated into the BamHI/XhoI sites of yeast expression vector pKN16 (Table 4). The \text{P}_{\text{GAL1/10}} \text{ promoter of the resulting plasmid was removed using SacI/SpeI and replaced with the endogenous promoter of } YBR074 \text{ (forward primer: ATG GAG AGC TCG ACA AGT GCG CTG GAT TTA CAA AAG AAA ATG AAT GA; reverse primer: TCA TAC TAG TGC GAT TCT TTA ACT TTA CCT ATC AAT TTA TTT CCT). This construct was verified by DNA sequence analysis (IDT, Coralville, IA).}

To generate the \textit{YBR074-M28HA} expression constructs a single HA tag was inserted at Pro45, within the M28 metalloprotease domain, using a two-stage PCR method (Wang & Malcolm, 1999). Secondary structure prediction programs, SSPRO version 4.5 (Cheng et al, 2005) and PSIPRED version 3.0 (Buchan et al, 2010), were used to identify a region for insertion of the HA tag without disrupting secondary structures. Specifically, the tag was inserted following residue Leu55, in a predicted coil region between transmembrane segment 1 and the first alpha helix predicted to compose the M28 protease domain (Pollastri et al, 2002). Briefly, the endogenous promoter and coding sequence of \textit{YBR074} were PCR amplified as two separate
fragments, PCR1 (forward primer A: ATG GAG AGC ACA AGT GCG CTG GAT TTA CAA AAG AAA ATG AAT GA; reverse primer B: TGC ATA GTC CGG GAC GTC ATA CGG ATA TGG TAG ATT GAG TTT ATA ACG TTC ATG ATC AAA GAT ATA G) and PCR2 (forward primer C: TAT CCG TAT GAC GTC CGG GAC TAT GCA AAA GAG GAT GAG CAC CCT GAA TTC AAT GAC; reverse primer D: AGA ACT CGA GTT ATA AAA TTA TAG CAT CCT TGA CAA TAA CTA ATC CTT TCT G). A third reaction, PCR3 (forward primer A, reverse primer D) was carried out in which a mixture of 5% PCR1 and 5% PCR2 (vol/vol) was used as a template for amplification. The resulting P\textsubscript{ENDG-}YBR074-M28HA cassette was inserted into pRS316 or pRS426 at the SacI/XhoI sites, and the constructs were verified by DNA sequence analysis, as above.

Sensitivity to killer toxin was tested based on previously described protocols (Boone et al, 1990; Santos et al, 2000). In brief, the killer toxin secreting strain 1368, described in Table 3, was grown to saturation overnight in Yeast Peptone Dextrose (YPD), pH 4.5, at room temperature. The growth medium was separated from cells by centrifugation at ~1000 g for 3 min, and filtered using a 0.22 μm diameter filter (Millipore, Franklin Lakes, NJ). The filtered medium was concentrated 200-fold using an ultrafiltration Amicon Ultra-15 unit with a 10 KDa molecular mass cut-off (Millipore, Billerica, MA). Test strains were grown in YPD to log phase, and 1 OD\textsubscript{600} cell equivalents were harvested and re-suspended in 100 μL Sterile Double Distilled Water (SDDW). The 2-fold concentrated YPD, pH 4.5, medium was incubated in a 37°C water bath before being added to an equal volume of autoclaved 4% Bacto agar, supplemented with 30 μg/mL methylene blue. Once equilibrated to approximately 37°C, the molten medium was seeded with the test strain, and the medium was poured and allowed to solidify. A sterile filter
disc spotted with 10 μL of the concentrated killer toxin medium was added to seeded plates and the plates were incubated for 4 days at room temperature.

Sensitivity to killer toxin was measured as the diameter of the halo using ImageJ software (Schneider et al., 2012). The diameter of each halo was taken as the average of four measurements. Each strain was tested in three individual replicates and the average halo diameter and standard error were calculated using Microsoft Excel 2010.
Table 3: Yeast strains used in this study.

<table>
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<td>BY4742</td>
<td>MAT α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>Brachmann C.B. <em>et al</em>. 1998</td>
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<td>OY209</td>
<td>MAT α cdc48-2::NATMX con1Δ::STE2pr-Sp_his5 lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0</td>
<td>C. Boone, University of Toronto, Toronto, Canada</td>
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<td>kre1Δ</td>
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<td>Open Biosystems Yeast Knockout Collection</td>
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<td>slt2A</td>
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<td>cdc48-3</td>
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<td>J.R. Tran, University of Pittsburgh, Pittsburgh, PA</td>
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<td>R. Wickner, NIH, Bethesda, MD</td>
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Table 4: Plasmids used in this study.

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<td>pGP564_1_b11</td>
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<td>LEU2</td>
<td>Jones G.M. et al. 2008</td>
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<td>pFA6a-HisMX6-PGAL1-GFP</td>
<td>$P_{GAL1}$-GFP integration plasmid</td>
<td>HIS3MX6</td>
<td>Longtine, M.S. et al. 1998</td>
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<td>Brachmann, C.B. et al. 1998</td>
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<td>NATMX integration vector</td>
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<td>This study</td>
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<td>Ybr074 expression</td>
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<td>This study</td>
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<td>URA3</td>
<td>This study</td>
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<td>Bhamidipati A. et al. 2005</td>
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<td>URA3</td>
<td>Bhamidipati, A. et al. 2005</td>
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<td>CPY*-DHFR(Pro)</td>
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<td>pSM1911</td>
<td>Ste6p*-HA overexpression vector</td>
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<td>PGAL-3HA-RHR2</td>
<td>Rhr2 overexpression vector</td>
<td>URA3</td>
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</table>
2.2.3 Protein localization

Immunofluorescence assays were performed, as described previously, with minor modifications (Adams et al, 1998). Spheroplasted cells were incubated in poly-lysine coated wells for 10 min at room temperature. Cells were then washed three times with 30 μL of sterile filtered blocking solution (PBS, pH 7.4, 0.5% BSA, 0.5% ovalbumin, 0.01% fish skin gelatin; Sigma, St. Louis, MO) supplemented with 0.1% Triton X-100 (Sigma, St. Louis, MO). Cells were then incubated in blocking solution, including Triton X-100, for 30 min in a humidified chamber, followed by an overnight incubation with 1:500 mouse anti-HA (Roche, Indianapolis, IN) and 1:500 rabbit anti-Kar2 (Brodsky et al, 1993), or 1:100 rabbit anti-HA (Cell Signaling Technology, Beverly, MA) and 1:750 mouse anti-Vph1 (Invitrogen/Molecular Probes, Carlsbad, CA) overnight at 4°C in a humidified chamber. The next day, cells were washed four times with 30 μL blocking solution, and incubated with 1/250 Alexa Fluor 488 goat anti-mouse IgG and 1:250 Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, Molecular Probes, Eugene OR). Images were visualized using a Leica TCS SP5 (Leica microsystems, Buffalo Grove, IL).

Live cell imaging of GFP-Ybr074 was examined in the BY4742 strain with chromosomally-integrated GFP-Ybr074 under the control of a galactose promoter, and selected using the HIS3MX gene as described above. Cells were grown in medium lacking histidine and supplemented with 2% galactose to induce expression, or 2% raffinose as a negative control. Cells were induced overnight at 30°C and harvested at an OD$_{600}$ of ~0.8. Approximately 0.4 OD$_{600}$ cell equivalents were harvested by centrifugation at 13,000 g for 30 sec and washed in 1mL PBS, pH 7.4. Cells were re-suspended in PBS, pH 7.4, to a concentration of 4 OD$_{600}$/mL.
and 5 μL were mounted on a glass slide for visualization. Cells were visualized using the Olympus BX60 fluorescence microscope and QED InVivo software. No expression of GFP-Ybr074 was observed when cells were grown in media supplemented with raffinose instead of galactose (data not shown).

Cell fractionation by sucrose density centrifugation was performed as described (Hong et al 1996). The wild type strain, BY4742, expressing HA-tagged YBR074 from its endogenous promoter on a 2μ plasmid was grown in 100 mL selective media to OD₆₀₀ of 0.6-0.8. Harvested cells were re-suspended in 400 μL 10% EDTA buffer (10 mM Tris-HCl, pH 7.6, 10 mM EDTA) or 10% Mg²⁺ buffer (10 mM Tris-HCl, pH 7.6, 2 mM MgCl₂) including protease inhibitors (PMSF, Leupeptin, Pepstatin A). Cells were lysed by agitation on a Vortex mixer with glass beads for four 30 sec pulses followed by 30 sec on ice. The homogenate was cleared of unbroken cells at 2000 rpm for 2 min at 4°C using a Microfuge 22R centrifuge (Beckman Coulter, Palo Alto, CA). A step gradient was made using 2 mL steps of 70%, 60%, 50%, and 40% sucrose followed by 1.5 mL steps of 30% and 20% sucrose in EDTA or Mg²⁺ buffer. The gradient was loaded with 400 μL of cleared cell homogenate and centrifuged at 139,000 g (28,500 rpm) for 18.5 h at 4°C in an SW41 Ti rotor using an L8-70M Ultracentrifuge (Beckman Coulter, Brea, CA). Fractions were collected by pipetting from the top of the gradient in 500 μL increments, and total protein was precipitated in 10% trichloroacetic acid (TCA) for 15 min on ice (Fischer Scientific, Fair Lawn, NJ). Precipitated proteins were pelleted, and were washed in acetone, spun down again, and re-suspended in 30 μL TCA buffer (80mM Tris-HCl, pH 8, 8mM EDTA, 120 mM DTT, 3.5% SDS, 10% glycerol, 0.08% Tris base, 0.01% Bromophenol Blue). Samples were immediately loaded onto a 10% SDS-polyacrylamide gel, and after electrophoresis proteins were transferred to nitrocellulose. Blots were incubated using the following primary antibodies:
1:5000 mouse anti-HA (Roche, Indianapolis, IN), 1:3000 rabbit anti-Pma1 (Dr. C. Slayman, Yale School of Medicine, New Haven, CT), 1:3000 rabbit anti-Anp1 (Dr. S. Munro, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK), 1:5000 rabbit anti-Sec61 (Stirling et al, 1992). The bound antibodies were visualized using Super Signal West Femto kit (Thermo Scientific, Rockford, IL) on a Kodak 440CF Image Station (Eastman Kodak, Rochester, NY).

Protease protection assays were performed using microsomes derived from wild type yeast expressing HA-tagged YBR074 from its endogenous promoter on a CEN plasmid. Microsomes were isolated using the “medium” preparation method (Nakatsukasa & Brodsky, 2010). Reactions were prepared in B88 (20mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol) using 100-500 μg of total microsomal protein, and were incubated in the presence or absence of 100 μg/mL proteinase K (Sigma, St. Louis, MO) and 1% Triton X-100. Reactions were incubated on ice and 25 μL fractions were taken at 0, 10, 30, and 60 min and precipitated in 10% TCA and processed as described above.

The presence of N-linked glycans on Ybr074 was tested by digestion with Endoglycosidase H (Roche, Indianapolis, IN). Microsomes were prepared as described above from a BY4742 strain expressing HA-tagged YBR074 from its endogenous promoter on a CEN plasmid. Approximately 100 μg of protein were incubated in 50mM citrate buffer, pH 5.5, 1 mM PMSF, 1 μg/mL leupeptin, and 0.5 μg/mL pepstatin A (Sigma, St. Louis, MO) in the presence or absence of 0.005 units of endoglycosidase H overnight at 4°C. Digested protein was resolved by 10% SDS-PAGE and proteins were visualized by western blot analysis using 1:5000 HRP-conjugated mouse anti-HA antibody (Roche, Indianapolis, IN) as described above. The molecular mass of the resulting protein bands was determined by Rf analysis.
Carbonate extraction was performed using microsomes prepared as described above from a BY4742 strain expressing HA-tagged YBR074 from its endogenous promoter on a CEN plasmid (Parlati et al, 1995). Approximately 200 μg of microsomal protein was re-suspended in 1 mL B88 or 0.1 M Na₂CO₃, pH 11.5, in the presence of protease inhibitors (PMSF, leupeptin, pepstatin A; Sigma, St. Louis, MO) at 4°C and incubated for 30 min. Soluble protein was separated from membrane bound protein by ultracentrifugation at 13,500 g (50,000 rpm) for 1 h at 4°C in a Sorvall LC M120-EX Micro Ultracentrifuge using a Sorvall S100AT5 fixed angle rotor. The supernatant was isolated and processed by TCA precipitation as described above. The pellet was washed with B88 containing protease inhibitors, and spun again at 198,000 g (60,000 rpm) for 10 min at 4°C. The supernatant from the second spin was removed and discarded, and the pellet was re-suspended in sample buffer. Samples were resolved by 10% SDS-PAGE and proteins were visualized by western blot analysis as described above.

2.2.4 Cycloheximide chase analysis of Ybr074 stability

To examine dependence on the 26S proteasome for degradation of tagged Ybr074 constructs, cells expressing tagged Ybr074 were grown to logarithmic phase (OD₆₀₀ = 0.4-0.8) and treated with 100 μM MG132 (Peptide institute, Inc., Osaka, Japan) for 30 min at 30°C before addition of 250 μg/mL cycloheximide (Sigma, St. Louis, MO). At the indicated times, 1 mL from each culture was transferred to a tube containing sodium azide at a final concentration of 20 mM. Cells were harvested by centrifugation at 18,000 g for 30 sec at 4°C and then washed with 1mL of ice cold 10 mM sodium azide to block respiration. Cells were centrifuged as above, the supernatant was aspirated, and the cell pellets were flash frozen in liquid nitrogen before being
processed the next day by TCA precipitation, according to an established protocol (Nakatsukasa et al, 2008).

2.3 RESULTS

When the genome of *Saccharomyces cerevisiae* was first sequenced, *YBR074W* and *YBR075W* were annotated as two separate open reading frames (ORFs) (Goffeau et al, 1996). When the genome of the closely related filamentous fungus *Ashbya gossypii* was sequenced years later, a comparison of these two genomes resulted in the fusion of *YBR074W* and *YBR075W*, and this fused ORF was re-named *YBR074W* (Figure 6) (Brachat et al, 2003; Dietrich et al, 2004; Goffeau et al, 1996).

![YBR074 locus](image)

Figure 6: The *YBR074W* locus.

The coding sequence of *YBR074W* (2931 bp) is drawn to scale, and each tick on the black line corresponds to 300 bp. The green and blue shaded regions represent the ORFs *YBR074W* and *YBR075W* prior to the gene being fused and re-annotated. The region underlined in orange represents the M28 metalloprotease domain boundaries as predicted by Pfam.
YBR074W is predicted to encode nine transmembrane segments based on a computational analysis of amino acid segments above a threshold level of hydrophobicity (Ikeda et al, 2002). In order to examine whether Ybr074 is a transmembrane protein, a sodium carbonate extraction experiment was conducted whereby a crude microsomal fraction from cells expressing N-terminally HA-tagged Ybr074 were treated with sodium carbonate at pH 11.5. Under these conditions sodium carbonate disrupts electrostatic interactions mediating the peripheral association of proteins with the lipid bilayer (Fujiki et al, 1982). Microsomes were then separated into membrane and soluble fractions by ultra-centrifugation. I found that 3HA-Ybr074 partitioned in the membrane fraction, as anticipated (Figure 7B). The peripherally membrane associated protein, Pdi1, was used as a negative control, and shown to transition from the membrane fraction to the soluble fraction upon treatment with sodium carbonate. The transmembrane protein, Sec61, was used as a positive control and remained in the membrane fraction even after treatment with sodium carbonate, as anticipated.
Figure 7: Ybr074 is a transmembrane protein with a conserved M28 metalloprotease domain that is predicted to reside in the ER lumen.

A) Computationally predicted topology of Ybr074 with putative lumenal glycosylation sites indicated by stars (Asn96, Asn121, Asn189, Asn217, Asn656, Asn768, Asn796, Asn811, Asn866, and Asn937). The N-terminus (N) and C-terminus (C) are labeled. The conserved M28 metalloprotease domain (Leu151-Leu314) is located in the first luminal loop between transmembrane segments 1 and 2. B) Ybr074 is an integral membrane protein. ER-derived microsomes were treated with 0.1M sodium carbonate, pH 11.5, and the pellet (P) and soluble (S) fractions were isolated. Ybr074 and Sec61 are transmembrane proteins, and Pdi1 is a peripheral ER membrane protein. * denotes a soluble non-specific protein recognized by rabbit anti-Sec61 antibody. C) Multiple sequence alignment between M28 metalloprotease domains of Ybr074, GI: 341941012; the human ortholog, ERMP1, GI: 55749804; and the rat ortholog,
FXNA, GI: 42476352. Boundaries for the protease domain sequence were determined using Pfam. Catalytic residues are underlined. Note that the conserved M28 catalytic motifs, His-Phe-Asp and Glu-Glu, are present in Ybr074, ERMP1, and FXNA.

Analysis of Ybr074 by multiple sequence alignment and domain searching using the Protein family database (Pfam) uncovered a conserved M28 family metalloprotease domain (Punta et al, 2012). The M28 family of metalloproteases contains both amino- and carboxy-peptidases whose enzymatic activities depend on a pair of co-catalytic zinc ions and the catalytic residues His-Xaa-Asp and Glu-Glu, which are required for catalysis (Fundoiano-Hershcovitz et al, 2004; Rawlings & Barrett, 1995; Rawlings et al, 2012).

Based on the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System version 7.2, YBR074W is a member of a gene family known as FXNA-related (PTHR12147), which includes 46 orthologous genes from 22 different species (Table 5) (Mi et al, 2010; Thomas et al, 2003). The human ortholog of YBR074W is known as Endoplasmic Reticulum MetalloPeptidase 1 (ERMP1). Its putative residence at the ER is inferred based on sequence similarity with the rat ortholog known as Felix-ina (FXNA). A C-terminally Flag tagged FXNA construct expressed in COS7 cells was shown by Garcia-Rudaz et al. to co-localize with the ER marker Protein Disulphide Isomerase (PDI). Furthermore, knock-down of FXNA using shRNA delivered using a lentivirus vector to rat ovaries was shown to lead to defects in ovarian follicle development by an unknown mechanism (Garcia-Rudaz et al, 2007). It was therefore hypothesized that Ybr074 would also be an ER-resident protein. In fact, PSORT, a program for the prediction of protein localization, suggested the Ybr074 most likely resided in the ER (Nakai & Horton, 1999). However, it is important to note that a proteomic
analysis of yeast vacuolar membranes found Ybr074 enriched there, suggesting Ybr074 may be a vacuolar protease (Wiederhold et al, 2009). In order to resolve this discrepancy, the localization of Ybr074 will be analyzed later in this chapter.

Table 5: Members of the FXNA-related gene family.

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As a first step toward testing the hypothesis that Ybr074 is an ER-resident protein, the presence of N-linked glycans on Ybr074 was examined, as it is known that these post-translational modifications occur within the ER (Lederkremer, 2009; Schwarz & Aebi, 2011). To test this hypothesis, microsomes containing 3HA-Ybr074 were treated with Endo H, which cleaves N-linked glycans that lack extended chains from proteins and causes a downward shift as observed by SDS-PAGE. Indeed, treatment of Ybr074 with Endo H produced a protein band shift of from ~147 kDa to ~123 kDa (Figure 8). Given that each N-linked glycan is estimated to contribute ~3 kDa to the molecular mass of a protein, this shift corresponds to removal of roughly 8 N-linked glycans (Helenius & Aebi, 2004). This is less than the 10 N-linked glycosylation sites on Ybr074, which are predicted to be lumenal based on ConPredII analysis. However, it is possible that some N-linked glycosylation sites are inaccessible to glycosylation because the required enzymes are sterically hindered from modifying Asn residues near the

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membrane, or because the tertiary structure precludes modification. It was also noted that the molecular mass of Endo H-treated Ybr074 was higher than predicted (114 kDa). It is possible that Ybr074 may bear additional post-translational modifications, such as O-linked glycosylation or phosphorylation, which were not examined. More trivially, membrane proteins often resolve by SDS-PAGE with migrations that do not correspond precisely to their masses. Furthermore, calculations of shifts in molecular mass may be limited by the resolution of the gel, and therefore should be considered as estimates rather than exact measurements.

Figure 8: Ybr074 is modified by N-linked glycans.

ER-derived microsomes were prepared from strains expressing an N-terminally HA-tagged Ybr074 from its endogenous promoter on a CEN/ARS plasmid. The microsomes were either Endo H treated or left in buffer, as indicated. The apparent molecular mass of glycosylated Ybr074 is 147 kDa. The apparent molecular mass of unglycosylated Ybr074 is 123 kDa. The difference in molecular mass between the glycosylated and unglycosylated forms of Ybr074 suggests that eight of the ten predicted glycosylation sites are modified by N-linked glycans.
In order to understand the biological function of Ybr074, it was important to determine on which side of the membrane its protease domain resides. The computationally predicted topology calculated using ConPredII suggested that the M28 protease domain of Ybr074 faces the ER lumen. In order to test this hypothesis, microsomes were isolated from yeast expressing tagged Ybr074 constructs. These microsomes represent crude membrane fractions containing both ER and vacuolar membranes. Microsomes isolated from strains expressing Ybr074 HA-tagged at the N-terminus, at the M28 protease domain, or at the C-terminus were subjected to a protease protection assay. Constructs in which the HA-tag is positioned outside the microsome should be susceptible to degradation by a protease. Conversely, constructs in which the HA-tag is positioned inside the microsome should be protected from degradation by proteinase K, unless Triton X-100 is included in the reaction to disrupt the integrity of the microsome membrane.

Western blot analysis of N-terminally HA-tagged Ybr074-containing microsomes indicated that the N-terminus of Ybr074 is mostly protected from degradation by Proteinase K (Figure 9). Proteinase K-treated 3HA-Ybr074 is observed to shift to a lower molecular mass as compared to microsomes that were not treated with proteinase K. This is indicative of partial degradation of Ybr074 in the presence of proteinase K, although the HA tag itself was not degraded. However, addition of Triton X-100 resulted in near complete degradation of the N-terminal HA tag after 30 min of Proteinase K treatment. Since the N-terminus is computationally predicted to face the cytosol, and therefore to be outside the microsome and susceptible to Proteinase K treatment, this result was surprising. However, the N-terminus, which is predicted to be only 14 amino acids in length, may be too closely associated with the membrane, resulting in its protection from Proteinase K. Although unlikely, an alternative explanation is that the first transmembrane segment (TM1) may not span the membrane as predicted, or it may be cleaved
by signal peptidase in the ER via a non-canonical cleavage site. To further test these possibilities, a strain expressing GFP-Ybr074 was also tested. This construct expresses a longer GFP moiety that is appended to the protein’s N-terminus, (238 amino acids, GI: 262348071). A protease protection assay using this construct showed the N-terminus of Ybr074 was not protected from Proteinase K digestion, consistent with the N-terminus facing the cytosol.

The Ybr074 construct tagged using HA within the M28 domain was also protected from degradation by proteinase K except when treated together with Triton X-100 to disrupt microsome integrity (Figure 9). This suggests that the M28 protease domain is lumenal, as predicted. However, it should be noted that the position of the HA tag is also predicted to be close to the membrane (approximately 10 amino acids), similar to the N-terminally HA-tagged Ybr074 construct. Therefore, protection from proteinase K treatment may also be the result of close association with the microsome membrane or association with Ybr074’s tertiary structure. Nevertheless, this region also harbors many of the predicted N-linked glycosylation sites, adding further evidence that this domain is lumenal.

The C-terminally HA-tagged Ybr074 construct appeared to be resistant to the protease even after treatment for 60 min in the presence of Triton X-100. This suggests that the C-terminal domain may assume a stable fold resistant to degradation. However, some degradation was observed in protease treated microsomes as compared to untreated microsomes, suggesting that the C-terminus of Ybr074 is also lumenal, as predicted.
Figure 9: Assignment of Ybr074 topology by protease protection assay

Samples were treated in the presence or absence of 100μg/mL proteinase K and Triton X-100, as indicated. The cytosolic membrane anchored protein, Bos1, and the lumenal protein, Kar2 were used as controls for protease sensitivity and protection, respectively. A) A protease protection assay was performed using ER-derived microsomes from yeast expressing N-terminally HA-tagged Ybr074 (CEN vector), Ybr074 HA-tagged within the M28 domain (2μ vector), and C-terminally HA-tagged Ybr074 (2μ vector). Each protein was expressed from its endogenous promoter. B) Protease protection of chromosomally integrated GFP-Ybr074 expressed from a galactose-inducible promoter.
Figure 10: Sub-cellular localization of Ybr074 as assessed using indirect immunofluorescence microscopy.

Yeast expressing HA-tagged Ybr074 in the putative M28 protease domain or at the N-terminus were processed as described in the Methods section. A) A mouse anti-HA antibody was used to detect Ybr074, and is shown in green in the merged images. A rabbit anti-Kar2 antibody was used to detect the ER-resident protein Kar2, and is shown in red in the merged images. Nuclear staining using DAPI is shown in blue. B) A rabbit anti-HA antibody was used to detect Ybr074, which is shown in red in the merged images. A mouse anti-Vph1 antibody was used to detect the vacuolar protein, Vph1, which is shown in green in the merged images.
As previously mentioned, although Ybr074 was computationally predicted to be ER-localized and its rat ortholog, FXNA, was found in the ER of COS7 cells, a proteomic analysis of yeast vacuolar membranes suggested Ybr074 was a vacuolar protein (Garcia-Rudaz et al, 2007; Wiederhold et al, 2009). In order to examine the sub-cellular localization of Ybr074, HA-tagged constructs were subjected to indirect immunofluorescence microscopy. I also examined the localization of the ER marker, Kar2, and the vacuolar membrane marker, Vph1. Both Ybr074 constructs tagged at the N-terminus or the putative M28 protease domain showed strong co-localization with the vacuolar membrane marker, Vph1, suggesting that Ybr074 is a vacuolar resident protein (Figure 10). Interestingly, a small proportion of Ybr074 was also observed to co-localize with Kar2, as exemplified in Figure 10. Specifically, a faint peri-nuclear ring, corresponding to the ER, is visible in the Ybr074 staining shown in the panels labeled “M28HA” and “N_{term}-HA” of Figure 10A, and in the panel labeled “M28HA” of Figure 10B. This is consistent with the Endo H sensitivity data indicating that Ybr074 traffics through the ER (Figure 8).
Figure 11: Live cell imaging of GFP-Ybr074 localization.

Live cell images are shown using a wild type strain, KH1, expressing a chromosomally-integrated form of GFP-tagged Ybr074 from a galactose-inducible promoter (see Table 3). GFP-Ybr074 expression was induced with galactose, and raffinose was used as a negative control. Differential Interference Contrast (DIC) images are shown on the left, with vacuoles appearing as a lighter shade due to their lower refractive index compared to the cytosol. The images on the right show GFP fluorescence only in galactose-treated cells.
As further support of Ybr074 vacuolar localization, a chromosomally-integrated GFP-tagged form of Ybr074 (GFP-Ybr074) was over-expressed from a galactose-inducible promoter. As shown in Figure 11, GFP-Ybr074 also showed vacuolar membrane localization. Furthermore, the proteomic study that identified Ybr074 in isolated vacuolar membranes did not rely on epitope tagging (Wiederhold et al, 2009).

In contrast to localization data shown in Figure 10, Ybr074 HA-tagged at the C-terminus appears to co-localize with the ER marker, Kar2 by indirect immunofluorescence microscopy (Figure 12). These data suggest that appending an HA epitope to the C-terminus of Ybr074 interferes with the vacuolar localization of Ybr074 (also see Discussion Chapter 5).
A) Ybr074-HA co-localizes with the ER marker, Kar2, forming at a peri-nuclear position. The vacuole appears as a circular depression by DIC imaging. In the merged image, DAPI signal, marking the nucleus, is shown in blue; Kar2 signal, corresponding to the ER, is shown in red; and Ybr074-HA signal is shown in green. The signal is yellow where Kar2 and Ybr074-HA signals overlap. B) Ybr074-HA signal was observed as a peri-nuclear ring, typical of ER protein staining. The vacuolar marker, Vph1, was localized to a ring coinciding with the vacuole observed by DIC imaging. The merged image shows the DAPI signal in blue, the Ybr074-HA signal in red, and the Vph1 signal in green.
If a C-terminally tagged Ybr074 construct is inappropriately retained in the ER, it might be expected to be less stable than a Ybr074 construct that is correctly targeted to the vacuole. To test this hypothesis, cycloheximide chase analyses were conducted to compare the stabilities of N-terminally and C-terminally HA-tagged Ybr074 constructs.

The C-terminally HA-tagged Ybr074 construct, found in the ER, was significantly less stable than the vacuolar N-terminally HA-tagged Ybr074 in wild type cells (Figure 13A). To determine if the vacuole plays a role in the degradation of Ybr074, the stability of Ybr074 was examined in a pep4Δ strain. Since Pep4 (proteinase A) is required for the proteolytic activation of many vacuolar proteases, disruption of Pep4 activity impairs vacuolar proteolytic function (van den Hazel et al, 1992). Results indicate that C-terminally tagged Ybr074 is dependent on vacuole activity for degradation, while N-terminally tagged Ybr074 stability is not affected by in the absence of Pep4 activity (Figure 13A).

To determine whether degradation of Ybr074 was dependent on the 26S proteasome, Ybr074 stability was examined in a pdr5Δ strain in which deletion of the multidrug transporter, Pdr5, allows cells to retain the 26S proteasome inhibitor MG132 (Collins et al, 2010). While C-terminally tagged Ybr074 was significantly stabilized when 26S proteasome activity was inhibited by MG132, stability of the N-terminally tagged Ybr074 was not dependent on 26S proteasome activity (Figure 13B).
A) Wild type (WT) and pep4Δ strains expressing N-terminally HA-tagged (HA-Ybr074) or C-terminally HA-tagged (Ybr074-HA) Ybr074 constructs were analyzed by cycloheximide chase analysis. B) A pdr5Δ strain expressing HA-Ybr074 or Ybr074-HA were treated for 30 min at 30°C with DMSO or 100 μM MG132 before addition of cycloheximide. * = p<0.05
2.4 CONCLUSIONS

Computational analyses indicated that Ybr074 is a member of the FXNA-related family of proteins, harbors a protease domain, and belongs to the M28 family of metalloproteases. Ybr074 was demonstrated to be a transmembrane protein and is glycosylated, indicating that Ybr074 trafficks through the ER. Protease protection assays suggest that the M28 and C-terminal domains of Ybr074 are lumenal, as expected. Surprisingly, the HA-tagged N-terminus appeared lumenal as well, either suggesting that the topological prediction was incorrect, or that the N-terminal epitope was not accessible to protease due to proximity to the membrane. A protease protection assay conducted using Ybr074 tagged with GFP at the N-terminus showed the N-terminus is cytosolic. Since the larger GFP tag is likely more accessible to digestion by proteinase K, this result may be more reliable and is consistent with the predicted topology of Ybr074. Furthermore, Ybr074 was shown both by indirect immunofluorescence microscopy and live cell imaging to be a vacuolar membrane protein. This is contrary to its computationally predicted ER localization. The localization of Ybr074 contrasts to that reported for FXNA, which was shown to be ER-localized in COS7 cells using a C-terminally FLAG-tagged FXNA construct (Garcia-Rudaz et al, 2007). However, it is worth noting that FXNA and Ybr074 may display cross-specifies differences. Furthermore, appending a tag at the C-terminus of Ybr074 causes this protein to be targeted for degradation in a 26S proteasome and vacuole dependent manner. Therefore, it is likely that Ybr074 is normally targeted to the vacuole. It is important to emphasize that an untagged Ybr074 was found enriched in the vacuolar membrane by proteomic analysis (Wiederhold et al, 2009). Therefore, appending a C-terminal tag to Ybr074 may destabilize the protein and cause the quality control machinery to prevent its trafficking to the vacuole by targeting Ybr074 for degradation. This scenario is discussed in Chapter 5.
3.0 ATTEMPTS TO DEFINE THE FUNCTION OF YBR074

3.1 INTRODUCTION

In parallel to discovering that Ybr074 is most likely a vacuolar protein, several approaches were taken to test the role of Ybr074 in ER quality control. In this chapter, I used molecular, genetic, phylogenomic, and proteomic methods to ask if Ybr074 participates in the degradation of ERAD substrates, to identify Ybr074’s genetic interactions, and to search for Ybr074 substrates. As my extensive data suggest, Ybr074 does not affect the degradation of any ERAD substrates tested. Genetic analysis indicated a potential role for Ybr074 is maintenance of cell wall integrity. Although proteins exhibiting evolutionary co-variance with Ybr074 using phylogenomic analysis did not produce statistically significant enrichment of any Gene Ontology (GO) terms, the data did support a potential role for Ybr074 is affecting trafficking of proteins involved in cell wall maintenance. Finally, a proteomic analysis revealed a potential Ybr074 substrate, Rhr2, but further analysis showed that Rhr2 is not a Ybr074 substrate.
3.2 MATERIALS AND METHODS

3.2.1 Strains

Deletion of *YBR074* in the BY4742 and BY4741 backgrounds were carried out using Polymerase Chain Reaction (PCR)-mediated gene disruption (Brachmann et al, 1998). Briefly, the BY4742 strain was transformed with a *ybr074::KANMX* cassette which was amplified from the pRS400 vector (forward primer: AAA TTA TCT ACA AGG AAA TAA ATT GAT AGG TAA AGT TAA AGA ATC ACG GCA GAT TGT ACT GAG AGT GCA C; reverse primer: CAG TAG GCG AAT TTG AGT TTA TAA AAA TTT ACA TTT AAA ACT AAT TAG AAC TGT GCG GTA TTT CAC ACC G). Transformants were selected on yeast-peptone containing 2% dextrose (YPD) and 250 μg/mL Geneticin (G-418; Invitrogen, Carlsbad, CA). Colonies incorporating the *ybr074::KANMX* cassette were verified by PCR using primers flanking the *YBR074* open reading frame (ORF) (upstream primer: CAA TGA CGC CAA ATA TGG ACA CT; downstream primer: AAG AGA GCA CCG TAG AAT GGT T). To generate the *ybr074::NATMX* strain, the *NATMX* cassette was PCR amplified from the *pFA6a-NAT-MX6* vector using Forward primer: CAA GGA AAT AAA TTG ATA GGT AAA GTT AAA GAA TCA CGG CCG GAT CCC CGG GTT AAT TAA and Reverse primer: ATT TGA GTT TAT AAA AAT TTA CAT TTA AAA CTA ATT AGA AGA GCT CGT TTA AAC TGG ATG (Vembar et al, 2010). The *NATMX* cassette was transformed into BY4742 and BY4741 strains as described (Adams et al, 1998). Cells were selected on YPD medium supplemented with nourseothricin (NAT), and integration of the *NATMX* cassette was verified by PCR using the same primers used to check for integration of the *KANMX* cassette.
3.2.1.1 Generating double mutant strains by yeast mating

To generate double mutant strains, such as the \( cdc48-2 \) \( ybr074Δ \) strain, yeast mating was conducted as in the example that follows. The \( cdc48-2 \) \( \text{MAT} \alpha \) strain (OY209) was mixed with \( ybr074::\text{KANMX} \) strain in a patch on solid YPD medium containing 2% Bacto agar. Parental strains were allowed to mate overnight at room temperature before selecting for diploids on YPD containing 250 μg/mL G-418 and 100 μg/mL NAT (clonNAT; Werner BioAgents, Jena, Germany) for 3 d at room temperature. Diploid colonies were patched onto GNA presporulation media (3% Difco nutrient broth, 1% Difco yeast extract, 5% dextrose, 2% Bacto agar; BD, Sparks, MD) overnight at room temperature before being transferred to liquid sporulation media (2% potassium acetate, 0.005% zinc acetate) and incubated in a roller drum for 3-7 d at room temperature. Spores were then dissected and genotyped as described (Adams et al, 1998). Strains KH01, KH02, and KH03 were isolated by this same method (Table 3).

3.2.2 Growth Assays

The growth curve assay was conducted using wild type \( \text{BY4742} \), \( ybr074Δ \), \( pdr5Δ \), and \( ybr074Δpdr5Δ \) strains, which were either grown in YPD or transformed with a plasmid engineered for the expression of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), and grown in selective media lacking uracil (Zhang et al, 2001). Cells were grown at 30°C to log phase before being diluted back to \( \text{OD}_{600} = 0.15 \). The cells were then incubated in the presence or absence of 25 μM MG132 (Peptides International, Louisville, KY) and \( \text{OD}_{600} \) readings collected every 40 min for 6 h.
Serial dilution growth assays were conducted by harvesting 0.5 OD$_{600}$ equivalents from logarithmic phase cultures grown in YPD. Cells were washed in sterile double distilled water (SDDW), re-suspended in SDDW at 1 OD$_{600}$/mL, and used to make 10x serial dilutions in SDDW. Samples were spotted onto media containing 50 μg/mL calcofluor white (CFW; Sigma, St. Louis, MO) or 50 μg/mL Congo Red (CR; Sigma, St. Louis, MO), with or without 1M sorbitol, and grown at the indicated temperatures for 4 d.

The assay for adhesion and invasion of agar was conducted using the wild type strain Y285 and the _ybr074Δ_ strain in the Y285 strain background, as described (Guldal & Broach, 2006). Briefly, cells were grown to saturation overnight in YPD medium at 30°C and diluted back to OD$_{600}$= 1.5 the next day. A total of 200 μL of cells were spotted onto synthetic complete solid medium supplemented with 2% dextrose, or 0.2% dextrose. Cells were grown at 30°C for 5 d and then rinsed with distilled water to observe adhesion onto agar medium. To observe invasion of the agar, cells on the surface of the agar medium were removed in distilled water by rubbing the surface of the plate with a gloved finger. Growth, adhesion, and invasion of agar were captured using the Epson Perfection 3490 photo scanner.

Qualitative mating assays were performed as described (Shei & Broach, 1995). Briefly, _MAT α_ and _MAT a_ strains were grown to logarithmic phase (OD$_{600}$= 0.4-0.8) and 0.5 OD$_{600}$ cell equivalents of each were collected, washed in SDDW, and re-suspended to 1 OD$_{600}$/mL in SDDW. Serial dilutions of _MAT α_ cells were mixed with 200 μL of _MAT a_ cells, and _vice versa_. The serial dilutions containing both _MAT α_ and _MAT a_ cell were plated on synthetic media lacking lysine and methionine to select for diploids, synthetic media lacking lysine to select for _MAT a_ cells, and synthetic media lacking methionine to select for _MAT α_ cells.
3.2.3 Molecular Techniques

The 3HA-Gas1* expression construct was generated by sub-cloning $P_{GAS1}$-3HA-GAS1-$T_{GAS1}$ from the pMF616 integration plasmid, described in Table 3, using the BamHi/SpeI sites and ligated into the pRS315 expression vector. The construct was verified by DNA sequence analysis (IDT, Coralville, IA).

$RHR2$ was PCR amplified from genomic DNA isolated from the BY4742 strain as described, using Forward primer: GAT CGG ATC CCC TTT GAC CAC AAA ACC TTT ATC TTT G, and Reverse primer: GAT CGA ATT CTT ACC ATT TCA ACA AGT CAT CCT TAG CGT (Adams et al, 1998). Amplified $RHR2$ was inserted into the pKN16 expression vector by ligation using BamHI/XhoI sites. The construct was verified by DNA sequence analysis (IDT, Coralville, IA).

3.2.4 Assays examining ER-Associated Degradation (ERAD)

The assays described in this section aim at testing the role of Ybr074 in ERAD, and assessing whether it acts in synergy with the 26S proteasome, as shown in Figure 12.

3.2.4.1 Cycloheximide chase analyses

Vectors expressing the indicated ERAD substrates were transformed into the specified yeast strains and grown in selective media lacking uracil or leucine, as required (see Table 6). Alternative transformation methods were used to transform the ybr074Δcdc48-2 strain, which could not be transformed using the standard LiAc method (Adams et al, 1998). The CPY*
expression vector was introduced by mating ybr074Δ and cdc48-2 strains containing the CPY*-3HA expression vector, detailed in Table 6. Strain construction by yeast mating, sporulation, tetrad dissection, and genotyping were conducted as described (Adams et al, 1998). To express Gas1* in the ybr074Δcdc48-2 strain, cells were transformed with both the LEU2-marked Gas1* expression vector (Table 6), and the URA3-marked 3HA-Ybr074 expression vector (Table 3). Cells were grown on selective media lacking both uracil and leucine, followed by growth on media lacking leucine and supplemented with 5-fluoroorotic acid (5-FOA) to counter-select for the URA3-marked 3HA-Ybr074 expression plasmid (Boeke et al, 1987). Finally, cells containing only the Gas1* expression vector were grown on media lacking leucine. Cells expressing the desired ERAD substrate were grown to logarithmic phase (OD600 = 0.4-0.8) and treated by one of three methods illustrated in Figure 14A. Briefly, cells were either concentrated by centrifugation at 1,000 g for 5 min, and re-suspended to 1 OD600/mL, or transferred directly to a water bath at the indicated temperature without concentrating the cells. At the various temperatures tested, cells were either immediately treated with 250 μg/mL cycloheximide, or pre-incubated for 90 min before the addition of cycloheximide (Sigma, St. Louis, MO). At the indicated times, 1 mL from each culture was transferred to a tube containing sodium azide at a final concentration of 20 mM. Cells were harvested by centrifugation at 18,000 g for 30 sec at 4°C and then washed with 1mL of ice cold 10 mM sodium azide to block respiration. Cells were centrifuged as above, the supernatant was aspirated, and the cell pellets were flash frozen in liquid nitrogen before being processed the next day by TCA precipitation, according to an established protocol (Nakatsukasa et al, 2008).
Table 6: ERAD substrate expression vectors

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Description</th>
<th>Selectable marker</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSM1152</td>
<td>CFTR-3HA overexpression vector</td>
<td><em>URA3</em></td>
<td>Zhang, Y. et al. 2002</td>
</tr>
<tr>
<td>3HA-Gas1p*</td>
<td>3HA-Gas1p* expression vector</td>
<td><em>LEU2</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMF616</td>
<td>3HA-Gas1p* integration vector</td>
<td><em>URA3</em></td>
<td>Fujita, M. <em>et al.</em> 2006</td>
</tr>
<tr>
<td>pSM1911</td>
<td>Ste6p*-HA overexpression vector</td>
<td><em>URA3</em></td>
<td>Huyer, G. <em>et al.</em> 2004</td>
</tr>
<tr>
<td>CPY*-3HA</td>
<td>CPY*-3HA expression vector</td>
<td><em>URA3</em></td>
<td>Bhamidipati, A.<em>et al.</em> 2005</td>
</tr>
<tr>
<td>CPY*-DHFR</td>
<td>CPY*-DHFR-3HA expression vector</td>
<td><em>URA3</em></td>
<td>Bhamidipati, A.<em>et al.</em> 2005</td>
</tr>
<tr>
<td>CPY*-DHFR(Pro)</td>
<td>CPY*-DHFR(Pro)-3HA expression vector with DHFR mutations A29P, W30P, F31P</td>
<td><em>URA3</em></td>
<td>Bhamidipati, A. <em>et al.</em> 2005</td>
</tr>
</tbody>
</table>
3.2.4.2 Cycloheximide pulse chase analyses

To examine whether Ybr074 contributes to the degradation of CPY*-DHFR, CPY*-DHFR(Pro), or CFTR, a cycloheximide pulse chase analysis was conducted using the indicated strains as described (Brodsky et al, 1998; Zhang et al, 2001). Briefly, cells transformed with CPY*-DHFR or CPY*-DHFR(Pro) were grown in selective medium lacking uracil and supplemented with glucose to an OD$_{600}$ ~ 0.5. Cells were concentrated to 10 OD$_{600}$ cell equivalents per mL and equilibrated to 30°C for 30 min. Cells were then labeled using Express $^{35}$S (Perkin-Elmer, Boston, MA) at a final concentration of 100 μCi/mL for 10 min at 30°C. Protein synthesis was quenched using cycloheximide at a final concentration of 200 μg/mL, and 4 OD$_{600}$ cell equivalents were collected at the indicated time points. The cells were washed in B88 and pellets were processed by glass bead lysis in extraction buffer (50 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM EDTA, 1 mM PMSF, 1 ug/mL pepstatin and leupeptin). The resulting cell homogenate, containing ~10$^7$ cpm in total, was incubated with mouse anti-HA (Roche, Indianapolis, IN) antibody overnight at 4°C and then washed before adding Protein A Sepharose (GE Healthcare, Uppsala, Sweden) and incubating for 1-2 h. Immunoprecipitates were washed and the proteins were resolved by 10% SDS-PAGE followed by phosphorimage analysis.
3.2.5 Analysis of phylogenetic profiling data

*Saccharomyces cerevisiae* genes whose Evolutionary Rate Covariation (ERC) clustered with that of Ybr074 were kindly calculated by Dr. Nathan L. Clark (Dept. of Computational and Systems Biology, University of Pittsburgh, Pittsburgh, PA) as described (Clark et al, 2012).

3.2.6 Proteomic analysis methods

Cell lysates were prepared on-site and subjected to 2D DiGE by Applied Biomics, Inc., Hayward, CA. The BY4741 and ybr074::NATMX MAT a strains were grown in YPD at 30°C to OD$_{600}$= 0.6. Cells were harvested by centrifugation at 4,900 g (5,500 rpm) for 5 min at 4°C, using a Sorvall RC 5B Plus centrifuge and a GSA rotor. Cell pellets were re-suspended in 10 mL PBS, pH 8, and harvested by centrifugation at 5,000 g (6,500 rpm) for 5 min at 4°C, using a Sorvall RC 5B Plus centrifuge and an SS-34 rotor. The final cell pellets were re-suspended in 1 mL standard lysis buffer containing 8M urea, 1% CHAPS, 10 mM Tris-HCl, pH 8, 5mM magnesium acetate at 4°C. Cells were sonicated using the VirTis VirSonic 475 sonicator at 10% output power with three 10 sec pulses with 1 min intervals at 4°C between each pulse. Cell lysate were flash frozen in liquid nitrogen and delivered to Applied Biomics, Inc., for further processing, as described (May et al, 2012). Briefly, cell lysates were labeled with size and charge matched CyDye DiGE fluorophores to enable separation of both samples on the same 2D gel. Cell lysate from the BY4741 strain was labeled with Cy2 dye, and cell lysate from the ybr074Δ strain was labeled with Cy3 dye. Cell lysates were separated by isoelectric focusing (IEF) in the first dimension, followed by SDS-PAGE in the second dimension. The gel was scanned with a Typhoon image scanner to visualize Cy2 dye and Cy3 dye signals. ImageQuant software was
used to generate images of each sample and the overlayed samples. Quantitative comparative analysis of all the protein spots on the gel were analyzed using DeCyder “in gel” software to calculate protein expression ratios across the two samples using a threshold value of 1.3. Protein spots chosen for identification by mass spectrometry were picked using the Ettan Spot Picker.

To test whether Rhr2 was a Ybr074 substrate, BY4742 and ybr074Δ strains containing the empty vector pKN16 or the 3HA-Rhr2 overexpression vector (see Table 4) were grown to logarithmic phase (OD₆₀₀= 0.4-0.8) at 30°C in synthetic complete media lacking uracil and supplemented with 2% glucose. Cells were harvested by centrifugation at 1000 g at room temperature and re-suspended in synthetic media lacking uracil and supplemented with 2% galactose to induce Rhr2 expression at 30°C for 6.5 h. Cells were harvested by centrifugation at 1000 g and processed by TCA precipitation, as above. Cell lysates were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose. Protein was visualized by Western blot analysis using HRP-conjugated anti-HA antibody at 1:5000 for 1 h at room temperature (Roche Diagnostics, Indianapolis, IN). Glycerol-3-phosphate dehydrogenase (G6PDH) was used as a loading control, and was detected using anti- G6PDH antibody at 1:5000 for 1 h at room temperature (Sigma, St. Louis, MO). The bound antibodies were visualized using Super Signal West Pico kit (Thermo Scientific, Rockford, IL) on a Kodak 440CF Image Station (Eastman Kodak, Rochester, NY).

To identify putative Ybr074 substrates, an analysis of a GFP-tagged protein library in the ybr074Δ strain background was conducted by members of the Schuldiner lab, Department of Molecular Genetics, The Weizmann Institute of Science Rehovot, Israel as described (Cohen & Schuldiner, 2011). Briefly, the ybr074Δ::NATMX strain was mated with the GFP-tagged MAT a collection using Synthetic Genetic Array (SGA) techniques as described (Cohen & Schuldiner, 2011; Tong & Boone, 2006). Haploid cells expressing a GFP-tagged protein in the ybr074Δ
background were selected and grown to logarithmic growth phase in synthetic complete medium at 30°C in 384-well growth plates. Cells were then transferred to 384-well microscope plates (Matrical Bioscience) and visualized using an automated inverted fluorescence microscope ScanR system (Olympus) using a swap robot (Hamilton). Images were then manually reviewed using ScanR analysis program and processed by Adobe Photoshop CS3 software.

3.3 RESULTS

3.3.1 Is Ybr074 a quality control protease?

Based on computational predictions, I initially hypothesized that Ybr074 is an ER-resident protease that may help degrade terminally misfolded proteins in the ER. To test this hypothesis, a number of assays to measure the degradation of ERAD substrates were employed. First, cycloheximide chase analyses were conducted, monitoring the degradation of CFTR, Ste6p*, CPY*, and Gas1p* in wild type and ybr074Δ strains. Second, pulse chase analysis of the ERAD substrate CPY* fused to a stably folded dihydrofolate reductase (DHFR) domain (CPY*-DHFR), or an unstable mutant form of DHFR (CPY*-DHFR(Pro)), were examined for Ybr074-dependent differences in their degradation fragment profiles. Third, a pulse chase analysis was used to examine the fragments resulting from the ERAD of CFTR in strains in which the function of Ybr074, the 26S proteasome, or both were disrupted. Fourth, to determine whether Ybr074 and the 26S proteasome function in parallel degradation pathways under conditions of ER stress, the growth of cells expressing CFTR was investigated in the presence or absence of the 26S proteasome inhibitor, MG132, in wild type or ybr074Δ strains.
Figure 14: The cytosolic 26S proteasome and lumenal Ybr074 putative protease

The 26S proteasome functions in the cytosol, while the putative proteasome domain of Ybr074 faces the lumen. Do these proteases cooperate in the degradation of integrated membrane ERAD substrate on either side of the ER membrane?

3.3.1.1 Does Ybr074 function as a general protease during ERAD?

One third of all proteins enter the secretory pathway through the ER, where nascent proteins undergo folding and maturation (Ghaemmaghami et al, 2003). These proteins are either retained in the ER, where their function is required, or they are targeted for vesicle-mediated transport to an alternative sub-cellular compartment (Marie et al, 2008; Mellman & Warren, 2000). Proteins that fail to fold correctly, due to mutations, environmental stress, or spontaneous misfolding, accumulate in the ER and are targeted for degradation via ERAD (Smith et al, 2011;
When this quality control mechanism was first described, it was thought that a degradation system consisting of one or more proteases residing in the ER was responsible for this phenomenon (Klausner et al, 1990; Lippincott-Schwartz et al, 1988). However, ER-lumenal proteases responsible for ER degradation were never identified, and in 1996, McCracken and Brodsky demonstrated that terminally misfolded proteins were selectively retrotranslocated from the ER and degraded in the cytosol (McCracken & Brodsky, 1996). Later, it was demonstrated that the cytosolic 26S proteasome was responsible for the degradation of ERAD substrates (Vembar & Brodsky, 2008; Werner et al, 1996). In this section, the hypothesis that Ybr074 represents an ER quality control protease was tested by comparing the ability of wild type and ybr074Δ strains to degrade a number of known ERAD substrates.

In order to optimize conditions for the cycloheximide chase protocol, several variables were tested using the ERAD substrate CFTR: strain background, temperature, and dilution. A study of genetic diversity in commonly used laboratory strains and wild strains of yeast has identified over 10,000 “single-feature polymorphisms” (SFPs) contributing to allelic variation across yeast strains. Among numerous genes associated with these SFPs, 160 ORFs were related to proteolytic degradation, and 59 ORFs were related to protein folding and stability (van Dijken et al, 2000; Winzeler et al, 2003). Therefore, Ybr074-dependent effects on the ERAD of CFTR were tested in BY4742, W303, and YPH499 strain backgrounds. Because no Ybr074-dependent effect on CFTR degradation was observed in these strain backgrounds, all subsequent experiments were conducted in the BY4742 background (Figure 15B).

Previous studies of CFTR degradation have been conducted at 30°C, 37°C, or 40°C because temperature affects the efficiency of CFTR folding, the expression of ERAD components, and temperature-sensitive mutant alleles that may be relevant to the experiment
Cells are typically concentrated prior to addition of cycloheximide in order to enhance protein detection. However, centrifugation of cells causes a brief period of glucose starvation which is associated with a transcriptional response and activation of autophagy (Yorimitsu & Klionsky, 2007). Therefore, degradation of CFTR was tested at varying temperatures, with or without concentrating the cells prior to addition of cycloheximide. My results showed no Ybr074-dependent effect on CFTR degradation under the conditions tested. Therefore, subsequent experiments were conducted at 37°C without concentrating cells prior to addition of cycloheximide (Figure 15B).

The factors required for degradation of ERAD substrates can vary depending on the location of the lesion in the ERAD substrate. Lesions located in the cytosol, membrane, or lumen may be recognized by different factors, and may, in principle, exhibit differential dependence on Ybr074. Therefore, a variety of well characterized ERAD substrates were chosen to test this hypothesis: CPY* is a soluble protein found in the lumen, Gas1* is glycerophosphoinositol-(GPI) anchored protein closely associated with the ER membrane on the luminal side, Ste6p* is multi-spanning transmembrane protein with a cytosolic lesion, and CFTR is an exogenous mammalian transmembrane protein predominantly exposed to the cytosol.

The degradation of each of these ERAD substrates was examined by cycloheximide chase analysis in both wild type and ybr074Δ strains. No statistically significant difference in the degradation of any of the ERAD substrates tested was found across these strains. These data suggest the Ybr074 is not involved in ER quality control.
Figure 15: Optimization of cycloheximide chase analysis using the substrate CFTR

A) Cycloheximide chase experiments were conducted using logarithmic phase cultures that were either concentrated by centrifugation or not. Cultures were shifted from room temperature to 30°C, 37°C, or 40°C and cycloheximide was added either immediately, or after a 90 min pre-incubation period. B) Using experimental methods 1, 2, or 3 described above, wild type or mutant strains derived from the indicated genetic backgrounds were tested. The temperatures and number of replicates for each experiment are indicated. The ybr074Δ strain refers to the disruption of the entire YBR074 ORF, while ybr075Δ refers to disruption of the YBR074 coding sequence shown in blue in Figure 6. Results indicated in the first row of (B) were reported by Dr. A. Ahner (Ahner & University of Pittsburgh. School of Arts and Sciences, 2005), while I obtained all other results.
Figure 16: Ybr074 has no effect on the degradation of known ERAD substrates.

Results are shown as percentage protein remaining over time, ± SE. No statistically significant YBR074-dependent effect was found using the ERAD substrates above. A representative western blot of each ERAD substrate and the loading control, glucose-6-phosphate dehydrogenase (G6PDH) is shown for both wild type and ybr074Δ strains. A) Strains expressing CPY* were shifted from room temperature to 37°C for 1 h prior to addition of cycloheximide
(n=4). B) Gas1* degradation was analyzed as above (n=7). C) Strains expressing Ste6p* were shifted from room temperature to 37°C for 2 h prior to addition of cycloheximide (WT, n=3; ybr074Δ, n=4). D) Strains expressing CFTR were shifted from 30°C to 40°C for 1.5 h prior to addition of cycloheximide (n=2).

3.3.1.2 Does Ybr074 help resolve ERAD substrates with large luminal domains?

In order to translocate from the ER for proteasome-mediated degradation, it is thought that ERAD substrates must remain soluble, which is assisted by chaperone activity, and that any residual quaternary and tertiary structures must be disassembled (Nishikawa et al, 2001; Romisch, 2005). Soluble ERAD substrates, such as CPY*, depend on retrotranslocation channels, such as Sec61, Hrd1 or Der1 for dislocation (Carvalho et al, 2010; Knop et al, 1996; Ng et al, 2000). However, multi-domain proteins containing stably folded luminal domains may be physically impeded from passing through these retrotranslocation channels. As an example of this scenario, Weissman and colleagues designed an ERAD substrate comprised of CPY* fused at the C-terminus with E.coli DHFR (Bhamidipati et al, 2005). DHFR is ~20 kDa protein with a well characterized structure that has been shown in numerous studies to assume a stable conformation in the ER of both yeast and mammalian cells (Bhamidipati et al, 2005; Lee et al, 2001; Tirosh et al, 2003). It was observed that the CPY*-DHFR fusion protein was significantly stabilized in yeast as compared to CPY*. Furthermore, the DHFR domain was clipped by an uncharacterized protease and accumulated in the ER lumen (Bhamidipati et al, 2005). Therefore, I hypothesized that Ybr074 may play a role in resolving multi-domain ERAD substrates in order to facilitate their retrotranslocation. To test this hypothesis, wild type and ybr074Δ strains
expressing CPY*-DHFR, or CPY* fused to a destabilized mutant form of DHFR (CPY*-DHFR(Pro)) were examined for differences in the clipping of the resident DHFR motif.

Accumulation of two degradation fragments of ~40 kDa is evident by cycloheximide chase analysis of CPY*-DHFR (Figure 17A, C). These fragments are absent from the degradation profile of the destabilized CPY*-DHFR(Pro) substrate. I noted that the DHFR-CPY* fragments observed were a higher molecular mass than the previously published ~20 kDa fragments. This suggests there may be strain-dependent variation in the degradation profile of this ERAD substrate. In any event, degradation of CPY*-DHFR(Pro) was not dependent on Ybr074. No statistically significant difference in the degradation of CPY*-DHFR was observed when comparing wild type and ybr074Δ strains, except at 40 min (Figure 17B,D; p=0.048). It is possible that disruption of Ybr074 function causes an initial delay in CPY*-DHFR degradation, which is resolved at later time points by other proteases, or there is delivery of the accumulated substrate to the vacuole. However, my data suggest that Ybr074 is not required for the degradation of CPY*-DHFR. Furthermore, no statistically significant difference between wild type and ybr074Δ was observed when accumulation of CPY*-DHFR degradation fragments was quantified (Figure 17E; p>0.4).
Figure 17: Degradation of CPY*-DHFR(Pro) and CPY*-DHFR is not dependent on Ybr074

A) CPY*-DHFR(Pro)-HA and C) CPY*-DHFR-HA degradation was examined by cycloheximide chase. Degradation of full length B) CPY*-DHFR(Pro) and D) CPY*-DHFR was quantified at the indicated time points as a percentage of initial protein. Data from wild type strains is shown as black circles, and data from the ybr074Δ mutant are shown as white squares.

E) Accumulation of the two ~40 kDa CPY*-DHFR fragments over time is shown. Error bars represent SE n=3.
3.3.1.3 Do Ybr074 and the 26S proteasome function in synergy during the degradation of CFTR?

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is a mammalian cAMP-regulated chloride channel that normally trafficks through the secretory pathway to the plasma membrane of epithelial tissues. In mammalian systems CFTR is co-translationally translocated into the ER, where it folds and assumes a core-glycosylated precursor form of ~140kDa. CFTR assumes a complex structure, consisting of two six-pass transmembrane domains, two cytosolic nucleotide binding domains, and a cytosolic regulatory domain. It is an intrinsically unstable protein, and ~75% of the wild type CFTR synthesized in mammalian cells is targeted for degradation by ERAD (Jensen et al, 1995; Ward & Kopito, 1994; Ward et al, 1995). A CFTR expression system in yeast was developed in the Brodsky lab and used to show that Hsp70, Hsp40, Hsp90, small heat shock proteins, and the peptidyl-prolyl-isomerase, FKBP8, are important factors modulating quality control of CFTR (Ahner et al, 2007; Hutt et al, 2012; Youker et al, 2004; Zhang et al, 2001). Yeast expressing exogenous wild type CFTR showed no growth defect when compared to yeast in which this mammalian protein is not expressed (unpublished observation). As occurs in mammals, CFTR is targeted for degradation by ERAD in yeast, demonstrating that yeast cells are able to respond to the introduction of this foreign and poorly folded protein (Zhang et al, 2001). Interestingly, the transcriptional response known as the Unfolded Protein Response (UPR), which is normally induced in response to ER stress is not triggered upon expression of CFTR in yeast (Zhang et al, 2001). This was somewhat surprising, as a prominent target of the UPR is ERAD. This result suggests there may be other factors that impact CFTR quality control in yeast. This section addresses the hypothesis that Ybr074,
functioning as a secretory pathway protease, may cooperate with the 26S proteasome to degrade complex transmembrane ERAD substrates, such as CFTR, from both sides of the ER membrane.

To test this hypothesis, a pulse chase analysis was conducted to examine CFTR peptide fragments resulting from its ERAD in strains in which Ybr074 function, 26S proteasome function, or both were disrupted.

First, in order to impair 26S proteasome function, its transcriptional activator, Rpn4, was disrupted, resulting in reduced proteasome levels (Xie & Varshavsky, 2001). Wild type, ybr074Δ, rpn4Δ, and ybr074Δ rpn4Δ strains expressing C-terminally HA-tagged CFTR were radiolabeled using 35S-Met for 5 min at 30°C, followed by treatment with cycloheximide. Samples were collected immediately after addition of cycloheximide. CFTR was then immunoprecipitated using an anti-HA antibody or the 3g11 antibody, which recognizes an epitope within NBD1. CFTR-derived fragments that appeared in CFTR-expressing strains and not in the empty vector control were compared in each strain. However, the presence of CFTR-derived fragments was not observed to depend exclusively on either Ybr074 activity or 26S proteasome activity (Figure 18).

Fewer CFTR-derived fragments were observed than were expected, suggesting that a longer incubation period after addition of cycloheximide may be required in order to detect CFTR-derived fragments. Nevertheless, these results indicate that CFTR-derived fragments are evident when there is reduced proteasome activity at this early time point, and that no Ybr074-dependent clipping of CFTR is taking place when comparing wild type and ybr074Δ strains along (Figure 18).
Figure 18: Comparison of CFTR-derived fragments in wild type, ybr074Δ, rpn4Δ, and ybr074Δ rpn4Δ strains.

Bands indicated by arrows appear in strains expressing CFTR and not in a wild type strain transformed with an empty vector (E.V.). Band 2 represents full-length CFTR, ~140 kDa. Strains expressing CFTR-HA were immunoprecipitated using an A) anti-HA antibody or B) the anti-NBD1 antibody, 3g11. All CFTR-derived fragments appear in all of the strains tested (n=2).

3.3.1.4 Does ablation of YBR074 and 26S proteasome function lead to synergistic effects on cell growth?

Accumulation of misfolded proteins in the ER can lead to cytotoxicity, as has been documented in cases of human endocrine ER storage diseases and liver disease (Guerriero & Brodsky, 2012; Kim & Arvan, 1998). As mentioned above, in yeast and in mammals, when the nascent protein
folding capacity of the ER is overwhelmed by the accumulation of misfolded proteins, a transcriptional stress response known as the UPR is induced. This transcriptional program results in the upregulation of genes involved in both ERAD and protein folding in response to ER stress (Travers et al, 2000). Overexpression of the ERAD substrate CPY* in yeast induces both the UPR and an “overflow” pathway that targets excess CPY* to the vacuole for degradation. However, when the gene encoding a vacuolar protease, Pep4, is disrupted in this strain, the accumulation of CPY* in the ER becomes toxic (Spear & Ng, 2003).

It was also noted above that the expression of CFTR in yeast is neither toxic nor does it induce the UPR (Zhang et al, 2001). Furthermore, immunofluorescence analysis has demonstrated that CFTR is localized in the ER and not the vacuole in yeast (Sullivan et al, 2003; Zhang et al, 2001). In the absence of UPR induction or a vacuolar “overflow” pathway, an alternative mechanism may be protecting cells from CFTR-induced toxicity. To address the hypothesis that Ybr074 is involved in mediating CFTR tolerance in yeast, strains expressing CFTR were examined for growth defects under conditions when Ybr074 function, the 26S proteasome function, or both were disrupted.

To impair 26S proteasome activity, the multidrug transporter, Pdr5, was disrupted in order to allow retention of the 26S proteasome peptide aldehyde inhibitor, MG132 (Lee & Goldberg, 1998). In the absence of MG132, wild type, ybr074Δ, pdr5Δ, and pdr5Δ ybr074Δ strains expressing CFTR exhibited similar growth rates. However, inhibition of 26S proteasome activity using MG132 slowed down the growth of both pdr5Δ, and pdr5Δ ybr074Δ strains. Since these strains were affected to a similar extent by the addition of MG132, it appears that Ybr074 and the 26S proteasome do not function in synergy to mediate the quality control of CFTR.
Figure 19: Growth curve of strains impaired for Ybr074 and/or 26S proteasome function.

All strains shown above were expressing CFTR and were grown in selective media lacking uracil. A) In the absence of the 26S proteasome inhibitor, MG132, the indicated strains exhibit similar growth curves at 30°C. B) In the presence of 50 μM MG132, pdr5Δ, and pdr5Δybr074Δ exhibit a growth curve lagging to a similar extent as compared to wild type and ybr074Δ strains (n=1).
3.3.2 Do genetic and chemical-genetic interactions between YBR074 and other genes link this protein to cellular processes?

A genetic approach toward understanding the function of Ybr074 was employed primarily using genetic interaction data from a synthetic genetic array (SGA) screen conducted by the Boone lab (University of Toronto, Toronto, Canada) (Tong & Boone, 2006). SGA is an automated, high-throughput method of double mutant yeast strain construction intended for analysis of genetic interactions. Robotic manipulations are used to systematically cross a query single deletion strain to an array of single gene deletion mutants (Tong & Boone, 2006). Genetic interactions are identified using colony size as an indicator of fitness. A genetic interaction is identified when double mutant progeny exhibit an unexpected phenotype compared to the fitness of each individual parental strain: a “negative” interaction refers to progeny exhibiting fitness lower than expected, and a “positive” interaction refers to progeny exhibiting fitness greater than expected. Genetic interactions are quantified using an SGA score ($\epsilon$), which is calculated by: $\epsilon = f_{ab} - (f_a \times f_b)$, where $f_{ab}$ represents progeny fitness, while $f_a$ and $f_b$ represent the fitness of individual parental strains (Koh et al, 2010).

When non-essential genes of the yeast genome were organized based on common genetic interaction profiles, genes with shared biological function clustered together. This resulted in the “guilt by association” hypothesis, which posits that the function of an uncharacterized gene can be inferred based on the function of known genes found in the same cluster (Tong et al, 2001).

Another method used to infer the function of uncharacterized genes involves characterizing drug sensitivity profiles. This method uses bioactive compounds with known targets to disrupt a specific biochemical pathway and identify genes linked to this pathway by
chemical-genetic interaction (Parsons et al, 2004). These chemical-genetic interactions were also used to examine biological functions of Ybr074.

To date, 1673 potential ORFs have been queried by SGA analysis against an array of strains representing 3885 ORFs. Although the ybr074Δ strain was not itself used as a query strain, it was represented on the array. Therefore, although this is not an exhaustive list of YBR074 genetic interactions, Figure 20A,B show that 34 out of 1673 query genes exhibited a genetic interaction with YBR074.

Using the Gene Ontology Term Finder, no significantly enriched GO function and GO process terms were identified among the 34 genes exhibiting genetic interaction with YBR074. However, ~85% of genes were categorized under the GO component term “membrane-bound organelle” (GO ID: 43227). In the absence of any clear links to any biological function, I manually curated the gene list and classified them into nine broad functional categories: lipid-related function, metabolic function, chromatin remodeling and transcription, ER quality control, translation, cell cycle, cell adhesion, secretion, and unknown function (Figure 20C). Of these candidates, FUS1, TMN3, and genes involved in lipid-related functions were selected for further analysis for the reasons detailed below.

A selection of genes shown in Table 7 was examined for chemical-genetic interactions using the compounds listed in Table 8. Double mutant strains grown on YPD or YPD supplemented with a stress-inducing chemical compound were compared against wild type and the parental single mutant strains. However, due to variability among tested colonies, no consistent phenotypes were observed. The only exception was an alleviating interaction observed upon treatment of cdc48-2 ybe074Δ strain with calcofluor white. However, this phenotype may
be caused by an unidentified third site mutation, as discussed in Section 4.2.1. Therefore, no chemical-genetic interactions can be said to have been identified by this method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Localization</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC63</td>
<td>sec63-1</td>
<td>ER</td>
<td>Essential subunit of the Sec63 post-translational translocation complex</td>
</tr>
<tr>
<td>CDC48</td>
<td>cdc48-2</td>
<td>cytosol, ER, nucleus, mitochondria, mating projection tip</td>
<td>AAA+ ATPase involved in retrotranslocation of ERAD substrates, autophagy, and cell wall maintenance</td>
</tr>
<tr>
<td>DEP1</td>
<td>dep1Δ</td>
<td>N/A</td>
<td>Transcriptional modulator involved in regulation of structural phospholipid biosynthesis genes</td>
</tr>
<tr>
<td>FUS1</td>
<td>fus1Δ</td>
<td>mating projection tip</td>
<td>Membrane protein localized to shmoo tip, required for cell fusion</td>
</tr>
<tr>
<td>GFA1</td>
<td>gfa1-97</td>
<td>N/A</td>
<td>Glutamine-fructose-6-phosphate amidotransferase catalyzes first step of chitin synthesis</td>
</tr>
<tr>
<td>NCR1</td>
<td>ncr1Δ</td>
<td>vacuole</td>
<td>Vacuolar membrane protein involved in sphingolipid metabolism; functional orthologue of human Niemann Pick C1 (NPC1) protein</td>
</tr>
<tr>
<td>OPI1</td>
<td>opi1Δ</td>
<td>ER, nucleus</td>
<td>Transcriptional regulator of phospholipid biosynthetic genes</td>
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<tr>
<td>STE24</td>
<td>ste24Δ</td>
<td>ER, nucleus, mitochondria</td>
<td>Highly conserved zinc metalloprotease that functions in two steps of a-factor maturation</td>
</tr>
<tr>
<td>VPS21</td>
<td>vps21Δ</td>
<td>late endosome, mitochondria, cytoplasm, nucleus</td>
<td>GTPase required for transport during endocytosis and correct sorting of vacuolar hydrolases</td>
</tr>
</tbody>
</table>
Table 8: Compounds used to test chemical-genetic interactions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium chloride</td>
<td>50 μM</td>
<td>Induces oxidative stress, leads to DNA damage, triggers heavy metal stress response</td>
</tr>
<tr>
<td>Caffeine</td>
<td>15 mM</td>
<td>Inhibits Tor1 kinase, activates Pkc1-dependent Cell Wall Integrity (CWI) pathway, inhibits Ras/cAMP pathway</td>
</tr>
<tr>
<td>Calcofluor white</td>
<td>50 μg/mL</td>
<td>Binds chitin, disrupts cell wall structure and morphogenesis, activates CWI pathway</td>
</tr>
<tr>
<td>Diamide</td>
<td>1 mM</td>
<td>Induces oxidative stress by depleting glutathion, triggers CWI pathway and High Osmolarity Glycerol (HOG) pathway</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>1 μg/mL</td>
<td>Inhibits N-glycosylation of nascent proteins in the ER, induces Unfolded Protein Response (UPR) and HOG pathways</td>
</tr>
</tbody>
</table>
Figure 20: Genes interacting with YBR074 by Synthetic Genetic Array (SGA) analysis

A) Genes that exhibit a negative genetic interaction with YBR074 by SGA analysis are shown. B) Genes that exhibit a positive genetic interaction with YBR074 by SGA analysis are shown. C) Manually curated functional categories representing all 34 genes found to interact with YBR074 by SGA analysis. Categories are based on descriptions of gene function obtained from the *Saccharomyces cerevisiae* Genome Database (SGD), www.yeastgenome.org.
3.3.2.1 Does YBR074 play a role in yeast mating?

*FUS1* encodes a membrane protein that localizes to the mating projection known as the shmoo tip, and facilitates membrane fusion between mating partners (Nolan et al, 2006). Because *FUS1* displayed the strongest genetic interaction with *YBR074*, a qualitative mating assay was conducted to determine whether Ybr074 plays a role in yeast mating.

Strains of one mating type were mixed with a serial dilution of the opposite mating type and spotted onto medium selective for diploids. As expected, *fus1*Δ strains of the opposite mating type did not fuse efficiently, and few diploids were recovered compared to wild type. However, *ybr074*Δ strains of the opposite mating type grew similarly to wild type on diploid selective medium (Figure 21). This suggests that *YBR074* is not required for efficient mating.
3.3.2.2 Does \textit{YBR074} function in the transition to pseudohyphal growth in nutrient-limited conditions?

When exposed to certain environmental cues, such as nitrogen starvation, \textit{S. cerevisiae} mounts a transcription response that alters the cell’s morphology and budding pattern. This promotes a transition from a unicellular morphology to a filamentous morphology that mediates...
substrate adhesion and invasion, and is thought to facilitate nutrient foraging. This adaptation is referred to as pseudohyphal growth, and depends largely on the activating transcription factor Flo8 (Froquet et al, 2008). However, the S288C-derived strain used predominantly in both this study and in the literature (BY4741 MAT a, and BY4742 MAT α), bears a mutation in the FLO8 gene that inhibits formation of pseudohyphae. Intriguingly, TMN3, a gene involved in mediating pseudohyphal growth, displayed a genetic interaction with YBR074, suggesting that there may be a role for YBR074 is pseudohyphal growth (Froquet et al, 2008).

To address this hypothesis, YBR074 was disrupted in the strain Σ1278b, known to exhibit extensive filamentous growth under well-defined growth conditions (Gimeno et al, 1992; Grenson, 1966). Because fusel alcohols are a byproduct of amino acid metabolism under nitrogen starvation conditions, filamentous growth was induced in liquid culture using 1% butanol to examine cellular morphology as described (Jin et al, 2008; Lorenz et al, 2000). Cell adhesion and invasion were examined under starvation conditions on solid medium as described (Guldal & Broach, 2006). As expected, the S288C-derived strain, BY4741, did not form pseudohyphae under pseudohyphal-inducing conditions. However, both wild type and ybr074Δ strains in the Σ1278b genetic background displayed filamentous growth, adhesion, and invasion of solid media under pseudohyphal-inducing conditions (Figure 22). This suggests that YBR074 is not required for transition to pseudohyphal growth.
Figure 22: Pseudohyphal growth does not require Ybr074

A) Morphological changes induced by supplementing YPD with 1% butanol (vol/vol) were observed in both wild type and ybr074Δ strains in the Σ1278b genetic background. B) Growth, adhesion, and invasion of solid media are shown under nutrient rich (SC + 2% glucose) and nutrient starvation (SC + 0.2% glucose) conditions. Wild type and ybr074Δ strains in the Σ1278b genetic background exhibit the adhesion and invasion phenotypes, while the S288C-derived strain, BY4741, does not.

3.3.3 Does the phylogenetic profile of YBR074 hint at its function?

Over the course of evolution, organisms experience environmental pressures that can accelerate or decelerate the mutability of their protein-coding genes. This produces a phylogenetic signature for each protein, which can be measured as its evolutionary rate of variation. Proteins that interact physically or functionally have been shown to exhibit similar phylogenetic signatures. Therefore, by measuring the evolutionary rate of covariation (ERC) of protein pairs across the
proteome of *Saccharomyces cerevisiae*, it may be possible to infer the function of Ybr074 by identifying its covarying proteins partners (Clark et al, 2012).

No statistically significant GO terms were enriched among the genes exhibiting evolutionary rate covariance with Ybr074 (Table 9) (Clark et al, 2012). However, it is interesting to note that six out of 30 genes were linked to vacuolar function (Vps41, Yhc3, Rcr2, Prb1, Vps13, Atg15), and seven out of 30 genes were linked to lipid or cell wall related function (Ymr086p, Rlm1, Yke4, Ydl109p, Sfl1, Lcl2, Gpi8).
Table 9: Genes related to *YBR074* by evolutionary rate of covariation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Localization</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMR086W</td>
<td>cell periphery</td>
<td>Protein of unknown function; possible component of the eisosome; expression is repressed by cAMP</td>
</tr>
<tr>
<td>YFL042C</td>
<td>N/A</td>
<td>Putative protein of unknown function; <em>YFL042C</em> is not an essential gene</td>
</tr>
<tr>
<td>VPS41</td>
<td>vacuolar membrane</td>
<td>Subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex</td>
</tr>
<tr>
<td>HSF1</td>
<td>nucleus</td>
<td>Trimeric heat shock transcription factor</td>
</tr>
<tr>
<td>LRP1</td>
<td>N/A</td>
<td>Nuclear exosome-associated nucleic acid binding protein; Involved in RNA processing, surveillance, degradation,</td>
</tr>
<tr>
<td>RLM1</td>
<td>cytoplasm, nucleus</td>
<td>MADS-box transcription factor, component of the protein kinase C-mediated MAP kinase pathway involved in the</td>
</tr>
<tr>
<td>YKE4</td>
<td>ER</td>
<td>Zinc transporter; localizes to the ER; null mutant is sensitive to calcofluor white, leads to zinc accumulation in cytosol</td>
</tr>
<tr>
<td>YHC3</td>
<td>vacuolar membrane</td>
<td>Protein involved in the ATP-dependent transport of arginine into the vacuole and possibly in balancing ion</td>
</tr>
<tr>
<td>PEX6</td>
<td>peroxisomes</td>
<td>AAA-peroxin, participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to</td>
</tr>
<tr>
<td>BOI1</td>
<td>bud, bud neck, cell periphery, cytoplasm</td>
<td>Protein implicated in polar growth, functionally redundant with Boi2p; interacts with bud-emergence protein Bem1p</td>
</tr>
<tr>
<td>MRP117</td>
<td>mitochondria</td>
<td>Mitochondrial ribosomal protein of the large subunit</td>
</tr>
<tr>
<td>YDL109C</td>
<td>N/A</td>
<td>Putative lipase; involved in lipid metabolism; <em>YDL109C</em> is not an essential gene</td>
</tr>
<tr>
<td>ICL2</td>
<td>mitochondria</td>
<td>2-methylisocitrate lyase of the mitochondrial matrix, functions in the methylcitrate cycle to catalyze the conversion of 2-methylisocitrate to succinate and pyruvate</td>
</tr>
<tr>
<td>FUN19</td>
<td>N/A</td>
<td>Non-essential protein of unknown function; expression induced in response to heat stress</td>
</tr>
<tr>
<td>RCR2</td>
<td>cytoplasm, vacuole</td>
<td>Vacular protein that presumably functions within the endosomal-vacuolar trafficking pathway</td>
</tr>
<tr>
<td>PRB1</td>
<td>vacuole</td>
<td>Vacuolar proteinase B (yseB), a serine protease of the subtilisin family; involved in protein degradation in the vacuole and required for full protein degradation during sporulation</td>
</tr>
<tr>
<td>SFL1</td>
<td>nucleus</td>
<td>Transcriptional repressor and activator; involved in repression of flocculation-related genes, and activation of stress</td>
</tr>
<tr>
<td>MLH3</td>
<td>N/A</td>
<td>Protein involved in DNA mismatch repair and crossing-over during meiotic recombination</td>
</tr>
<tr>
<td>MDV1</td>
<td>mitochondria</td>
<td>Peripheral protein of the cytosolic face of the mitochondrial outer membrane, required for mitochondrial fission</td>
</tr>
<tr>
<td>SRC1</td>
<td>nuclear periphery</td>
<td>Inner nuclear membrane protein that functions in regulation of subtelomeric genes and is linked to TREX</td>
</tr>
<tr>
<td>YOR296W</td>
<td>cytoplasm</td>
<td>Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm; expressed during copper starvation</td>
</tr>
<tr>
<td>VPS13</td>
<td>cytoplasm, endosomes</td>
<td>Involved in sporulation, vacuolar protein sorting and protein-Golgi retention; peripherally associated with membranes</td>
</tr>
<tr>
<td>YBP1</td>
<td>cytoplasm</td>
<td>Protein required for oxidation of specific cysteine residues of the transcription factor Yap1p, resulting in the nuclear localization of Yap1p in response to stress</td>
</tr>
<tr>
<td>LCL2</td>
<td>N/A</td>
<td>Putative protein of unknown function; mutant is deficient in amounts of cell wall mannosylphosphate; genetic interactions suggest a role in ERAD</td>
</tr>
<tr>
<td>ATG15</td>
<td>vacuole</td>
<td>Lipase required for intravacuolar lysis of autophagic bodies and Cvt bodies; targeted to intravacuolar vesicles during autophagy via the multivesicular body (MVB) pathway</td>
</tr>
<tr>
<td>SAM37</td>
<td>mitochondria</td>
<td>Component of the Sorting and Assembly Machinery (SAM or TOB complex) of the mitochondrial outer membrane</td>
</tr>
<tr>
<td>AIM11</td>
<td>N/A</td>
<td>Protein of unknown function; null mutant is viable but shows increased loss of mitochondrial genome and synthetic interaction with prohibitin (phb1)</td>
</tr>
<tr>
<td>GPI8</td>
<td>ER</td>
<td>ER membrane glycoprotein subunit of the glycosylphosphatidylinositol transamidase complex that adds glycosylphosphatidylinositol (GPI) anchors to newly synthesized proteins</td>
</tr>
<tr>
<td>SLD3</td>
<td>cytoplasm, nucleus</td>
<td>Protein involved in the initiation of DNA replication, required for proper assembly of replication proteins at the origins of replication; interacts with Cdc45p</td>
</tr>
<tr>
<td>DYN1</td>
<td>cytoplasm</td>
<td>Cytoplasmic heavy chain dynein, microtubule motor protein, required for anaphase spindle elongation</td>
</tr>
</tbody>
</table>
3.3.4 Can a substrate for Ybr074 be identified using proteomic approaches?

The following proteomic approaches were based on the notion that disrupting a protease would result in accumulation of its substrate(s). Two methods were used to attempt to identify the substrate(s) of Ybr074: 2D in-Gel Electrophoresis (DiGE), and a fluorescence-based proteomic screen using a library of yeast strains each expressing a different GFP-tagged protein (Cohen & Schuldiner, 2011; Huh et al, 2003; May et al, 2012; Rimon & Schuldiner, 2011).

DiGE is an approach whereby the total protein content of a wild type and a ybr074Δ strain is extracted and differentially labeled using unique fluorescent probes, most commonly cyanine-3 (Cy3) and cyanine-5 (Cy5). Labeled proteins from each strain are resolved on a single gel by 2D-gel electrophoresis and compared for differences in fluorescence intensity associated with each protein spot (Unlu et al, 1997). Protein spots can then be cut out of the gel and identified by mass spectrometry. This method informs on the average protein content of a population of cells, and has been used to detect changes in protein levels associated cancer-specific markers, for example (Zhou et al, 2002). This method can also be used to identify substrates of enzymes mediating post-translational modifications, as was achieved for the serine protease, granzyme B (Bredemeyer et al, 2004).

The relative abundance of each protein spot in wild type and ybr074Δ strains was compared by calculating the ratio of each ybr074Δ protein spot to its corresponding wild type protein spot. The majority of protein content (88.6%) visualized by this method was similar across both strains. The results did show 112 spots (5%) decreased in the ybr074Δ strain as compared to wild type, and 145 spots (6.4%) that had accumulated in the ybr074Δ strain as compared to wild type. As mentioned above, proteins that accumulated in the ybr074Δ strain
Figure 23: 2D DiGE comparing wild type and \textit{ybr074\textDelta} protein levels

The relative abundance of protein from the A) wild type strain and B) \textit{ybr074\textDelta} strain are quantitatively compared in the C) merge image, with protein spots showing noticeable changes in level of expression circled and numbered for identification.
may represent substrates of Ybr074. Alternatively, these proteins may have accumulated as an indirect effect of disrupting Ybr074 function. In order to differentiate between these possibilities, candidate protein spots were selected for identification and further investigation by western blot analysis in wild type and ybr074Δ strains.

Ten protein spots were selected for identification by mass spectrometry based on a high ratio of ybr074Δ to wild type levels, high molecular mass, or being in a close proximity to a protein spot of lower molecular mass that displayed a decrease in ybr074Δ to wild type levels, suggesting a Ybr074-dependent shift from a high molecular mass product, to a lower molecular mass product as exemplified by spots 14 and 35, shown in Figure 24.

![Figure 24: Apparent shift of spots 14 and 35 from a higher molecular weight in the ybr074Δ sample to a lower molecular weight in the wild type sample.](image)

Protein spots 14 and 35 are labeled in the left panel. Spots neighboring 14 and 35, denoted by asterisks, appear more abundant in the wild type sample and have a lower molecular mass than spots 14 and 35.
The genes encoding the protein spots selected for identification are shown in Table 10. It is interesting to note that the vacuolar protease, Pep4, accumulated in a ybr074A strain. It is possible that loss of Ybr074 function at the vacuole is partially compensated for by increased abundance of Pep4.

Among the proteins identified in this assay, Rhr2 was intriguing because of its role in glycerol synthesis as a response to osmotic stress. Osmotic stress can be caused by damage to the cell wall upon treatment with the cell wall digesting enzyme, zymolyase. Cell wall damage caused in this manner results in the induction of two parallel signaling pathways known as the Cell Wall Integrity (CWI) and High-Osmolarity Glycerol (HOG) signaling pathways (Rodriguez-Pena et al, 2010). Since genetic interactions of YBR074 with genes related to maintenance of cell wall integrity have been reported by the Boone lab, as previously mentioned, I was interested in verifying whether the accumulation of Rhr2 in the ybr074Δ strain resulted from loss of Ybr074-dependent degradation of Rhr2, or whether the accumulation might be attributed to an indirect effect of disrupting Ybr074 function.

To do this, N-terminally 3HA-tagged Rhr2 was constructed and expressed from a galactose promoter in wild type cells. 3HA-Rhr2 isolated from these cells was examined by Western blot analysis for Ybr074-dependent fragments that might result from degradation. However, no Ybr074-dependent Rhr2 fragments were observed (Figure 25). This suggests that accumulation of Rhr2 in ybr074Δ cells may have been an indirect effect of loss of Ybr074 function. (It is important to note that accumulation of Rhr2 was not expected in this experiment because the protein was highly overexpressed in order to better visualize any generated fragments).
Figure 25: Rhr2 expression in wild type and ybr074Δ strains.

A) Total protein from cells expressing 3HA-Rhr2, which was induced by galactose for 6.5 h at 30°C, was separated by 12.5% SDS-PAGE. 3HA-Rhr2 and the loading control glyceraldehyde-6-phosphate dehydrogenase (G6PDH) were detected by Western blot. B) Relative abundance of full length Rhr2 was quantified and the SE is shown (n=4; p>0.05).
Table 10: Candidate substrates identified by 2D DiGE

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Systematic name</th>
<th>Standard name</th>
<th>CI #</th>
<th>Molecular Weight (kDa)</th>
<th>Protein PI</th>
<th>Description</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>YJR045C</td>
<td>SSC1</td>
<td>151945113</td>
<td>70.9</td>
<td>5.48</td>
<td>Hsp70 family ATPase, constituent of the import motor component of the Translocase of the Inner Mitochondrial membrane (TIM23 complex)</td>
<td>mitochondria</td>
</tr>
<tr>
<td>6</td>
<td>YFL018C</td>
<td>LPD1</td>
<td>15826394</td>
<td>51.5</td>
<td>8.54</td>
<td>Dihydrolipoamide dehydrogenase, the lipoamide dehydrogenase component (E3) of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multi-enzyme complexes</td>
<td>mitochondria</td>
</tr>
<tr>
<td>8</td>
<td>YPL154C</td>
<td>PEP4</td>
<td>7766834</td>
<td>35.7</td>
<td>4.35</td>
<td>Vacuolar aspartyl protease (proteinase A), required for the posttranslational precursor maturation of vacuolar proteinases; important for protein turnover after oxidative damage</td>
<td>vacuole</td>
</tr>
<tr>
<td>13</td>
<td>YGR167W</td>
<td>CLC1</td>
<td>1945336</td>
<td>25.5</td>
<td>4.31</td>
<td>Clathrin light chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis</td>
<td>cytosol</td>
</tr>
<tr>
<td>14</td>
<td>YIL053W</td>
<td>RHR2</td>
<td>96658007</td>
<td>27.9</td>
<td>5.35</td>
<td>Constitutively expressed isoform of DL-glycerol-3-phosphatase, involved in glycerol biosynthesis</td>
<td>cytosol, nucleus</td>
</tr>
<tr>
<td>18</td>
<td>YDR399W</td>
<td>HPT1</td>
<td>6320007</td>
<td>25.2</td>
<td>5.46</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase, catalyzes formation of purine nucleotides guanosine monophosphate and inosine monophosphate</td>
<td>cytosol nucleus</td>
</tr>
<tr>
<td>21</td>
<td>YK0.056C</td>
<td>TMA19</td>
<td>151941490</td>
<td>18.7</td>
<td>4.41</td>
<td>Protein that associates with ribosomes, GFP-fusion protein localizes to the cytoplasm and relocates to the mitochondrial outer surface upon oxidative stress</td>
<td>cytosol, nucleus</td>
</tr>
<tr>
<td>25</td>
<td>YPR102C</td>
<td>RPL11A</td>
<td>6325359</td>
<td>19.7</td>
<td>9.92</td>
<td>Protein of the large 60S ribosomal subunit involved in ribosomal assembly</td>
<td>nucleas</td>
</tr>
<tr>
<td>29</td>
<td>YOL121C</td>
<td>RPS19A</td>
<td>6324451</td>
<td>15.9</td>
<td>9.61</td>
<td>Protein component of the small (40S) ribosomal subunit, required for assembly and maturation of pre-40 S particles</td>
<td>cytosol</td>
</tr>
<tr>
<td>35</td>
<td>YOL086C</td>
<td>ADH1</td>
<td>6324486</td>
<td>36.8</td>
<td>8.21</td>
<td>Alcohol dehydrogenase, required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway</td>
<td>cytosol</td>
</tr>
</tbody>
</table>
In a second approach, the fluorescence intensity was measured *in vivo* by high-content fluorescent microscopy in screening a collection of yeast strains each expressing a different GFP-tagged protein, both in the wild type or *ybr074Δ* genetic background. Using this approach, data are collected from individual cells rather than a population. This work was performed by Dr. Maya Schuldiner’s group (Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel). The Schuldiner lab has validated this approach in a related study aimed at identifying substrates dependent on the cargo receptor, Erv14, for ER exit. This was achieved by crossing an *erv14Δ* mutant strain to the GFP-tagged yeast library, and identifying GFP-tagged substrates that accumulated in the ER of mutant strains as compared to wild type strains (Herzig et al, 2012). Similarly, it was hypothesized that substrates of Ybr074 might accumulate in a *ybr074Δ* strain as compared to a wild type strain.

Results of the GFP-tagged yeast library screen identified 72 proteins that accumulated in the absence of Ybr074 activity, and 28 proteins with decreased levels in the absence of Ybr074 (Tables 11-12). No statistically significant GO terms were enriched among these candidates. It is interesting to note that the majority of proteins, ~70%, which accumulated in the *ybr074Δ* strain were annotated using the GO-Slim Component term “cytoplasm”. However, it is important to keep in mind that ~60% of the yeast genome is annotated to this term. No vacuolar proteases were found among the proteins identified in this screen. However, the vacuolar protein sorting factor, Vps70 was among the proteins that accumulated in the absence of Ybr074 activity.
### Table 11: GFP-tagged proteins exhibiting increased levels in ybr074Δ strain compared to wild type

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio</th>
<th>Localization</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDA1</td>
<td>1.23</td>
<td>nucleus</td>
<td>Highly conserved nuclear protein required for actin cytoskeleton organization</td>
</tr>
<tr>
<td>SNZ1</td>
<td>1.25</td>
<td>N/A</td>
<td>Protein involved in vitamin B6 biosynthesis</td>
</tr>
<tr>
<td>ERR3</td>
<td>1.30</td>
<td>cytosol</td>
<td>Protein of unknown function, has similarity to enolases</td>
</tr>
<tr>
<td>YAL069W</td>
<td>1.32</td>
<td>N/A</td>
<td>Dubious open reading frame unlikely to encode a protein</td>
</tr>
<tr>
<td>YHR022C</td>
<td>1.39</td>
<td>N/A</td>
<td>Putative protein of unknown function</td>
</tr>
<tr>
<td>YIL169C</td>
<td>1.40</td>
<td>N/A</td>
<td>Putative protein; similar to YOL155C, a putative glucan alpha-1,4-glucosidase</td>
</tr>
<tr>
<td>EMI1</td>
<td>1.52</td>
<td>N/A</td>
<td>Required for induction of the early meiotic-specific transcription factor IME1</td>
</tr>
<tr>
<td>PUS5</td>
<td>1.53</td>
<td>mitochondria</td>
<td>Pseudouridine synthase, catalyzes formation of pseudouridine in mitochondrial 21S rRNA</td>
</tr>
<tr>
<td>SAG1</td>
<td>1.57</td>
<td>cell wall</td>
<td>Alpha-agglutinin of alpha-cells, binds to Aga1p, C-terminal half is GPI anchored</td>
</tr>
<tr>
<td>AVO1</td>
<td>1.89</td>
<td>cytosol, plasma membrane</td>
<td>Component of a membrane-bound complex containing the Tor2p kinase and other proteins</td>
</tr>
<tr>
<td>STE3</td>
<td>1.94</td>
<td>plasma membrane</td>
<td>Receptor for a factor pheromone, mediates pheromone response</td>
</tr>
<tr>
<td>VPS70</td>
<td>2.05</td>
<td>N/A</td>
<td>Protein of unknown function involved in vacuolar protein sorting</td>
</tr>
<tr>
<td>YDL218W</td>
<td>2.12</td>
<td>N/A</td>
<td>Putative protein; transcription is induced by starvation and aerobic conditions</td>
</tr>
<tr>
<td>DIN7</td>
<td>29.41</td>
<td>mitochondria</td>
<td>Mitochondrial nuclease functioning in DNA repair and replication</td>
</tr>
<tr>
<td>THI20</td>
<td>1.39</td>
<td>cytosol</td>
<td>Multifunctional protein with kinase involved in thiamine biosynthesis and degradation</td>
</tr>
<tr>
<td>FLC1</td>
<td>1.49</td>
<td>ER, bud neck, cell periphery, vacuole</td>
<td>Putative FAD transporter; required for uptake of FAD into ER; involved in cell wall maintenance</td>
</tr>
<tr>
<td>TNA1</td>
<td>1.39</td>
<td>plasma membrane, ER, mitochondria</td>
<td>High affinity nicotinic acid plasma membrane permease</td>
</tr>
<tr>
<td>HXT2</td>
<td>1.37</td>
<td>plasma membrane, vacuole</td>
<td>High-affinity glucose transporter of the major facilitator superfamily</td>
</tr>
<tr>
<td>Gene</td>
<td>Ratio</td>
<td>Localization</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>-----------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CPA1</td>
<td>1.21</td>
<td>cytosol</td>
<td>Small subunit of carbamoyl phosphate synthetase; synthesis of citrulline, an arginine precursor</td>
</tr>
<tr>
<td>TEF1</td>
<td>1.24</td>
<td>cytosol, mitochondria</td>
<td>Translational elongation factor EF-1 alpha</td>
</tr>
<tr>
<td>RPL40B</td>
<td>1.25</td>
<td>cytosol, mitochondria</td>
<td>Fusion protein, cleaved to yield ubiquitin and a ribosomal protein of the 60S ribosomal subunit</td>
</tr>
<tr>
<td>YAT2</td>
<td>1.26</td>
<td>cytosol</td>
<td>Carnitine acetyltransferase; similar to Yat1p, a carnitine acetyltransferase associated with mitochondria</td>
</tr>
<tr>
<td>RPS19B</td>
<td>1.27</td>
<td>cytosol</td>
<td>Protein component of the 40S ribosomal subunit, required for assembly and maturation of pre-40S</td>
</tr>
<tr>
<td>YLR364W</td>
<td>1.27</td>
<td>cytosol</td>
<td>Glutaredoxin that employs a dithiol mechanism of catalysis</td>
</tr>
<tr>
<td>ADE17</td>
<td>1.29</td>
<td>cytosol, plasma membrane</td>
<td>Enzyme of 'de novo' purine biosynthesis</td>
</tr>
<tr>
<td>STM1</td>
<td>1.30</td>
<td>cytosol</td>
<td>Protein required for optimal translation under nutrient stress; involved in TOR signaling</td>
</tr>
<tr>
<td>SER1</td>
<td>1.33</td>
<td>cytosol</td>
<td>3-phosphoserine aminotransferase, required for serine and glycine biosynthesis</td>
</tr>
<tr>
<td>YML131W</td>
<td>1.34</td>
<td>cytosol</td>
<td>Putative protein of unknown function with similarity to medium chain dehydrogenase/reductases</td>
</tr>
<tr>
<td>SIP18</td>
<td>1.36</td>
<td>cytosol</td>
<td>Phospholipid-binding protein; expression is induced by osmotic stress</td>
</tr>
<tr>
<td>GCD11</td>
<td>1.45</td>
<td>cytosol</td>
<td>Gamma subunit of the translation initiation factor eIF2, involved in the identification of the start codon</td>
</tr>
<tr>
<td>ATX1</td>
<td>1.47</td>
<td>cytosol</td>
<td>Cytosolic copper metallochaperone; transports copper to the secretory vesicle copper transporter Ccc2p</td>
</tr>
<tr>
<td>RPS30B</td>
<td>1.50</td>
<td>cytosol</td>
<td>Protein component of 40S ribosomal subunit; nearly identical to Rps30Ap and similar to rat S30 ribosomal protein</td>
</tr>
<tr>
<td>GRE1</td>
<td>2.04</td>
<td>cytosol</td>
<td>Hydrophilin of unknown function; stress induced; regulated by the HOG pathway</td>
</tr>
<tr>
<td>PUB1</td>
<td>2.81</td>
<td>cytosol, nucleus</td>
<td>Poly (A)+ RNA-binding protein; required for stability of many mRNAs</td>
</tr>
<tr>
<td>SOL1</td>
<td>1.22</td>
<td>cytosol, nucleus</td>
<td>Protein with a possible role in tRNA export</td>
</tr>
<tr>
<td>Gene</td>
<td>Ratio</td>
<td>Localization</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>--------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ADH5</td>
<td>1.22</td>
<td>cytosol, nucleus</td>
<td>Alcohol dehydrogenase isoenzyme V; involved in ethanol production</td>
</tr>
<tr>
<td>ADE1</td>
<td>1.24</td>
<td>cytosol, nucleus</td>
<td>N-succinyl-5-aminoimidazole-4-carboxamide ribotide (SAICAR) synthetase; purine biosynthesis</td>
</tr>
<tr>
<td>GSP2</td>
<td>1.26</td>
<td>cytosol, nucleus</td>
<td>GTP binding protein; maintenance of nuclear organization, RNA processing and transport</td>
</tr>
<tr>
<td>SER2</td>
<td>1.26</td>
<td>cytosol, nucleus</td>
<td>Phosphoserine phosphatase of the phosphoglycerate pathway; serine and glycine biosynthesis</td>
</tr>
<tr>
<td>TDH2</td>
<td>1.27</td>
<td>cytosol, nucleus, plasma membrane, cell wall, lipid droplet, mitochondria</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase; glycolysis and gluconeogenesis</td>
</tr>
<tr>
<td>SHP1</td>
<td>1.29</td>
<td>cytosol, nucleus</td>
<td>UBX domain-containing protein; degradation of ubiquitylated substrate with Cdc48</td>
</tr>
<tr>
<td>TKL2</td>
<td>1.34</td>
<td>cytosol, nucleus</td>
<td>Transketolase, functions in the pentose phosphate pathway; synthesis of aromatic amino acids</td>
</tr>
<tr>
<td>YNL313C</td>
<td>1.44</td>
<td>cytosol, nucleus</td>
<td>Essential protein of unknown function; GFP-tagged protein localizes to cytoplasm and nucleus</td>
</tr>
<tr>
<td>PIR1</td>
<td>1.50</td>
<td>cell wall, cytosol, nucleus</td>
<td>O-glycosylated protein required for cell wall stability</td>
</tr>
<tr>
<td>NTO1</td>
<td>1.58</td>
<td>cytosol, nucleus</td>
<td>Subunit of the NuA3 histone acetyltransferase complex that acetylates histone H3</td>
</tr>
<tr>
<td>HSH155</td>
<td>1.70</td>
<td>cytosol, nucleus</td>
<td>U2-snRNP associated splicing factor</td>
</tr>
<tr>
<td>SNF7</td>
<td>1.22</td>
<td>endosomes, cytosol</td>
<td>Subunit of the endosomal sorting complex required for transport III (ESCRT-III)</td>
</tr>
<tr>
<td>SEC65</td>
<td>1.34</td>
<td>ER, cytosol</td>
<td>Subunit of the signal recognition particle (SRP), involved in protein targeting to the ER</td>
</tr>
<tr>
<td>RTN2</td>
<td>1.29</td>
<td>ER</td>
<td>Protein of unknown function; similarity to mammalian RTNLA (reticulon-like A) subfamily</td>
</tr>
<tr>
<td>SED1</td>
<td>1.34</td>
<td>cell wall, ER, mitochondria</td>
<td>Major stress-induced structural GPI-cell wall glycoprotein in stationary-phase cells</td>
</tr>
<tr>
<td>Gene</td>
<td>Ratio</td>
<td>Localization</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>--------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SEC24</td>
<td>3.41</td>
<td>ER, Golgi</td>
<td>Component of the COPII vesicle coat; cargo selection in ER to Golgi transport</td>
</tr>
<tr>
<td>LSC1</td>
<td>1.23</td>
<td>mitochondria</td>
<td>Alpha subunit of succinyl-CoA ligase; mitochondrial enzyme of the TCA cycle</td>
</tr>
<tr>
<td>MRPS5</td>
<td>1.26</td>
<td>mitochondria</td>
<td>Mitochondrial ribosomal protein of the small subunit</td>
</tr>
<tr>
<td>YPR011C</td>
<td>1.33</td>
<td>mitochondria</td>
<td>Putative transporter, member of the mitochondrial carrier family</td>
</tr>
<tr>
<td>SEN54</td>
<td>1.39</td>
<td>mitochondria, cytosol</td>
<td>Subunit of the tRNA splicing endonuclease</td>
</tr>
<tr>
<td>ILV6</td>
<td>1.60</td>
<td>mitochondria</td>
<td>Regulatory subunit of acetolactate synthase; branched-chain amino acid biosynthesis</td>
</tr>
<tr>
<td>PDB1</td>
<td>8.26</td>
<td>mitochondria</td>
<td>E1 beta subunit of the pyruvate dehydrogenase (PDH) complex found in mitochondria</td>
</tr>
<tr>
<td>YGR283C</td>
<td>1.26</td>
<td>nucleolus, nucleus</td>
<td>Protein of unknown function; predicted to be involved in ribosome biogenesis</td>
</tr>
<tr>
<td>MAK5</td>
<td>1.22</td>
<td>nucleolus, nucleus</td>
<td>Essential nucleolar protein, putative DEAD-box RNA helicase; biogenesis of 60S ribosomal subunits</td>
</tr>
<tr>
<td>TAF6</td>
<td>1.20</td>
<td>nucleus</td>
<td>Subunit of TFIID and SAGA complexes; transcription initiation of RNA pol II and chromatin modification</td>
</tr>
<tr>
<td>LYS20</td>
<td>1.22</td>
<td>nucleus, mitochondria</td>
<td>Homocitrate synthase isozyme; lysine biosynthesis</td>
</tr>
<tr>
<td>TAL1</td>
<td>1.26</td>
<td>nucleus, cytosol</td>
<td>Transaldolase, enzyme in the non-oxidative pentose phosphate pathway</td>
</tr>
<tr>
<td>UTP21</td>
<td>1.26</td>
<td>nucleolus, nucleus</td>
<td>Subunit of U3-containing 90S preribosome and Small Subunit (SSU) processome complexes</td>
</tr>
<tr>
<td>SSL2</td>
<td>1.26</td>
<td>nucleus</td>
<td>Component of the holoenzyme form of RNA polymerase transcription factor TFIH</td>
</tr>
<tr>
<td>RSC3</td>
<td>1.38</td>
<td>nucleus</td>
<td>Component of RSC chromatin remodeling complex; regulation of ribosomal genes and cell wall/stress response</td>
</tr>
<tr>
<td>MED4</td>
<td>1.42</td>
<td>nucleus</td>
<td>Subunit of the RNA polymerase II mediator complex</td>
</tr>
<tr>
<td>YGR043C</td>
<td>1.51</td>
<td>nucleus</td>
<td>Transaldolase of unknown function</td>
</tr>
<tr>
<td>TAF5</td>
<td>3.06</td>
<td>nucleus, cytosol</td>
<td>Subunit (90 kDa) of TFIID and SAGA complexes; RNA Pol II transcription initiation and chromatin modification</td>
</tr>
<tr>
<td>Gene</td>
<td>Ratio</td>
<td>Localization</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>-------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NCE102</td>
<td>1.22</td>
<td>plasma membrane, cytosol, vacuole, ER, mitochondria, mating projection, bud membrane</td>
<td>Protein of unknown function; involved in secretion of proteins that lack classical secretory signal sequences</td>
</tr>
<tr>
<td>RET3</td>
<td>1.47</td>
<td>N/A</td>
<td>Zeta subunit of the coatamer complex (COPI); involved in retrograde transport between Golgi and ER</td>
</tr>
<tr>
<td>GTR1</td>
<td>1.23</td>
<td>vacuole, cytosol, late endosome, nucleus</td>
<td>Cytoplasmic GTP binding protein; component of GSE complex, which is required for sorting of Gap1p</td>
</tr>
<tr>
<td>GNT1</td>
<td>1.24</td>
<td>Golgi, vacuole</td>
<td>N-acetylglucosaminyltransferase capable of modification of N-linked glycans in the Golgi apparatus</td>
</tr>
</tbody>
</table>
### Table 12: GFP-tagged proteins exhibiting reduced levels in ybr074Δ strain compared to wild type

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio</th>
<th>Localization</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTF2</td>
<td>0.47</td>
<td>nuclear envelope</td>
<td>Nuclear envelope protein, essential player in nucleocytoplasmic transport</td>
</tr>
<tr>
<td>YLR122C</td>
<td>0.47</td>
<td>N/A</td>
<td>Dubious open reading frame unlikely to encode a protein; partially overlaps the dubious ORF YLR123C</td>
</tr>
<tr>
<td>ARP9</td>
<td>0.15</td>
<td>nucleus</td>
<td>Component of SWI/SNF and RSC chromatin remodeling complexes</td>
</tr>
<tr>
<td>ILV5</td>
<td>0.50</td>
<td>mitochondria</td>
<td>Acetohydroxyacid reductoisomerase, mitochondrial protein; branched-chain amino acid biosynthesis</td>
</tr>
<tr>
<td>OPI9</td>
<td>0.41</td>
<td>N/A</td>
<td>Dubious open reading frame unlikely to encode a protein; partially overlaps the verified ORF VRP1/YLR337C</td>
</tr>
<tr>
<td>HSP30</td>
<td>0.47</td>
<td>plasma membrane</td>
<td>Hydrophobic plasma membrane localized, stress-responsive protein that negatively regulates the H(+)–ATPase Pma1p</td>
</tr>
<tr>
<td>RPL21B</td>
<td>0.31</td>
<td>cytosol</td>
<td>Protein component of the 60S ribosomal subunit, nearly identical to Rpl21Ap; similarity to rat L21 ribosomal protein</td>
</tr>
<tr>
<td>RPS6B</td>
<td>0.07</td>
<td>cytosol</td>
<td>Protein component of the 40S ribosomal subunit; identical to Rps6Ap; similarity to rat S6 ribosomal protein</td>
</tr>
<tr>
<td>PYC1</td>
<td>0.11</td>
<td>cytosol</td>
<td>Pyruvate carboxylase isoform, cytoplasmic enzyme that converts pyruvate to oxaloacetate</td>
</tr>
<tr>
<td>RPL19A</td>
<td>0.32</td>
<td>cytosol</td>
<td>Protein component of the large (60S) ribosomal subunit</td>
</tr>
<tr>
<td>RSE1</td>
<td>0.50</td>
<td>cytosol</td>
<td>Protein involved in pre-mRNA splicing; component of the pre-spliceosome; involved in ER to Golgi transport</td>
</tr>
<tr>
<td>SHM2</td>
<td>0.35</td>
<td>cytosol, nucleus, plasma membrane, mating projection tip</td>
<td>Cytosolic serine hydroxymethyltransferase; generating precursors for purine, pyrimidine, amino acid, and lipid biosynthesis</td>
</tr>
<tr>
<td>CDC48</td>
<td>0.46</td>
<td>cytosol, nucleus, ER, mating projection tip, mitochondria</td>
<td>ATPase in ER, nuclear membrane and cytosol; in a complex with Npl4p and Ufd1p participates in ERAD</td>
</tr>
<tr>
<td>DBP2</td>
<td>0.08</td>
<td>cytosol, nucleus, mitochondria</td>
<td>ATP-dependent RNA helicase of the DEAD-box protein family; nonsense-mediated mRNA decay and rRNA processing</td>
</tr>
<tr>
<td>Gene</td>
<td>Ratio</td>
<td>Localization</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>-------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MFA1</td>
<td>0.10</td>
<td>extracellular space</td>
<td>Mating pheromone a-factor; interacts with alpha cells to induce cell cycle arrest and other responses leading to mating</td>
</tr>
<tr>
<td>CAR2</td>
<td>0.35</td>
<td>cytosol, nucleus</td>
<td>L-ornithine transaminase (OTase), catalyzes the second step of arginine degradation</td>
</tr>
<tr>
<td>RAD51</td>
<td>0.19</td>
<td>cytosol, nucleus</td>
<td>Strand exchange protein; involved in repair of double-strand breaks in DNA</td>
</tr>
<tr>
<td>KAP104</td>
<td>0.43</td>
<td>cytosol, nucleus, bud neck, bud tip</td>
<td>Transportin or cytosolic karyopherin beta 2; regulates asymmetric protein synthesis in daughter cells during mitosis</td>
</tr>
<tr>
<td>DIP2</td>
<td>0.49</td>
<td>cytosol, nucleus</td>
<td>Nucleolar protein of the small subunit (SSU) processome, required for 18S rRNA biogenesis</td>
</tr>
<tr>
<td>GYP1</td>
<td>0.47</td>
<td>cytosol, mitochondria, Golgi</td>
<td>Cis-golgi GTPase-activating protein (GAP) for the Rab family members; involved in vesicle docking and fusion</td>
</tr>
<tr>
<td>FAT1</td>
<td>0.19</td>
<td>lipid droplet, ER, peroxisome, plasma membrane</td>
<td>Very long chain fatty acyl-CoA synthetase and long chain fatty acid transporter; activates imported fatty acids</td>
</tr>
<tr>
<td>YJR041C</td>
<td>0.37</td>
<td>nucleolus, nucleus</td>
<td>Nucleolar protein required for metabolism of the rRNA primary transcript; ribosome biogenesis</td>
</tr>
<tr>
<td>SIK1</td>
<td>0.16</td>
<td>nucleolus, nucleus</td>
<td>Essential nucleolar protein component of the box C/D snoRNP complexes</td>
</tr>
<tr>
<td>HHF2</td>
<td>0.46</td>
<td>nucleus</td>
<td>Histone H4, core histone protein required for chromatin assembly and chromosome function</td>
</tr>
<tr>
<td>YRA1</td>
<td>0.22</td>
<td>nucleus</td>
<td>RNA binding protein required for export of poly(A)+ mRNA from the nucleus</td>
</tr>
<tr>
<td>RET1</td>
<td>0.47</td>
<td>nucleus</td>
<td>Second-largest subunit of RNA polymerase III, responsible for the transcription of tRNA and 5S RNA genes</td>
</tr>
<tr>
<td>STE2</td>
<td>0.18</td>
<td>plasma membrane, vacuole</td>
<td>Receptor for alpha-factor pheromone; GPCR that initiates the signaling response during mating</td>
</tr>
<tr>
<td>STE6</td>
<td>0.35</td>
<td>plasma membrane, mating projection tip, Golgi, vacuole</td>
<td>Plasma membrane ATP-binding cassette (ABC) transporter required for the export of a-factor</td>
</tr>
</tbody>
</table>
3.4 CONCLUSIONS

Ybr074 was not implicated in the ER quality control of any of the ERAD substrates tested, including CFTR, Ste6p*, Gas1*, CPY* and CPY*-DHFR. Similarly, Ybr074 does not act in synergy with the 26S proteasome in the degradation of ERAD substrates. This was evidenced by the absence of distinct Ybr074-dependent CFTR degradation fragments in the absence of 26S proteasome activity. Furthermore, no synergistic growth defect was observed when both Ybr074 and the 26S protease activity were disrupted in cells expressing CFTR. In hindsight, this is not surprising in light of the evidence discussed in Chapter 2, which indicated that Ybr074 is a vacuolar protein as opposed to an ER-localized protein. In fact, this may support the hypothesis presented in Chapter 2, suggesting that the ER accumulation of Ybr074 observed in cells expressing Ybr074-HA may represent an aberrantly localized, and perhaps misfolded, gene product.

Multiple methods designed to associate Ybr074 with a group of genes sharing a common biological function, including Synthetic Genetic Array (SGA) analysis, phylogenetics, and proteomics, did not place Ybr074 in any clear functional category. However, the presence of nine genes related to lipid metabolism and cell wall function, which were identified by SGA analysis, merited further investigation (see Chapter 4). Only five of 72 GFP-tagged proteins (Flc1, Hxt2, Nce102, Gtr1, Gnt1) that accumulated in a ybr074Δ strain are described as having vacuolar localization. It is interesting to note that none of these candidates are resident vacuolar proteins, but may be targeted to the vacuole for degradation under certain conditions. For example, high glucose levels trigger internalization and vacuolar targeting of the plasma
membrane glucose transporter, Hxt2 (Kruckeberg et al, 1999). An alternative strategy to identify endogenous substrates of Ybr074 will be discussed in Chapter 5.
4.0 CELL WALL INTEGRITY

The cell wall in yeast and other fungi forms a physical barrier between the cell and its environment. It is comprised of an inner network of linear and branched sugars, and an outer layer of highly glycosylated cell wall proteins (CWP) s. The primary functions of the cell wall are to stabilize osmotic conditions within the cell, to provide mechanical strength and elasticity required for protection from physical stress, and to maintain cell shape and regulate morphogenesis (Lesage & Bussey, 2006).

The sugar polymers at the base of the cell wall are primarily responsible for providing the cell with tensile strength, and consist of the β-1,4-linked N-acetylglucosamine (GlcNAc) linear polymer known as chitin, a continuous network of helical β-1,3-glucan polymers that interact by hydrogen bonding, and highly branched, water-soluble β-1,6-glucan chains (Klis et al, 2006).

Mannoproteins forming the outer layer of the cell wall reduce its porosity and protect the polysaccharide layer from degrading enzymes. CWP functions include adhesion, iron uptake, cell wall remodeling, and signaling. The majority of CWPs are attached via a GPI anchor to β-1,6-glucan, which is attached in turn to β-1,3-glucan or chitin. Proteins with Internal Repeats-(PIR) CWPs are attached directly to β-1,3-glucan via an unidentified alkali-sensitive linkage. Other CWPs are non-covalently attached to neighboring CWPs via disulphide linkages.

Sugars and CWPs must be delivered to the cell wall to support growth and respond to changes in environmental conditions. CWPs are delivered via the secretory pathway, β-glucans
are delivered via poorly characterized, non-canonical pathways, and chitin is delivered via specialized vesicles known as chitosomes (Ziman et al, 1996).

The composition of the cell wall is known to vary with the cell cycle, and is subject to changes in oxygen levels, nutrient availability, and osmotic conditions (Jendretzki et al, 2011; Ziman et al, 1996). Two major mitogen-activated protein kinase (MAPK) signaling pathways are required for responding to these environmental cues and mounting transcriptional responses that allow cells to adapt to changing conditions. The Cell Wall Integrity (CWI) pathway is activated under condition of cell wall instability, while the High-Osmolarity Glycerol (HOG) pathway responds to osmotic stress (Rodriguez-Pena et al, 2010).

As was discussed in Chapter 3, YBR074 exhibits genetic interactions with genes related to cell wall function: FUS1, involved in cell fusion during mating; GFA1, involved in the first step of chitin biosynthesis; CDC48, involved in numerous cellular functions including ERAD and cell wall maintenance; TMN3, involved in cellular adhesion and filamentous growth; and GUP1, involved in remodeling GPI anchors. YBR074 also exhibits a chemical-genetic interaction under conditions of cell wall stress when deleted in the cdc48-2 background (Figure 27, discussed below). In this chapter, the role of Ybr074 in cell wall integrity and its interaction with CDC48 is examined.
4.1 MATERIALS AND METHODS

4.1.1 Strains and Molecular Techniques

Strains used in this chapter are described in Table 3. Serial dilutions were conducted as described in Chapter 3, using 50 μg/mL CR or 50 μg/mL CFW.

4.1.2 Zymolyase sensitivity Assay

Cells were grown in YPD at 30°C to logarithmic phase (OD\textsubscript{600} = 0.4-0.8) and 0.5 OD\textsubscript{600} equivalents of cells were harvested by centrifugation at ~13,000 g. Cells were washed in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA buffer and re-suspended in the same buffer to a final concentration of 1 OD\textsubscript{600}/mL. Cells were treated with 1 mg/mL zymolyase 20-T (MP Biomedicals, Solon, OH) for 1 h at 37°C. The OD\textsubscript{600} of each strain was measured using a 10 fold dilution of cells into water at 0 min and 60 min. Sensitivity to zymolyase treatment was measured as a percentage of the initial OD\textsubscript{600}.

4.1.3 β-1,3-glucan quantification assay

Quantification of 1,3-β-glucan was carried out as described (Watanabe et al, 2001). In brief, logarithmic phase cultures were washed twice and re-suspended in 10 mM TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) at 1.0 OD\textsubscript{600} cell equivalents in 0.5 mL. Sodium hydroxide was added to a final concentration of 1 M, and the reactions were incubated at 80°C for 30 min to solubilize the 1,3-β-glucan. To the solubilized sample, 2.1 mL of aniline blue mix (40 volumes
of 0.1% aniline blue; Electron Microscopy Science, Hatfield, PA, in water, 21 volumes of 1 M HCl, and 59 volumes of 1 M glycine/NaOH, pH 9.5) was added, and incubated at 50°C for 30 min followed by a further incubation at room temperature for 30 min. Fluorescence was measured using an Aminco-Bowman Series 2 spectrometer and AB2 series 2 software at an excitation wavelength of 400 nm/16 nm bandpass, and an emission wavelength of 460 nm/16 nm bandpass (Thermo Spectronic, Rochester, NY). Curdlan was used to determine the linear range for fluorescence intensity (0-20 ug/mL) (InvivoGen, San Diego, CA).

4.1.4 Killer toxin halo assay

Sensitivity to killer toxin was tested based on previously described protocols (Boone et al, 1990; Santos et al, 2000). K1 killer toxin secreting strain, 1368, described in Table 3, was grown to saturation in YPD, pH 4.5, overnight at 25°C. Cells were separated from K1 killer toxin secreted into the medium by centrifugation at ~2000 g for 4 min at room temperature. The supernatant containing K1 killer toxin was filtered using a 0.22 μm filter (Millipore, Franklin Lakes, NJ). The filtered medium was concentrated 200-fold using an ultrafiltration Amicon Ultra-15 unit with a 10 kDa molecular mass cut-off (Millipore, Billerica, MA). Test strains were grown in YPD to log phase, and 1 OD₆₀₀ cell equivalents were harvested and re-suspended in 100 μL sterile double distilled water. Two fold concentrated YPD, pH 4.5, medium was incubated in a 37°C water bath before being added to an equal volume of autoclaved 4% Bacto agar, supplemented with 30 μg/mL methylene blue. Once equilibrated to approximately 37°C, the molten medium was seeded with the test strain, and the medium was poured and allowed to solidify. A sterile filter disc spotted with 10 μL of the concentrated killer toxin medium was added to seeded plates and the plates were incubated for 4 d at room temperature. Sensitivity to
killer toxin was measured as the diameter of the halo using ImageJ software. The diameter of each halo was taken as the average of four measurements. Each strain was tested in three individual replicates and the average halo diameter and standard error were calculated using Microsoft Excel 2010.

### 4.2 RESULTS

Cdc48 is a homohexameric yeast ATPase Associated with diverse cellular Activities (AAA) protein, whose mammalian homolog is p97, also known as Valosin-Containing Protein (VCP). Broadly, Cdc48 and its cofactors, are required to segregate ubiquitinated substrates from protein complexes, membranes, and chromatin in order to facilitate their degradation (Meyer et al, 2012). Cdc48 facilitates ERAD, Golgi and ER membrane fusion, nuclear membrane re-assembly, cell cycle regulation, DNA repair, and autophagosome maturation. A distinct set of co-factors binds to the Cdc48 N-terminal domain in order to mediate each of these cellular functions. Cdc48 also contains a D1 ATPase domain required for hexamerization and heat-induced ATPase activity, as well as a D2 ATPase domain, which contributes most of the ATPase activity under physiological conditions (Yamanaka et al, 2012).

Recently, a role for Cdc48 in the maintenance of cell wall integrity during the cell cycle was described (Hsieh & Chen, 2011). Although the mechanism for Cdc48-dependent maintenance of cell wall integrity is undefined, these data are intriguing in light of the cell wall stress-dependent interaction between *CDC48* and *YBR074* observed in this study (see Figure 27).
Figure 26: CDC48 mutant alleles.

A) CDC48 mutant alleles are shown along with the corresponding yeast and human nucleotide changes associated with each allele. When a converted amino acid is not indicated, it was not naturally occurring in this organism B) Amino acids associated with each mutant allele are highlighted using the corresponding colors shown in A). The N-terminal domain, D1 ATPase domain, and D2 ATPase domain, respectively, are highlighted in yellow according to the domain
In order to elucidate which of the Cdc48 functions is affected by disruption of Ybr074 function, ERAD and cell wall integrity phenotypes were examined in various CDC48 mutant alleles (Figure 26) in the presence or absence of Ybr074 activity. CDC48 has been linked to a human degenerative disorder known as Inclusion Body Myopathy associated with Paget disease of bone and Frontotemporal Dementia (IBMPFD) (Watts et al, 2004). Two mutant alleles associated with IBMPFD were used in this study: cdc48 A232E and cdc48 R155H. The A232E mutation affects the D1 ATPase domain. The R155H mutation occurs within the linker region between the N-terminal domain and D1 ATPase domain, and disrupts an important interaction with N387 in the D1 ATPase domain. The R155-N387 interaction normally allows conformational changes induced by ATP hydrolysis to be transmitted to the N-terminal domain (DeLaBarre & Brunger, 2003; Hubbers et al, 2007).

The thermosensitive mutant allele, cdc48-3, was isolated in yeast and bears two mutations in the D1 ATPase domain. The cdc48-3 allele is associated with defects in ERAD at the non-permissive temperature of 37°C (Nakatsukasa et al, 2008). The cdc48-2 allele bears two mutations, one in the D2 ATPase domain, and the second near the C-terminus. Although this allele is less well represented in the literature, it has been shown to cause an ERAD defect at 30°C (Garza et al, 2009).

These alleles were used to further examine the genetic interaction between YBR074 and CDC48. Specifically, I assessed how cdc48 mutants in a ybr074Δ background affect sensitivity to the cell wall stress inducing agents calcofluor white (CFW) and Congo red (CR), as well as ERAD efficiency. My results suggest that Ybr074 is not involved in modulating the ERAD function of Cdc48. Secondly, while Ybr074 does not appear to affect the composition of
polysaccharides in the cell wall, it may contribute to a cdc48-2 allele-specific cell wall phenotype in the presence of CFW and Congo red.

4.2.1 Does YBR074 interact with the cell wall integrity function of CDC48?

As previously mentioned above, CFW and CR induce cell wall stress by preferentially binding to chitin in the cell wall (Karreman & Lindsey, 2007; Ram & Klis, 2006). A serial dilution of wild type, ybr074A, cdc48-2, and ybr074Acdc48-2 cells was plated on YPD supplemented with 50 μg/mL CFW or CR. To differentiate between cell wall-related defects and other defects associated with the CDC48 mutant strain, a serial dilution of cells was also plated on YPD supplemented with CFW or CR and with 1 M sorbitol, which acts as an osmotic stabilizer (Klis F.M. 2002). The krelΔ strain was used as a positive control for CFW and CR sensitivity because it is involved in β-1,6-glucan assembly. Disruption of KRE1 results in activation of the CWI pathway, which leads to a 2% to 20% compensatory increase in cellular chitin levels (Boone et al, 1990; Klis et al, 2002; Lesage et al, 2005). Strains with higher chitin content are more sensitive to CFW and CR, while strains with reduced chitin levels are more resistant to CFW and CR as compared to wild type yeast.
The positive control strain, *kre1Δ*, exhibits sensitivity to both CR and CFW as compared to wild type. This phenotype is rescued upon addition of sorbitol as an osmotic stabilizer, demonstrating that this is a cell wall specific defect (Figure 27). While *ybr074Δ* shows no sensitivity to CR or CFW as compared to the wild type, the *cdc48*−2 strain is sensitive to these cell wall stress-inducing agents at 30°C. Interestingly, the sensitivity of the *cdc48*−2 strain was partially rescued by addition of sorbitol, suggesting that much of the observed growth defect is induced by treatment with CR and CFW. The slight growth defect observed in the presence of sorbitol can be attributed to the temperature sensitivity observed in this strain at 30°C, and may be associated with defects in other functions of Cdc48 discussed above (Garza et al, 2009). Intriguingly, the *ybr074Δ cdc48*−2 strain exhibits no growth defect compared to wild type upon treatment with either CR or CFW. This suggests the presence of an alleviating genetic interaction between *YBR074* and *CDC48*. The *ybr074Δ* and *cdc48*−2 alleles in this double mutant strain are

![Figure 27: Sensitivity of *ybr074Δ* and *cdc48*-2 to cell wall stress inducing agents CFW and CR.](image)

A serial dilution of cells was spotted on YPD, YPD supplemented with CFW or CR, and YPD supplemented with CFW or CR and 1 M sorbitol, and incubated at 30°C. Although only one set is shown, this experiment was confirmed in five different colonies obtained from a single cross.
marked with the drug resistance genes *KANMX* and *NATMX*, and their presence has been confirmed multiple times by plating the cells on YPD medium supplemented with G418 or NAT. The *cdc48-2* allele in this double mutant strain was also confirmed by DNA sequence analysis.

To confirm that the alleviating interaction observed in the *ybr074Δcdc48-2* strain on CFW and CR was attributed to mutations in only *YBR074* and *CDC48*, and not to a third mutation elsewhere in the genome, two more *ybr074Δcdc48-2* isolates were obtained from independent yeast matings, sporulation, tetrad dissection, and genotyping. Parental strains were mated on three separate occasions to produce three individual *ybr074Δcdc48-2* progeny. However, while the double mutant isolate shown in Figure 27, displayed an alleviating phenotype, the remaining two isolates exhibited sensitivity to CR and CFW similar to the *cdc48-2* strain alone (Figure 29). This result suggests that a third, suppressor mutation is responsible for conferring resistance to CR and CFW in the initial *ybr074Δcdc48-2* strain tested in Figure 27, or that a spontaneous gene duplication event occurred that restored Cdc48 function.

In order to confirm and remove this third suppressor mutation, the *ybr074Δcdc48-2* strain will be backcrossed to a wild type strain several times in order to restore the genetic background of this strain to wild type. If the third suppressor mutation is responsible for CR and CFW resistance, its elimination should result in a *ybr074Δcdc48-2* strain with similar susceptibility to CR and CFW.

Serial dilutions of double mutant strains, in which the *ybr074Δ* allele was combined with the remaining *CDC48* mutant alleles shown Figure 26, were not consistent with the alleviating phenotype observed for the *ybr074Δcdc48-2* strain (Figure 28). This supports the hypothesis that a third suppressor mutation is responsible for conferring resistance to CR and CFW. However, it is formally possible that the genetic interaction between *YBR074* and *CDC48* is allele specific.
Figure 28: cdc48-3, cdc48 R155H, and cdc48 A232E do not exhibit a chemical-genetic interaction in the ybr074Δ genetic background.

Serial dilutions of the indicated strains were plated on YPD in the presence or absence of 50 μg/mL CFW. The chs3Δ strain, defective in biosynthesis of chitin, was used as a positive control for CFW resistance.
Individual ybr074Δcdc48-2 isolates do not exhibit a consistent chemical-genetic interaction of CFW.

ybr074Δcdc48-2 isolate A, derived from cross 56, spore 7B, shows a phenotype similar to the wild type strain on YPD containing 50 μg/mL CFW. Isolate B, derived from cross 71, spore 11C, and isolate C, derived from cross 73, spore 9A are both more sensitive to CFW than the wild type strain.
4.2.2 Does the loss of \textit{YBR074} affect the function of \textit{CDC48} during ERAD?

The best characterized function of Cdc48 is its role in ERAD, where it couples the chemical energy derived from ATP hydrolysis to produce the mechanical force necessary to extract ubiquitinated ERAD substrates from the ER membrane into the cytosol for degradation by the 26S proteasome (Wolf & Stolz, 2012). In order to ascertain whether the ERAD-related function of Cdc48 might be affected by disruption of \textit{YBR074}, cycloheximide assays were conducted using the ERAD substrates CPY* and Gas1*, which were discussed in the previous chapter. The soluble substrate CPY* has been previously shown to depend on Cdc48 function for ERAD, and this dependence is re-capitulated in Figure 30A (Jarosch et al, 2002). It was hypothesized that Ybr074 may be compensating for the loss of Cdc48 function, since accumulation of CPY* upon its overexpression has been demonstrated to target CPY* to the vacuole (Spear & Ng, 2003). However, instead of the expected increase in CPY* stabilization in a \textit{ybr074Δ cdc48-2} strain, my results demonstrate increased degradation of CPY* in this strain. This suggests that deletion of both \textit{CDC48} and \textit{YBR074} may be inducing a stress response that either facilitates delivery of CPY* to the vacuole, or induces expression of vacuolar proteases or an alternative degradation pathway.

The ERAD substrate Gas1* represents the misfolded form of the wild type Gas1 protein, which is responsible for the formation and maintenance of the β-1,3-glucan network of the yeast cell wall (Carotti et al, 2004). Gas1* bears the amino acid substitution, G291R, within a hydrophobic region of the protein. This mutation leads to Gas1* misfolding, ER retention, and 26S proteasome-dependent degradation (Fujita et al, 2006).
Gas1* is a unique ERAD substrate, in that it is subject to three different forms of quality control involving recognition of its protein, glycan and lipid elements. The misfolded protein is recognized by the ER chaperone Kar2, N-linked glycans are modified by the ER mannosidases Htm1 and Mns1 to promote ERAD, and O-linked glycans are appended by the O-mannosyltransferases Pmt1 and Pmt2 to mediate ER retention and association with Der1 (Goder & Melero, 2011; Hirayama et al, 2008). Finally, quality control of the GPI lipid anchor is mediated by the inositol GPI deacylase, Bst1, and members of the p24 complex, which function as an adaptor linking GPI-anchored substrates to the COPII coat (Castillon et al, 2011; Hirayama et al, 2008).

Intriguingly, Cdc48 has been found to be associated with Pmt1, Pmt2, and Der1 in a complex promoting the ERAD of Gas1* (Goder & Melero, 2011). However, Cdc48 has never been directly shown to contribute to Gas1* ERAD. Furthermore, although the function of the vacuolar protease, Pep4, is dispensable for Gas1*p degradation, it was shown to be required when in the absence of O-mannosylation was absent. This suggests that Gas1* is targeted for vacuolar degradation in Pep4-dependent manner when ERAD is impaired (Hirayama et al, 2008). It was therefore hypothesized that simultaneously crippling both ERAD and vacuolar degradation pathways in a ybr074Acdc48-2 mutant strain would result in exacerbated stabilization of Gas1*.
Figure 30: ERAD efficiency of *cdc48*-2 strains is not exacerbated by disruption of Ybr074 function.

A) CPY* degradation in wild type (n=4), *ybr074Δ* (n=5), *cdc48*-2 (n=6), and *ybr074Δ cdc48*-2 (n=6) at 37°C. B) Gas1* degradation in wild type, *ybr074Δ* (n=5), *cdc48*-2 (n=6), and *ybr074Δ cdc48*-2 (n=6) at 37°C. *ybr074Δ, cdc48*-2, and *ybr074Δ cdc48*-2 at 37°C (n=7). * = p < 0.05
To test this hypothesis, Gas1* degradation was monitored using cycloheximide chase analysis in wild type, ybr074Δ, cdc48-2, and ybr074Δcdc48-2 strains. Degradation of Gas1* was not significantly different from wild type in either ybr074Δ or cdc48-2 strains, indicating that Cdc48 is not required for degradation of Gas1* (Figure 30B). Formally, this may be the result of a compensatory vacuolar degradation pathway. Intriguingly, Gas1* was degraded to a greater extent in the ybr074Δ cdc48-2 strain than in the wild type strain, suggesting that a compensatory degradation mechanism may be induced in this strain, and that Ybr074 is not specifically involved in quality control of GPI-anchored substrates. As a control for this experiment, I also examined CPY* dependence in each individual mutant strain and found that CPY* was stabilized in the cdc48-2 strain, as expected (Rabinovich et al, 2002). However, CPY* was degraded slightly better in the ybr074Δcdc48-2 strain as compared to the cdc48-2 strain (Figure 30A). This indicates that Ybr074 is not involved in ERAD.

4.2.3 Are β-glucan levels affected by disruption of Ybr074 and Cdc48 function?

Given the results in Figure 27 suggesting Ybr074 may be affecting levels of chitin in the cell wall, I hypothesized that the more abundant β-glucan components of the cell wall may also be affected in a ybr074Δ strain. There are several ways to examine β-glucan levels in the yeast cell wall. In this section, enzymatic treatment using zymolyase and a quantitative fluorescence-based assay were used to assess β-1,3-glucan levels, while a killer toxin halo assay was used to detect β-1,6-glucan levels.
Zymolyase is an enzyme isolated from *Arthrobacter luteus* that specifically hydrolyses β-1,3-glucosidic bonds (Kitamura & Yamamoto, 1972). Yeast strains in which assembly of the β-glucan network is impaired are sensitive treatment with zymolyase, which eventually results in cell lysis (de Nobel et al, 2000; Garcia et al, 2009). As an example, the *kre1Δ* strain exhibits a cell wall weakened by a lack of β-1,6-glucan-mediated cross-linking of the β-1,3-glucan network. Consequently, the *kre1Δ* strain was observed to be approximately twice as sensitive to zymolyase treatment compared to wild type (Figure 31A). However, the *cdc48-2* and *ybr074Δ* strains did not display significantly different sensitivities to zymolyase compared to wild type. This result may be explained if the β-glucan levels in these strains remained unaltered compared to wild type, or if a reduction in β-glucan levels has been compensated for by an increase in mannoprotein levels. Increased mannoprotein levels may mask a zymolyase sensitivity phenotype by decreasing cell wall porosity and therefore, limiting access of zymolyase to the β-1,3-glucan layer of the cell wall (Shimoi et al, 1998). Surprisingly, the *ybr074Δ* strain was found to have a subtle, yet statistically significant increase in resistance to zymolyase treatment. This suggests *ybr074Δ* may have either increased levels of β-1,3-glucan or other cell wall components, which may mediate resistance to zymolyase compared to wild type yeast.

In order to examine β-1,3-glucan levels directly using a more quantitative approach, I employed the fluorescent dye, aniline blue, which binds specifically to β-1,3-glucans (Dubois et al, 1956). Purified β-1,3-glucan from yeast, known as Curdlan, was used to generate a standard curve for quantification of β-1,3-glucan levels in test strains. The results of this assay differed from the zymolyase sensitivity assay. In this case, the *ybr074Δ* strain did not exhibit any difference in β-1,3-glucan levels compared to wild type yeast, while both the *cdc48-2* and the
ybr074Δcdc48-2 strains had a statistically significant reduction in β-1,3-glucan levels to ~20% of wild type levels. This result suggests that there may be a zymolyase-specific stress response altering the cell wall structure in these strains via the HOG and/or CWI signaling pathways (Rodriguez-Pena et al., 2010). It is also possible that treatment with zymolyase for 1 h is sufficient to increase chitin levels in the cdc48-2 strain, protecting it from zymolyase-induced cell lysis and increasing its sensitivity to CFW and CR. However, this explanation is inconsistent with the observation that the ybr074Δ cdc48-2 strain is more resistant to CR and CFW. In this case, it is possible that an increase in mannoprotein levels at the cell wall may be contributing to CR and CFW resistance in this strain by reducing cell wall porosity (de Nobel et al., 1990). The cell wall protein Sed1 is a strong candidate for mediating resistance of the ybr074Δ cdc48-2 strain to CFW. Sed1 was shown by Bermejo et al. to be strongly up-regulated in response to cell wall stress-inducing agents such as CR and zymolyase (Bermejo et al., 2010). Furthermore, Sed1 is known to mediate resistance to zymolyase and rescue the sensitivity of cell wall mutant strains to CFW (de Nobel et al., 2000; Shimoi et al., 1998). Intriguingly, Sed1 was observed to accumulate in a ybr074Δ strain, as shown in Table 11. Therefore, I hypothesize that Sed1 is upregulated in the ybr074Δ cdc48-2, and is responsible for mediating resistance to CFW in this strain. Examining SED1 mRNA or protein levels in these strains may confirm this hypothesis if the ybr074Δ cdc48-2 strain exhibits increased levels of Sed1 as compared to ybr074Δ or cdc48-2 single mutant strains.
Figure 31: Analysis of β-1,3-glucan levels

A) Zymolyase sensitivity was measured as the percent OD$_{600}$ of a culture after 1 h of treatment with zymolase compared to the initial OD$_{600}$ (n=9; *, p < 0.05). B) Aniline blue fluorescence-based quantification of β-1,3-glucan levels are shown (n=2; *, p < 0.05).
In order to specifically examine β-1,6-glucan levels in these strains, a killer toxin halo assay was employed. In this experiment sensitivity to the K1 killer toxin protein was used as a measure of β-1,6-glucan levels in the cell wall of the test strain. K1 killer toxin binds specifically to β-1,6-glucans, forms pores in the plasma membrane, and results in lethality by impairing cellular ion homeostasis (Bussey, 1991).

Molten medium was seeded with test strains, and a filter disc containing K1 killer toxin was placed on the surface of the solidified medium. As the K1 killer toxin diffuses through the medium, it creates a circular region around the filter disc in which the test strain is killed. The diameter of this “halo” is used as a measure of K1 killer toxin sensitivity, and correlates with β-1,6-glucan levels. The kre1Δ strain, impaired in β-1,6-glucan synthesis, was used as a negative control, and exhibited no halo in this assay (Figure 32). The slt2Δ strain showed a slightly increased sensitivity to K1 killer toxin because the function of the MAPK, Slt2, normally mediating the CWI response, was disrupted. In hindsight, a hog1Δ strain would have been a stronger positive control, since this mutant strain displayed a stronger phenotype in a genome-wide mutant screen than slt2Δ, although the reason for this is not understood (Page et al., 2003). The ybr074Δ and ybr074Δ cdc48-2 strains both exhibited a subtle, but statistically significant decrease in sensitivity to K1 killer toxin. This may be explained by reduced levels of β-1,6-glucan in the cell wall. Alternatively, it has been shown that increased cell wall thickness can result in resistance to killer toxin by blocking K1 killer toxin access to the plasma membrane. Increased resistance to K1 killer toxin is difficult to interpret because increased cell wall thickness can be attributed to a number of reasons, including impaired cortical actin assembly or impaired endocytosis (Li et al., 2002; Page et al., 2003). Paradoxically, over-production of β-1,6-glucan has also been linked to K1 killer toxin resistance. It is thought that increased K1 killer
toxin binding may reduce its effective concentration and inhibit pore formation (Page et al, 2003). Therefore, it may be informative to examine cell wall thickness in ybr074Δ and ybr074Δcdc48-2 strains compared to wild type cells using electron microscopy, as previously published by our lab (Wright et al, 2007).

Figure 32: K1 killer toxin halo assay shows slight resistance of ybr074Δ and ybr074Δ cdc48-2 strains compared to wild type.

A) K1 killer toxin halos and B) halo diameters in arbitrary units (AU), normalized to wild type, are shown for the indicated strain along with the standard error associated with each (n=3, * = p < 0.05).
4.3 CONCLUSIONS

An isolated ybr074Δcdc48-2 strain displayed resistance to CR and CFW, as well as increased ERAD efficiency of CPY* and Gas1*p. Although these data suggest that the loss of both Ybr074 and Cdc48 function may be triggering a compensatory stress response that contributes to cell wall maintenance and ERAD, this conclusion is approached with caution because the possible presence of a third site mutation in this strain remains to be investigated.

The slight increase in zymolyase resistance of the ybr074Δ strain, as well as the data showing no difference in β-1,3-glucan levels in ybr074Δ compared to wild type yeast, suggest that a cell wall stress response independent of polysaccharide levels at the cell wall is protecting this strain from zymolyase treatment. This may be explained by an increase in mannoprotein levels at the cell wall, which would reduce its porosity and restrict access of zymolyase to the β-glucan substratum of the cell wall. In support of this hypothesis, the ybr074Δ strain was observed to be slightly resistance to K1 killer toxin, a phenomenon which has been linked to increased cell wall thickness and that is attributed to the accumulation of a mannoprotein layer. Therefore, future work should address this possibility using electron microscopy to examine cell wall thickness. Furthermore, results showing GFP-Sed1 accumulation in the ybr074Δ strain merit further investigation since Sed1 may be contributing to the accumulation of mannoproteins in the ybr074Δ strain as part of a response to cell wall stress. The ybr074Δcdc48-2 phenotype will be confirmed by back-crossing to the BY4742 wild type strain, and these recently established cell wall assays will be repeated.
To assign a more descriptive standard name for Ybr074, I propose Protein in FXNA-related Family 1, or Pff1. This name is derived from the PANTHER classification of this putative protease in the FXNA-related family of proteases whose members are listed in Table 5.

The finding that Pff1 is a vacuolar protease was surprising because published data demonstrate that its mammalian counterpart, FXNA, is ER-localized (Garcia-Rudaz et al, 2007). With only 11% sequence identity between Pff1 and FXNA, it may be that their differing subcellular localization is attributed to a legitimate cross-species difference, perhaps reflective of a divergence in function as well. In considering the reason for this difference, it is interesting to note that Pff1 has a 16 amino acid C-terminal sequence that is absent from both rat and human FXNA. The C-terminus of transmembrane proteins trafficking through the secretory pathway can be associated with di-basic or di-hydrophobic ER exit signals that mediate interaction with the COPII coat (Nufer et al, 2002; Watanabe & Riezman, 2004). Pff1 contains a C-terminal Ile-Leu, which in the case of Erv41, has been shown to serve as an ER exit signal (Otte & Barlowe, 2002). FXNA has a C-terminal Val-Phe, which is not a proven ER exit signal, but the composition of ER exit signals does display some flexibility and Val and Phe feature prominently within dihydrophobic motifs (Nufer et al, 2002). However, the topological analysis of Pff1 in this study indicates that the C-terminus is lumenal, and therefore cannot interact with the COPII coat in the cytosol to serve as an ER exit signal. Together, the simplest view is that the
C-terminus of Pff1 is not used as an ER-exit module. A more likely explanation is that an epitope tag at the C-terminus of Pff1 may disrupt protein folding and result in ER retention. Indeed, cycloheximide chase analysis comparing N- and C-terminally tagged Ybr074 constructs supports this hypothesis.

The localization data available for FXNA was obtained by immunofluorescence using a FXNA construct bearing a C-terminal FLAG tag (Garcia-Rudaz et al, 2007). This is striking when taken together with immunofluorescence data obtained from Pff1 constructs used in this study. Pff1 constructs bearing an HA tag either at the N-terminus or within the M28 protease domain were localized at the vacuolar membrane (Figure 10). In contrast, Pff1 tagged with HA at the C-terminus was localized to the ER, in congruence with FXNA-FLAG localization. This suggests that placing a tag at the C-terminus may influence the structure of these proteins and cause ER retention (Figure 12). In support of vacuolar localization, untagged Pff1 was identified as a component of the vacuolar membrane in a proteomic screen (Wiederhold et al, 2009). A re-examination of FXNA localization using an epitope tag placed at the N-terminus may be required to resolve this question. I plan to address this issue by examining the stabilities of Pff1-HA and HA-Pff1 by cycloheximide chase analysis. If Pff1 is retained in the ER due to misfolding, it may be targeted for degradation by the ER quality control system, and therefore be less stable than Pff1 that is successfully transported to the vacuole.

The mechanism by which Pff1 may be targeted to the vacuole also merits further investigation. The N-linked glycosylation of Pff1 (Figure 8), and the sub-population of Pff1 somewhat visible in the ER by immunofluorescence (Figure 10), suggest that Pff1 is not targeted to the vacuole by the Cvt pathway. However, transport of Pff1 to the vacuole either via the CPY pathway or the ALP pathway is theoretically possible (Bowers & Stevens, 2005). Since many of
the factors mediating these pathways have been worked out, identifying which transport pathway relays Pff1 to the vacuole may provide clues to some of its interaction partners.

To examine whether Pff1 sorts to the vacuole via the CPY pathway, Pff1 localization can be assayed in class E vacuolar sorting mutants (Raymond et al, 1992). This class of mutants is associated with defects in protein sorting to the MVB, and includes members of the ESCRT complexes. Mutant strains such as \textit{vps27Δ} (ESCRT 0) and \textit{vps23Δ} (ESCRT I) exhibit an accumulation of cargo dependent on the CPY pathway in the MVB, while cargo targeted by the ALP pathway bypasses the MVB and is targeted directly to the vacuole (Bowers & Stevens, 2005; Van Den Hazel et al, 1996).

To investigate the potential dependence of Pff1 on the ALP pathway, strains expressing a mutant allele of the AP3 adaptor complex binding partner, Vps41, can be examined. Cargo sorted by the ALP pathway requires the AP3 adapter complex and Vps41 for vesicle budding from the Golgi and for fusion with the vacuole in a clathrin-independent manner (Bowers & Stevens, 2005). The \textit{vps41Δ-231} allele is characterized by a Gly171Arg mutation in its N-terminal domain, which disrupts binding to the Apl5 subunit of the AP-3 adaptor complex (Darsow et al, 2001). Although \textit{vps41Δ} strains are defective for both CPY and ALP transport, the \textit{vps41Δ-231} mutant allele exhibits a specific defect in ALP trafficking. If Pff1 were found to accumulate in a pre-vacuolar compartment in a \textit{vps41Δ-231} mutant strain or in AP3 mutant strains, this would indicate that Pff1 must be transported to the vacuole via the ALP pathway.

As discussed in Chapter 1, there are seven known proteases in the yeast vacuole. This raises the question of why an eighth protease, such as Pff1, is expressed in this compartment. To answer this question it helps to consider the features of Pff1 that make it unique in comparison to other vacuolar proteases. The most striking feature of Pff1 is that it is the only multi-pass
transmembrane protease identified in the vacuole. As such, Pff1 has the potential to interact with, and possibly influence the activity of other proteins found in the vacuolar membrane. These include vacuolar sorting and fusion proteins, transporters, and the cell wall proteins required for the biogenesis of this structure, such as the GPI-anchored protein, Gas1, and the multi-pass transmembrane protein Fks1, which were discussed in Chapter 4. These interacting proteins may represent substrates of Pff1 or non-substrate partners of Pff1. Identifying vacuolar proteins that physically interact with Pff1 may be informative of its function.

Furthermore, it will be interesting to test whether Pff1 is ubiquitinated. The ubiquitin ligase, Rsp5, is involved in the transport of both endocytic cargo and cargo trafficking to the vacuole via the CPY pathway (Bowers & Stevens, 2005). Intriguingly, Pff1 was found in a large-scale pull down of ubiquitinated proteins (Peng et al, 2003). In addition, this study showed that Endo H-treated Pff1 had a higher molecular mass than was predicted based its amino acid sequence. Therefore, it is possible that Pff1 is ubiquitinated by Rsp5, or the Golgi-associated ubiquitin ligase, Tul1 (Reggiori & Pelham, 2002). It will be interesting to examine the dependence of vacuolar Pff1 and Pff1 retained in the ER on different ubiquitin ligases. It is possible that the E3 ligase interaction between these two forms of Pff1 may shift from the Golgi-associated E3 ligases, mentioned above, to ER-associated E3 ligases such as Hrd1 and Doa10 (Vembar & Brodsky, 2008).

In order to identify proteins that physically interact with Pff1, I will conduct immunoprecipitation experiments using purified vacuoles. Methods for isolating vacuoles from spheroplasted cells by Ficoll gradient centrifugation have been described (Ohsumi & Anraku, 1981; Wiederhold et al, 2009). In order to distinguish Pff1 substrates from non-substrate proteins interacting with Pff1, wild type Pff1 and a catalytically inactive Pff1 mutant will be examined.
Bonafide substrates of Pff1 may be partially or completely degraded in the presence of wild type Pff1, or may accumulate in a strain expressing a catalytically inactive Pff1 mutant.

The structures of select M28 metalloproteases have been solved, including that of the human glutamate carboxypeptidase II (GCP II; PDB ID 3RBU) (Tykvart et al, 2012). Site-directed mutagenesis of the GCP II active site was used to identify mutations that disrupted its protease activity (Speno et al, 1999). These mutations map to zinc-binding residues, which in Pff1 correspond to His156Gly, His256Gly, and Asp168Glu, shown in Figure 33. These amino acid substitutions will be used to generate catalytically inactive Pff1 mutants for immunoprecipitation experiments.
Figure 33: Schematic of Ybr074 (Pff1) active site

The active site contains two Zn$^{2+}$ ions denoted Zn1 and Zn2. Zn1 is coordinated to His300 and Glu200, Zn2 is coordinated to His156 and Glu266, while Asp168 takes part in a bidentate interaction, coordinating both Zn1 and Zn2. Only zinc-binding residues are shown, and the Glu201 residue putatively involved in catalysis is omitted.
Since autophagy is a major catabolic process associated with the vacuole, a role for Pff1 in autophagy must be considered. While many vacuolar proteases are upregulated in response to nitrogen starvation, this was not found to be true in the case of Pff1 (Gasch et al, 2000). Nonetheless, Pff1 may still interact with Atg proteins en route to the vacuole to facilitate sorting of autophagosomes to the vacuolar compartment. To test this hypothesis, wild type and pff1Δ strains expressing GFP-Atg8 will be constructed and the localization and cleavage of the protein will be assessed as described (Nair et al, 2011). Specifically, GFP-Atg8 can be used as a reporter for proper formation of the autophagosome by fluorescence microscopy. GFP-Atg8 may also be used to examine internalization of the phagocytic body. In wild type cells, the inner membrane of the autophagosome, containing GFP-Atg8, fuses with the vacuole and GFP and degradation of GFP-Atg8 will be visible by Western blot analysis. In the pff1Δ strain, GFP-Atg8 may accumulate, indicating a defect in fusion of the autophagosome with the vacuole.

The vacuole is a dynamic buffering compartment that responds to numerous environmental cues by regulating cytosolic concentration of nutrients (He & Klionsky, 2009). The vacuole responds to nutrient stress, osmotic stress, stages of the cell cycle, oxidative stress, and the presence of toxic metals and drugs (Li & Kane, 2009). The proteases responsible for recycling amino acids in this compartment have broad specificities, allowing for non-selective degradation of cargo targeted to the vacuole. Some of these proteases, such as CPY and CpS have partially redundant functions, but are targeted to the vacuole in slightly different ways; as a soluble protease or anchored to the membrane, respectively. Others have catalytic activities that can be modulated by the presence of certain cellular factors; for example, Ape3 activity is affected by Co²⁺ (Yasuhara et al, 1994). These data suggest that the importance of the vacuole’s buffering capacity requires an arsenal of proteases, which may interact with different partners.
and whose activities may be fine-tuned in response to cellular conditions and environmental stresses. Understanding the cues that influence Pff1 activity will be important to place its function in the context of both cellular physiology and that of other vacuolar proteases. Expression of Pff1, by Northern blot analysis or quantitative RT-PCR, under conditions that trigger the cell wall integrity response, such as via heat stress, calcofluor white treatment, or excess Ca\textsuperscript{2+}, may address this question (Hohmann, 2009; Ram & Klis, 2006; Rodriguez-Pena et al, 2010; Zhao et al, 1998). This experiment might also address the potential role of Pff1 in the maintenance of cell wall integrity, which was raised in this study. However, the effect of Pff1 on the cell wall cannot be affirmed until cell wall related phenotypes of \textit{cdc48-2pff1Δ} yeast have been re-examined, as discussed in Chapter 4.

Overall, this study has shed light on a novel putative protease that adds to the repertoire of the yeast vacuole. Further studies demonstrating Pff1 proteolytic activity and substrate specificity, as well as those breaking down the biological function of Pff1 await further research, and are greatly anticipated.
APPENDIX A

ATTEMPTS TO PURIFY THE PROTEASE DOMAIN OF YBR074 (Pff1)

In order to determine whether Pff1 has proteolytic activity, attempts were made to purify its putative M28 metalloprotease domain in order to conduct proteolytic assays \textit{in vitro}. This section outlines the various cloning, expression, purification, and enzymatic assays used in an effort to elucidate the predicted enzymatic activity of Pff1.

A.1 ASSIGNING PROTEASE DOMAIN BOUNDARIES

An initial attempt at expressing the Pff1 protease domain was made by Douglas Placais using the sequence shown in Table 13. This construct was N-terminally GST-tagged and inserted into a pGEX2T vector with a P\textsubscript{tac} promoter. However, cloning attempts resulted in rearrangements of the insert, and it was hypothesized that this was the result of toxicity induced by leaky expression of the Pff1 protease domain. To overcome the leaky expression of the Pff1 protease domain, a pET21a(+) vector was tested. In this vector, expression of the Pff1 protease domain, containing a C-terminal 6 x His tag, was under control of a P\textsubscript{T7} promoter, and expression of T7 polymerase was controlled by a P\textsubscript{tac} promoter.

Expression of the Pff1 protease domain from the pET21a(+) vector in BL21 (DE3) \textit{E. coli} cells was impeded by low codon usage bias. When Rosetta Gami B (DE3) cells containing the pRARE vector encoding rare tRNAs was used instead, high molecular weight bands were
observed upon expression of the Pff1 protease domain. This suggested that this Pff1 protease domain construct was prone to aggregation. Aggregation of the protein encoding this construct may have been caused by the presence of a cysteine residue near the C-terminus, or by the presence of sequence predicted to constitute the adjacent hydrophobic transmembrane segments on either side of the Pff1 protease domain (Table 13).

In order to increase solubility of the Pff1 protease domain for the purpose of purification, alternative constructs were designed, as part of this dissertation work, using the following rationale. Construct PD₀ was designed to exclude transmembrane segment (TM) 1 based on ConPred II topology prediction, using Glu38 as the first residue. The PD₀ construct also excludes TM 2 and a flanking cysteine residue, leaving Ala341 as the C-terminal residue. Construct PD₁ boundaries are based on those of secondary structure elements within the protease domain. The secondary structure of the *Streptomyces griseus* M28 metalloprotease known as aminopeptidase S (PDB ID: 1CP7) was compared to a secondary structure of the Pff1 protease domain predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (Figure 34). The PD₁ construct begins at Thr75; three amino acids upstream of a predicted alpha helix, and ends at Ser332; three amino acids downstream of a predicted alpha helix (Figure 34). Construct PD₂ was designed based on the Pff1 M28 domain boundaries assigned by MEROPS (http://merops.sanger.ac.uk/). Finally, construct PD₃ was designed based on the Pff1 M28 domain boundaries assigned by Pfam (http://pfam.sanger.ac.uk/).
Table 13: Pff1 protease domain constructs designed for purification

The name, amino acid boundaries, predicted molecular weight, and amino acid sequence of each construct is shown.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Boundaries</th>
<th>Molecular Weight</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD_Piecelsis</td>
<td>Tyr33-Cys364</td>
<td>38 kDa</td>
<td>YFDHREYKLNLPSREDEHPNFLLELETANGDLQITASPHYTSKENDKHVDYL LEKRLVLTLCTGGHSSFASTTSDDKREESERSLFFQQDDDFNLESSRFSEFVTFESSNLVLFK LERGNNPEEEGLLLSAHDFSVPFGYGTDDDMGVSLANLYHKHRPNRTLLF NPNNHSEFFLGLLSDYTFHNSWNLTKYVINLEGTGAGGRFLFRSTDSTARYQQSVLK ENSPNCSYYQQGSFKYSRYVRSYTEDKLYEEENGMSRGWDAFYKPRNLNYH2IKDGQ YTSFA8LHMTLSLQSLAYVANN5LDTADQTPA</td>
</tr>
<tr>
<td>PD0</td>
<td>Glu38-Ala341</td>
<td>35 kDa</td>
<td>ERYKLNLPSREDEHPNFLLELETANGDLQITASPHYTSKENDKHVDYLLEKRLVL TLTGNSFSAVSDDEKREESERSLFFQQDDDFNLESSRFSEFVTFESSNLVLFK LERGNNPEEEGLLLSAHDFSVPFGYGTDDDMGVSLANLYHKHRPNRTLLF NPNNHSEFFLGLLSDYTFHNSWNLTKYVINLEGTGAGGRFLFRSTDSTARYQQSVLK ENSPNCSYYQQGSFKYSRYVRSYTEDKLYEEENGMSRGWDAFYKPRNLNYH2IKDGQ YTSFA8LHMTLSLQSLAYVANN5LDTADQTPA</td>
</tr>
<tr>
<td>PD1</td>
<td>Thr75-Ser332</td>
<td>29 kDa</td>
<td>TSNDKVDYHQLLREKTLTGGHSSFASTTSDDKREESERSLFFQQDDDFNLESSRFSEFVTFESSNLVLFK LERGNNPEEEGLLLSAHDFSVPFGYGTDDDMGVSLANLYHKHRPNRTLLF NPNNHSEFFLGLLSDYTFHNSWNLTKYVINLEGTGAGGRFLFRSTDSTARYQQSVLK ENSPNCSYYQQGSFKYSRYVRSYTEDKLYEEENGMSRGWDAFYKPRNLNYH2IKDGQ YTSFA8LHMTLSLQSLAYVANN5LDTADQTPA</td>
</tr>
<tr>
<td>PD2</td>
<td>Asn96-Ala341</td>
<td>28 kDa</td>
<td>N88PSALV8DDEKREESERSLFFQQDDDFNLESSRFSEFVTFESSNLVLFK LERGNNPEEEGLLLSAHDFSVPFGYGTDDDMGVSLANLYHKHRPNRTLLF NPNNHSEFFLGLLSDYTFHNSWNLTKYVINLEGTGAGGRFLFRSTDSTARYQQSVLK ENSPNCSYYQQGSFKYSRYVRSYTEDKLYEEENGMSRGWDAFYKPRNLNYH2IKDGQ YTSFA8LHMTLSLQSLAYVANN5LDTADQTPA</td>
</tr>
<tr>
<td>PD3</td>
<td>Glu149-Ser332</td>
<td>21 kDa</td>
<td>E5LLLAMFDSVPFGYGTDDDMGVSLANLYHKHRPNRTLLF NPNNHSEFFLGLLSDYTFHNSWNLTKYVINLEGTGAGGRFLFRSTDSTARYQQSVLK ENSPNCSYYQQGSFKYSRYVRSYTEDKLYEEENGMSRGWDAFYKPRNLNYH2IKDGQ YTSFA8LHMTLSLQSLAYVANN5LDTADQTPA</td>
</tr>
</tbody>
</table>
Key
Conf: Confidence (0=low, 9=high)
Pred: Predicted secondary structure (H=helix, E=strand, C=coil)
Pff1: Pff1 amino acid sequence

# PSIPRED HFORMAT (PSIPRED V3.0)

Conf: 90677855321466642678999999999999999643210145345799998989887759999
Pred: CHHHHHHHHCCCCCHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCC
Pff1: MKLKSVFRSVLKYRKNLSLLLLITYSIITLLYTIFDHHERYKLNLPEDEHPEFNDLLETA
       10     20     30     40     50     60
Conf: 9999995359878999659999999998614899863455322201343037887
Pred: HHHHHHHHCCCCCHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCC
Pff1: WGDQLIITASFHPYTSKENDKVKHDYLLKRVLEITGNSSFSVSSKESERSILFCQDPE
       70     80     90    100    110    120
Conf: 6566520223311005999983789999589990105889999876821699999999
Pred: CCCCCCEEEEEECCCCCCCCEEEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHHHHHHHHH
Pff1: NESSRFSRVTYSNSSNILVKLEGKNNEEGLLLSAHFDSVPTGYGATDGMVYVSLLANI
       130    140    150    160    170    180
Conf: 970039999419999729633550115999845355655579987225889921688719
Pred: HHHHHHHCCCCEEEEEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
Pff1: KYHIXCHRPNRTL1IFNENNEFGLLGASTYFDHWSNLTKYVINLEGTGAGGKAVLFRST
       190    200    210    220    230    240
Conf: 85289999840158888721112334788889972075764799434752315999798
Pred: CCHHHHHHHCHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
Pff1: DTSTARIYQQSVKENPFNGSIYQQGFYRSRTYVRSSETDVKIYEENMRGWDVAFYKPRNLYH
       250    260    270    280    290    300
Conf: 96898247999999999999999999999999809999999999928983057269999365036
Pred: CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
Pff1: TIKDSIQYTSKASLWHMLHTSLOLSAYVASNAIHDATDQTPACYFDGLKFFVISAKTLE
       310    320    330    340    350    360
The transmembrane segments of Pff1 predicted using ConPredII are highlighted in grey. The M28 domain as predicted by the MEROPS database is highlighted in yellow, with conserved metal-binding residues in bold and underlined.

**A.2 CLONING AND EXPRESSION OF THE PFF1 PROTEASE DOMAIN**

The protease domain constructs described above were cloned into the vectors shown in Table 14. Numerous attempts to clone Pff1 protease domain into these vectors failed, with the exception of the pBAD/gIII A vector. The tightly regulated pBAD promoter is dependent on the presence of L-arabinose for induction, and can be repressed by the presence of glucose. Constructs PD₀, PD₁, PD₂ and PD₃ were successfully cloned into the pBAD vector and induced in TOP10 *E. coli* cells using 0.2% L-Arabinose at 37°C. TOP10 cells were used because they do not metabolize L-arabinose. Although protease domain expression increased over time, the optical density of the
bacterial culture expressing the protease domain began to decrease after 4 h of induction (Figure 35A). This observation supports the hypothesis that Pff1 protease domain expression is toxic in *E. coli*.

Table 14: Vectors used in attempts to clone Pff1 protease domain for purification

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter</th>
<th>Tag</th>
<th>Tag location</th>
<th>Cleavage site</th>
<th>Secretion Signal</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMAL-p4X</td>
<td>P<em>bad</em></td>
<td>6xHIS-MBP</td>
<td>N-terminal</td>
<td>Factor Xa</td>
<td>mali signal sequence</td>
<td>periplasmic space</td>
</tr>
<tr>
<td>pMAL-c4X</td>
<td>P<em>bad</em></td>
<td>MBP</td>
<td>N-terminal</td>
<td>Factor Xa</td>
<td>None</td>
<td>cytosol</td>
</tr>
<tr>
<td>pLC3</td>
<td>T7</td>
<td>6xHIS-MBP</td>
<td>N-terminal</td>
<td>TEV</td>
<td>None</td>
<td>cytosol</td>
</tr>
<tr>
<td>pBAD/gIII A</td>
<td>P<em>bad</em></td>
<td>myc-6xHIS</td>
<td>C-terminal</td>
<td>N/A</td>
<td>Gene III secretion signal</td>
<td>periplasmic space</td>
</tr>
</tbody>
</table>

![Figure 35](image)

Figure 35: Expression of PD$_2$ from the pBAD vector

A) Optical density of TOP 10 *E. coli* expressing PD$_2$ at 37°C over the course of 6 h, induced by the indicated amounts of L-Arabinose. O.D$_{600}$ values are tabulated on the right (n=1)
B) Cells expressing PD$_2$ were collected at the indicted time points, lysed, and separated into
supernatant and pellet fractions. Protein samples were normalized to the culture O.D.\textsubscript{600} and equal quantities of protein were resolved by 10\% SDS-PAGE and visualized using Coomassie brilliant blue stain. PD\textsubscript{2} had an apparent molecular weight of ~30 kDa and fractionated with the insoluble pellet fraction.

A.3 PILOT PURIFICATION OF THE PFF1 PROTEASE DOMAIN

A pilot purification of PD\textsubscript{2} was conducted using native purification buffer, or denaturing purification buffers containing 6 M urea, 1\% Triton X-100, or 6 M guanidine hydrochloride (GnCl).

Native purification buffer: \begin{itemize} 
\item 50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.8
\item 0.5 M NaCl
\end{itemize}

6M GnCl lysis buffer: \begin{itemize} 
\item 6 M guanidine hydrochloride
\item 20 mM sodium phosphate pH 7.8
\item 500 mM NaCl
\end{itemize}

6M urea lysis buffer: \begin{itemize} 
\item 6M urea
\item 20 mM sodium phosphate pH 7.8
\item 500 mM NaCl
\end{itemize}

1\% Triton X-100 lysis buffer: \begin{itemize} 
\item 1\% Triton X-100
\item 20 mM sodium phosphate pH 7.8
\item 500 mM NaCl
\end{itemize}

A 250mL culture was induced using 0.2\% L-Arabinose for 4 h at 37°C. Cells were harvested by centrifugation at 5000 g for 10 min at 4°C in a GSA rotor. The pellet was re-suspended 32 mL
native purification buffer and split into four 8 mL samples. Each 8 mL sample was treated using 8 mg of chicken egg lysozyme for 30 min on ice and in the presence of protease inhibitors (PMSF, Leupeptin, and Pepstatin A). Cells were then lysed by sonication on ice in four 30 sec pulses with 1 min intervals on ice between pulses. Lysates were cleared by centrifugation at 3000 g for 15 min at 4°C in an SS-34 rotor. Cleared lysates were re-suspended in 8 mL of native binding buffer, 6M GnCl lysis buffer, 6M urea buffer, or 1% Triton X-100 buffer. Cells were incubated on ice for 20 min with occasional mixing to ensure thorough lysis. Lysates were centrifuged at 3000 g for 15 min at 4°C. Supernatants were added to 2 mL of equilibrated Ni$^{2+}$-NTA resin and allowed to bind in batch on a nutator for 60 min at 4°C. The resin was allowed to settle, the flow through was collected, and the resin was washed twice with 8 mL wash buffer (10 mM imidazole, 20 mM sodium phosphate pH 7.8, 500 mM NaCl). SDS-PAGE sample buffer was added to 10 µL of resin from each in batch purification and protein bound to the resin was resolved on 10% SDS-polyacrylamide gel and visualized by Coomassie brilliant blue staining (Figure 36). Purification using 6 M guanidine hydrochloride lysis buffer appeared to produce the best combination of yield and purity.
Cells were grown in 1 L Luria broth (LB) supplemented with 200 μg/mL ampicillin at 37°C to an O.D.₆₀₀ = 0.7-0.9. Pff1 protease domain expression was induced with 0.2% L-Arabinose for 4 h at 37°C. Cells were spun down in two 500 mL batches at 5000 g for 6 min at 4°C in an F7S rotor. The cell pellet was re-suspended in 10 mL 6 M GnCl lysis buffer supplemented with protease inhibitors (PMSF, Leupeptin, Pepstatin A). Cells were then lysed by sonication in three 30 sec pulses with 1 min intervals on ice between pulses to prevent heat-induced protein denaturation. Lysates were cleared by centrifugation at 12 000 g for 15 min at 4°C using an SS-34 rotor. The cleared lysate was added to 5 mL of equilibrated Ni²⁺-NTA resin and the His-

**Figure 36: Pilot purification of PD2**

PD2 (~30 kDa) is shown by coomassie blue staining of a 10% SDS-PAGE gel. PD₂-myc-6xHIS purified in batch using native binding buffer, 1% Triton X-100 lysis buffer, 6 M urea lysis buffer, or 6 M guanidine hydrochloride (GnCl) lysis buffer.

### A.4 PURIFICATION PROCEDURE FOR PPF1 PROTEASE DOMAIN

Cells were grown in 1 L Luria broth (LB) supplemented with 200 μg/mL ampicillin at 37°C to an O.D.₆₀₀= 0.7-0.9. Pff1 protease domain expression was induced with 0.2% L-Arabinose for 4 h at 37°C. Cells were spun down in two 500 mL batches at 5000 g for 6 min at 4°C in an F7S rotor. The cell pellet was re-suspended in 10 mL 6 M GnCl lysis buffer supplemented with protease inhibitors (PMSF, Leupeptin, Pepstatin A). Cells were then lysed by sonication in three 30 sec pulses with 1 min intervals on ice between pulses to prevent heat-induced protein denaturation. Lysates were cleared by centrifugation at 12 000 g for 15 min at 4°C using an SS-34 rotor. The cleared lysate was added to 5 mL of equilibrated Ni²⁺-NTA resin and the His-
tagged Pff1 protease domain was allowed to bind the resin in batch on a rocker for 60 min at 4°C. The resin was allowed to settle and the flow-through was removed. The resin was washed four times using 15 mL of 6 M GnCl lysis buffer by incubation for 5 min at 4°C. Protein was then eluted in 8-10 steps from the resin using 1 mL washes with elution buffer. This protocol typically yielded purified Pff1 protease domain at a concentration of approximately 0.5 mg/mL.

Since SDS precipitates in the presence of guanidine hydrochloride, 50 μL of each protein purification sample was TCA precipitated and acetone washed prior to re-suspension in SDS-PAGE sample buffer. Protein purification samples were resolved by 10% SDS-PAGE gels and protein was visualized by Coomassie brilliant blue staining.

6M GnCl lysis buffer: 6 M guanidine hydrochloride
10 mM imidazole
20 mM sodium phosphate pH 7.8
500 mM NaCl

Elution buffer: 6 M guanidine hydrochloride
500 mM imidazole
50 mM NaH₂PO₄, pH 7.8
0.5 M NaCl
10% SDS-polyacrylamide gel in which protein is visualized by Coomassie brilliant blue staining. Samples shown are protein from uninduced cells (UN), cells induced for 4 h (4), whole lysate (WL), pellet (P), cleared lysate (CL), flow through (FT), washes (W1-4), and elutions (E1-8). PD$_2$, with an apparent molecular mass of ~30 kDa, is enriched in elution fractions.

**A.5 REFOLDING THE PFF1 PROTEASE DOMAIN**

In order to use the purified Pff1 protease domain in an *in vitro* enzymatic assay, the protease domain must be re-folded back into its native structure. To do this, the guanidine hydrochloride had to be removed from the eluted protein sample by dialysis. Solubility of PD$_2$ could be maintained in as low as 2M guanidine hydrochloride, but any further reduction in guanidine hydrochloride concentration resulted in PD$_2$ crashing out of solution (Figure 37).

This result was observed after overnight dialysis at 4°C of 500 μL of purified PD$_2$ into 4 M GnCl, 150 mM NaCl, 250 mM sorbitol, 10% glycerol, 400 mM arginine pH 7.8. In a second
step, the protein was dialyzed for 5.5 h at 4°C into a solution containing 2 M GnCl, 150 mM NaCl, 250 mM sorbitol, 10% glycerol, 400 mM arginine pH 7.8 (Figure 37). Finally, the protein was dialyzed overnight at 4°C into 1 M GnCl, 150 mM NaCl, 250 mM sorbitol, 10% glycerol, 400 mM arginine pH 7.8 in the presence or absence of 1 mM ZnCl$_2$. Since zinc is a cofactor for M28 family metalloproteases, I hypothesized that the presence of zinc in the dialysis buffer might facilitate refolding. However, zinc did not have an effect on PD$_2$ solubility under these conditions.

![Figure 38: Refolding of PD$_2$ by dialysis](image)

The left panel shows a Coomassie brilliant blue stained 10% SDS-polyacrylamide gel of PD$_2$ samples. At 6 M GnCl the whole (W) protein sample was loaded, while at 4 M, 2 M, and 1 M GnCl each sample was fractionated by centrifugation into an insoluble pellet (P) and soluble (S) fraction. Zinc chloride was omitted from all dialysis buffers except the 1 M GnCl buffer, as indicated. The right panel shows the same samples analyzed by western blot analysis using an anti-5XHis antibody to visualize PD$_2$ protein.
In addition to dialysis, fast dilution of PD$_2$ into various refolding buffers was attempted based on previous successes of this method (Vincentelli et al, 2004). Purified PD$_2$ was diluted 20-fold into various refolding buffers, listed in Table 15, in a 96-well plate. Isopropanol was used as a negative control and elution buffer was used as a positive control for PD$_2$ solubility. Differences among the refolding buffers in maintaining PD$_2$ solubility could not be reliably ascertained by measuring absorbance because precipitated protein settled to the bottom of the wells and resulted in spurious readings. To overcome this problem, samples were collected from the 96-well plates and separated by centrifugation into insoluble and soluble fractions. Protein in these fractions was resolved by 10% SDS-PAGE and visualized by silver staining. Although there may have been a tendency towards more protein partitioning to the soluble fraction in buffers containing arginine at pH 8 or 9, these results were also inconsistent.
Table 15: Refolding buffers tested on PD2 in a 96-well plate format

<table>
<thead>
<tr>
<th>Row</th>
<th>Column</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>isopropanol</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>solution buffer</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>50 mM sodium acetate pH 4</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>50 mM sodium acetate pH 4, 100 mM NaCl</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>50 mM sodium acetate pH 4, 200 mM NaCl</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>50 mM sodium acetate pH 4, 20% glycerol, 50 μM CaCl2, 50 μM MgCl2, 50 μM ZnCl2, 50 μM NiCl2, 50 μM CuSO4</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>50 mM sodium acetate pH 4, 0.05% PEG, 500 mM glucose</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>50 mM sodium acetate pH 4, 1 mM EDTA</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>50 mM sodium acetate pH 4, 800 mM arginine</td>
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<tr>
<td>B</td>
<td>2</td>
<td>50 mM MES pH 5</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>50 mM MES pH 5, 100 mM NaCl</td>
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<tr>
<td>D</td>
<td>2</td>
<td>50 mM MES pH 5, 200 mM NaCl</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>50 mM MES pH 5, 20% glycerol, 50 μM CaCl2, 50 μM MgCl2, 50 μM ZnCl2, 50 μM NiCl2, 50 μM CuSO4</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>50 mM MES pH 5, 0.05% PEG, 500 mM glucose</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>50 mM MES pH 5, 1 mM EDTA</td>
</tr>
<tr>
<td>H</td>
<td>2</td>
<td>50 mM MES pH 5, 800 mM arginine</td>
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<td>50 mM MES pH 6, 200 mM NaCl</td>
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<tr>
<td>D</td>
<td>3</td>
<td>50 mM MES pH 6, 20% glycerol, 50 μM CaCl2, 50 μM MgCl2, 50 μM ZnCl2, 50 μM NiCl2, 50 μM CuSO4</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>50 mM MES pH 6, 0.05% PEG, 500 mM glucose</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>50 mM MES pH 6, 1 mM EDTA</td>
</tr>
<tr>
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<td>50 mM Tris pH 7, 20% glycerol, 50 μM CaCl2, 50 μM MgCl2, 50 μM ZnCl2, 50 μM NiCl2, 50 μM CuSO4</td>
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<td>E</td>
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<td>50 mM Tris pH 7, 0.05% PEG, 500 mM glucose</td>
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<td>F</td>
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<td>50 mM Tris pH 7, 1 mM EDTA</td>
</tr>
<tr>
<td>G</td>
<td>4</td>
<td>50 mM Tris pH 7, 800 mM arginine</td>
</tr>
<tr>
<td>H</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>5</td>
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</tr>
<tr>
<td>C</td>
<td>5</td>
<td>50 mM Tris pH 8, 20% glycerol, 50 μM CaCl2, 50 μM MgCl2, 50 μM ZnCl2, 50 μM NiCl2, 50 μM CuSO4</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>50 mM Tris pH 8, 0.05% PEG, 500 mM glucose</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>50 mM Tris pH 8, 1 mM EDTA</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>50 mM Tris pH 8, 800 mM arginine</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>50 mM CHES pH 9</td>
</tr>
<tr>
<td>H</td>
<td>5</td>
<td>50 mM CHES pH 9, 100 mM NaCl</td>
</tr>
<tr>
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<td>6</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
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<tr>
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<tr>
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Arginine has been used successfully to refold nucleotide binding domain (NBD) 1 of the cystic fibrosis transmembrane conductance regulator (CFTR) from a purification buffer containing 6 M guanidine hydrochloride (Qu & Thomas, 1996). It has been proposed that the guanidinium group of arginine helps solubilize polar residues, while its aliphatic 3-carbon chain may protect hydrophobic regions of the protein from aggregation. In order to assess whether arginine may be an effective refolding reagent for PD$_2$, a similar re-folding buffer was tested. Refolding buffer containing 100 mM Tris-HCl pH 8.0, 400 mM L-arginine-HCl, 2 mM EDTA, 1 mM DTT, and 1 mM ZnCl$_2$500 μL was used to dilute 500 μL of purified PD$_2$ in 6 M GnCl-containing elution buffer 50-fold. The diluted protein was incubated on a nutator overnight at 4°C. Soluble PD$_2$ was isolated by centrifugation at 16 000 g for 20 min at 4°C in an SS-34 rotor. The supernatant fraction was TCA precipitated and the protein resolved on a 10% SDS-polyacrylamide gel and visualized by Coomassie brilliant blue staining and silver staining. Using the same treatment in which L-arginine in the refolding buffer was replaced with L-histidine also produced some soluble PD$_2$ but at a lower level. This suggests that L-arginine promotes PD$_2$ solubility in the course of protein refolding.
In order to test the proteolytic activity of the Pff1 protease domain two different colorimetric assays were employed: an azocasein cleavage assay and an L-leucine p-nitroanilide (LeuNA) cleavage assay. The azocasein assay is targeted towards endopeptidases, while the LeuNA assay is targeted towards exopeptidases.

The azocasein cleavage assay was performed as follows. Azocasein powder (Sigma) was dissolved in a 0.5% (w/v) sodium carbonate solution to produce a 2.5% (w/v) azocasein solution.

A.6 ASSAYS FOR PROTEOLYTIC ACTIVITY
500 μL of this azocasein solution was mixed with 300 μL of 0.5% (w/v) sodium carbonate and allowed to equilibrate at 37°C. The equilibrated mixture was supplemented with 200 μL of purified Pff1 protease domain and incubated with shaking in a 37 °C water bath for 30 min. The reaction was stopped using 4 mL of 5% TCA, forming a yellow precipitate. This precipitate was separated from the solution by centrifugation at 12 000 g for 10 min at 4°C in an SS-34 rotor. 1 mL of the clear supernatant was transferred to a fresh borosilicate culture tube and mixed with 3 mL of 0.5 M NaOH, producing a yellowish color. Absorbance was measured at 440 nm. Trypsin was used as a positive control for this assay.

It was found that imidazole in the elution buffer interfered with the azocasein assay by resulting in absorbance at 440 nm even in the absence of any protein. Therefore, for future reference it is important to note that Pff1 protease activity cannot be tested without dialyzing out the imidazole first.

Although the possibility that Pff1 is an endopeptidase cannot be excluded, it is important to note that the M28 family of metalloproteases is comprised of exopeptidases. Among these are the *S. griseus* aminopeptidase, SGAP, and the *Vibrio proteolyticus* (also known as *Aeromonas proteolytica*) aminopeptidase, AAP. Both of these aminopeptidase exhibit a specificity for leucine at their substrate binding site and have shown proteolytic activity in the presence of the substrate LeuNA (Arima et al, 2006).

The LeuNA cleavage assay was modified from the Sigma protocol and performed as a 96-well plate assay in the following manner. In each well, 933 μL of 50 mM sodium phosphate buffer, pH 7.2 was mixed with 33 μL of 24 mM LeuNA solution prepared in methanol. The mixed solution was equilibrated at 37°C and the absorbance at 405 nm was monitored until it remained constant. Then 33 μL of enzyme was added, the solution was mixed and the A$_{405}$ was
read every minute for 6 min. Leucine aminopeptidase, microsomal, porcine kidney (Sigma) was used as a positive control, and buffer was used as a negative control. Although a steady increase in A405 was observed for the positive control, leucine aminopeptidase, none was observed for purified PD2 or PD0 (data not shown). Furthermore, it was observed that L-arginine inhibited the positive control reaction completely. This suggests that the activity of Pff1 protease domain refolded in the presence of L-arginine cannot be determined using this assay.
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