The Role of Ubx2p in ER-Resident Protein Retention

by
Andrew Trivette

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Thesis Committee:

Dr. Matt Elrod-Erickson, Chair

Dr. James Robertson

Dr. Lynn Boyd

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I did all of the research and all of the writing by myself, and thus have no real need to acknowledge others.

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And with the committee out of the way, I suppose I could also mention numerous instances of assistance from Drs. Tony and Mary Farone, Dr. Altman, and the members of both labs, all of whom made my life much easier through advice, access to equipment, and in the case of the Farones and the TRIAD program, money. There were also several occasions on which I received material and ideological contributions from the Jessen and Nelson labs, so they deserve my thanks as well.

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ABSTRACT

Protein folding in the endoplasmic reitculum (ER) happens with the help of ER-resident proteins called chaperones. However, when UBX2 is deleted in *Saccharomyces cerevisiae*, some of these chaperones, namely Kar2p and Pdi1p, are secreted outside the cell at uncommonly high levels. This is noteworthy since none of the other proteins involved in Ubx2p's primary known function demonstrate the same secretion phenotype when deleted. Ubx2p is also known to regulate desaturated lipids in the cell's membranes, so in order to investigate this unusual secretion phenotype, *ubx2Δ* mutant cells, WT cells, and *ubx2Δ* transformed with either full-length UBX2 or a construct lacking one of its two primary domains were assayed for secretion of chaperone proteins after growth on YPD and YPD with oleate. Oleate reduced differences in chaperone secretion between *ubx2Δ* and WT cells, suggesting that it is Ubx2p's role in lipid regulation that results in the secretion defect when it is removed.

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INTRODUCTION

Most research conducted in cell and molecular biology is focused on understanding and mitigating human diseases; it can be nice to imagine that knowledge is an end unto itself, but if it can also lead to improvements in human lives, then government-funded agencies will inevitably direct most of their money toward those goals, and researchers will direct their efforts to receive that funding.

Thus, when speaking of organisms that we wish to study, we most often are talking about either human cells or "model" organisms, through which we hope to learn things about cells and systems that can be applied to ourselves. In studying exactly how a disease can affect the body and how the body responds, it is most helpful to examine organisms more similar to us, like other mammals, in which the mechanisms of disease and defense are likely to be highly analogous to ours.

But when it comes to the discovery and characterization of genes and proteins thought to be involved in various processes, it can be far more convenient to study a model that lends itself to quick and inexpensive examination, even if the actual homology between the model and the target is limited. One model commonly studied for a variety of reasons, and the organism whose inner workings will be the primary topic of the remainder of this document, is *Saccharomyces cerevisiae*, a budding yeast also well known for its use in baking and brewing.

S. cerevisiae is a single-celled eukaryotic fungus, known as a budding yeast due to the way a mother cell produces a new daughter cell by simply "budding" the new cell out from its own membrane and then separating from it by essentially tying it off with a

filamentous protein called Actin (Balasubramanian et al., 2004). This is not so dissimilar from the way that a human cell undergoing mitosis will split into two cells during the final step. The more basic and essential a function is to the cell, the more likely it is to be conserved from one organism to the next, and this is as true for the endoplasmic reticulum as for almost any other cellular structure.

Endoplasmic Reticulum

For eukaryotic cells, the endoplasmic reticulum, or ER, marks the first stop along the secretory pathway, by which proteins initiated or completed in the cytoplasm can reach certain other destinations like the lysosome (the vacuole in yeast), endosomes, or—as the name suggests—the cell membrane or beyond (Barlowe and Miller, 2013; Trombetta and Parodi, 2003). Like other organelles, the ER is a region of the cell separated from the rest by a double membrane of phospholipids, which allows the inside, or lumen (and the interior membrane), to contain different molecules at different levels than the outside. This enables the ER to perform specialized functions related to sending proteins along to their next destination.

One primary function of the ER is to receive nascent proteins, simple chains of amino acids, and transform them into their functional, three-dimensional shapes. This remodeling of proteins is, of course, performed by *other* proteins located in the ER, called chaperones, and is referred to as "folding". Enzymes, for example, often rely on an "active site", a portion of the protein which has a precise shape and arrangement and which binds specifically to their target molecules, to perform their functions. Unless they are folded properly, this active site is not placed where it should be, and the protein is useless.

In fact, misfolded and unfolded proteins can even be a detriment to the cell. Some misfolded proteins can aggregate, potentially leading to a disease state. For example, while the exact causes of Parkinson's and Alzheimer's disease are unknown, both are characterized by the aggregation of specific proteins, potentially a result of misfolding in the ER (Hashimoto et al., 2003). When misfolded into precise—if incorrect—conformations, some proteins, as in the case of prion diseases, can subsequently alter other, correctly-folded versions of themselves, propagating the faulty form (Aguzzi, 2008). Even if most misfolded proteins do not result in consequences quite so severe, they can still have a negative impact on the cell by leading to general ER stress (Belden and Barlowe, 2001).

Chaperones in the ER continually identify un- and misfolded proteins and attempt to contort them into useful shapes, but when certain proteins fail to fold properly and must receive further attention, this can leave chaperones unavailable to attend new proteins entering the ER. While under normal circumstances the ER would be packaging freshly folded proteins into transport vesicles and sending them to the Golgi apparatus, with its machinery otherwise occupied the ER instead begins to fill up with proteins waiting to be folded and the risk of misfolding disorders greatly increases (Bernales et al., 2006). This illustrates an extreme scenario, and one unlikely to occur in normal cells, but only because eukaryotic cells are equipped with mechanisms to deal with just this type of problem.

Even when everything is proceeding as expected, errors in transcription, translation, or even transport result in a constant low level of protein misfolding. The cell is an incredibly complex and effective machine, but it is far from perfect. Fortunately,

there are two primary cellular mechanisms for addressing the ER stress caused by misfolding proteins, both highly conserved in eukaryotic organisms from humans to plants to yeast: ER-associated degradation (ERAD) and the unfolded protein response (UPR).

Unfolded Protein Response

The UPR is exactly what it sounds like. In response to unfolded proteins in the ER, the cell attempts to do various things to relieve mounting ER stress, primarily through changes in gene expression. There are three known pathways by which the UPR can be activated: the PERK, ATF6, and Ire1p pathways. Of these, the Ire1p pathway is the most highly conserved between organisms, and it is the only one found in yeast (Bernales et al., 2006).

Among the lineup of chaperones in the ER of yeast cells is a protein called Kar2p. Its chaperone duties come in addition to its other roles, including karyogamy, or the fusion of two haploid cells (also how it got its name), and even ERAD (Nishikawa et al., 2001; Normington et al., 1989). If there existed no unfolded proteins in the ER, Kar2p would spend its time bound to another protein, straddling the ER membrane, called Ire1p. However, since there are always unfolded proteins that require chaperones, some fraction of the ER's total Kar2p is bound to these, preventing them from misfolding until other chaperones can do the job of properly folding them (Bernales et al., 2006).

As a result, the more unfolded proteins there are in the ER, the more Kar2p molecules are occupied with these instead of being bound to Ire1p. This allows any two unbound Ire1p molecules to form a dimer, activating their cytosolic domains, which are then able to splice an unprocessed HAC1 messenger RNA (mRNA) molecule in the

cytoplasm. Once spliced, this mRNA can be translated by ribosomes into the protein Hac1p, which makes its way to the nucleus where it acts as a transcription factor (Cox and Walter, 1996).

A variety of genes relevant to relieving ER stress contain UPR elements (UPREs) in their promoter regions to which Hac1p can bind, turning on or increasing the transcription of those genes into mRNAs which are then processed and translated into more proteins. These assorted proteins help attenuate ER stress by expanding the ER membrane to increase its capacity, adding more chaperones to the ER lumen, relaxing restrictions on what can and can't leave the ER to help clear excess buildup, and increasing ERAD activity (Bernales et al., 2006; Travers et al., 2000). Because its regulation by Kar2p/Ire1p is sensitive to small changes in the levels of unfolded proteins, the UPR is able to react quickly to their buildup.

ER-Associated Degradation

Regulated in part by the UPR, ERAD is another useful tool at the cell's disposal for dealing with the folding issue. While UPR activity is a general gauge of total unfolded protein levels and ER stress, ERAD is more of a surgical instrument. In mammals, immature proteins which are not yet ready to move on from the ER are bound and delayed by the chaperones calnexin (CNX) and calreticulin (CRT). Another ER protein, ER mannosidase I, periodically makes modifications to carbohydrate groups on the proteins bound by CNX/CRT (regardless of whether these proteins are correctly folded), which allows EDEM (ER degradation-enhancing α-mannosidase-like protein) to recognize them and direct them to ERAD. Thus, faulty or problematic proteins which

remain bound to CNX/CRT for too long are targeted for degradation (Meusser et al., 2005).

The actual degradation step of ERAD makes use of the ubiquitin proteasome system (UPS), which is a large-scale protein degradation pathway throughout the cytoplasm of eukaryotic cells. The UPS relies on a series of enzymatic reactions, typically in three stages. First a ubiquitin activating, or E1, enzyme primes the ubiquitin (Ub) molecule. Next, a ubiquitin conjugating enzyme (E2) receives the Ub molecule and may be associated with the substrate which will be the final acceptor for it. Finally, the ubiquitin ligating enzyme (E3) locates the substrate and the relevant E2 with high specificity and transfers the Ub molecule from the E2 to the substrate (Heinemeyer et al., 2004).

Some proteins are monoubiquitinated, while others can be polyubiquitinated in different ways. Recognition and degradation by the UPS usually requires a protein that has received a ubiquitin chain with each Ub molecule linked to the next at its 48^{th} amino acid, a lysine, which pattern is referred to as a K48 linkage. The final step in the process occurs when the proteasome, a complex of proteins that forms a barrel shape with two caps, recognizes the Ub chain. The tagged protein then enters the proteasome, where it is broken down into small peptides that can be recycled by the cell into new proteins (Heinemeyer et al., 2004).

Different E2 and E3 enzymes are used by different processes which are linked to the UPS. In the case of ERAD, the E2 enzymes include Ubc1p, Ubc6p, and Ubc7p, while the E3s thought to be involved are Hrd1p and Doa10p (Meusser et al., 2005; Neuber et al., 2005; Schuberth and Buchberger, 2005). The obvious problem that arises, however, is

that the UPS machinery is located primarily in the cytoplasm, while the misfolded proteins which need to be degraded are in the ER lumen. On their way into the ER, these proteins must pass through the membrane at a pore composed of several specialized pore proteins, like Sec61p (Barlowe and Miller, 2013). Likewise, they must traverse another pore to exit, although its components are not entirely certain. The proteins Der1p and Dfm1p may be involved (Schuberth and Buchberger, 2005), and the ERAD-associated ubiquitin ligases Hrd1p and Doa10p are other possibilities (Meusser et al., 2005; Ye et al., 2004).

At this point, we have a protein which has been marked as misfolded and begun its way through a pore complex from the ER lumen to the cytoplasm. On the cytosolic face of the membrane, it will meet the ERAD-specific ubiquitin ligases, which will add the appropriate Ub molecules to mark the protein for degradation by the UPS. It will also encounter a protein complex composed of Cdc48p, Ufd1p, and Npl4p (which I will refer to as the Cdc48 complex or simply Cdc48) (Jarosch et al., 2002; Mouysset et al., 2006). The exact role of this complex is not clear, but it is required for ERAD to function properly. One possible role for Cdc48 is to act as a sort of motor to pull the tagged substrate the rest of the way through the pore into the cytoplasm. It is also possible that it acts to bring the substrate from the pore to the proteasome where it will finally meet its end (Baek et al., 2013; Römisch, 2005; Tsai et al., 2002).

At the site of the pore complex, associated with the ERAD ubiquitin ligases, and largely responsible for recruiting Cdc48 to the membrane, is a protein called Ubx2p (Schuberth et al., 2004; Wilson et al., 2006). Ubx2p (Figure 1) is a transmembrane

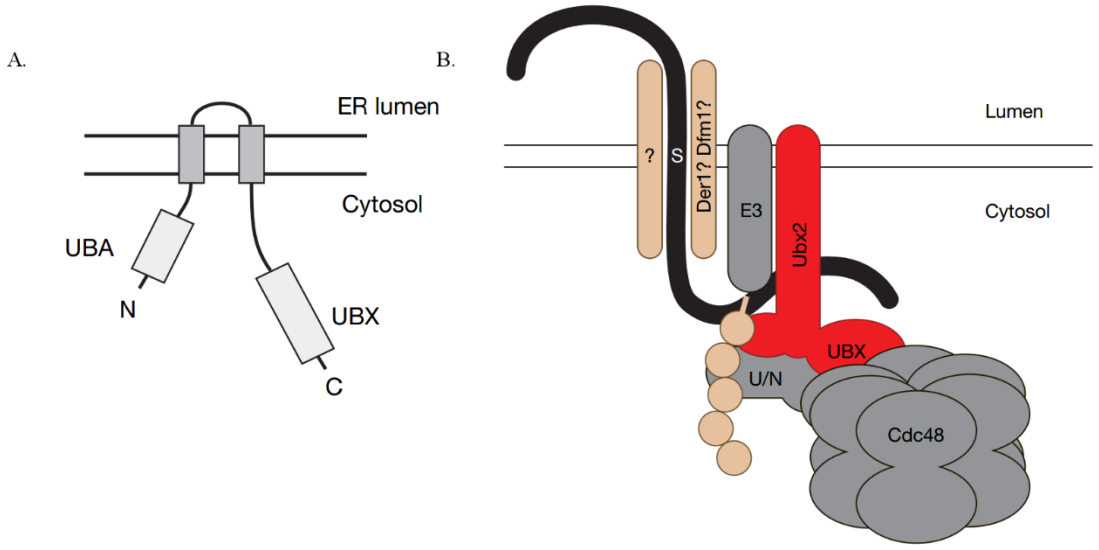

Figure 1. Ubx2p in ERAD. These diagrams show Ubx2p as it is thought to exist in the cell. Both the N-terminus, containing the UBA domain, and the C-terminus with its UBX domain are found on the cytosolic face of the ER membrane (A). In ERAD, the UBX domain is responsible for recruiting Cdc48p, and Ubx2p is also believed to interact with the pore and/or E3 ubiquitin ligases to draw the various components of the system together (B).

Adapted from Neuber et al., 2005 (A), and Schuberth and Buchberger, 2005 (B).

protein found in the ER membrane which has two cytosolic domains: an N-terminal UBA domain, and a C-terminal UBX domain (Schuberth and Buchberger, 2005). The UBX domain is a feature of many proteins in yeast and mammals, and serves to recruit the highly conserved Cdc48 complex (Buchberger et al., 2001). The role of the UBA domain in ERAD is unclear, but both of these domains are required for proper ERAD function, so it is likely that it helps bind to the ubiquitin or pore machinery involved (Meusser et al., 2005; Neuber et al., 2005).

Ubx2p has another known function: it is involved in the regulation of lipid droplets (Wang and Lee, 2012). Lipid droplets are organelles, bound by a single-layer membrane, which have a core of neutral lipids and proteins. They function as storage bodies for these neutral lipids, largely triacylglycerol and sterol ester, and also play a role in lipid homeostasis. They are thought to form when neutral lipids accumulating between the layers of the ER membrane bulge out and eventually bud off (Suzuki et al., 2011). Though Ubx2p is normally found associated with the ER membrane, when yeast are grown to diauxic shift or stationary phase, it relocalizes to lipid droplets. In *ubx2Δ* cells, Lro1p, a protein partially responsible for manufacturing these neutral lipids, is mislocalized and lipid droplets are smaller and sparser (Sandager et al., 2002; Wang and Lee, 2012). Ubx2p's role in this seems to be independent of its ERAD responsibilities, and it is with Ubx2p that we finally arrive at the basis for my own research.

The Secretion Deletions

Proteins destined to reach the cell membrane or to be secreted frequently have tags which help the ER sort them into vesicles, while chaperones and other ER resident proteins not only lack this signal, but have another signal that tells the Golgi apparatus to

send them back to the ER if they happen to wander off (Barlowe, 2015; Barlowe and Miller, 2013). By way of these and other mechanisms, though there are always very low levels of ER resident secretion, cells for the most part reliably secrete proteins which must be secreted and retain those whose duties are inside the cell. Therefore, elevated secretion of ER resident proteins can be taken as a sign of trouble that may indicate ER stress of one kind or another.

A pair of screens examined the collection of *S. cerevisiae* single-deletion mutants by probing for the secretion of ER resident proteins Kar2p and Pdi1p. They found that for many different genes, removal of the gene resulted in elevated secretion of these chaperone proteins. Among the high secretors identified by both screens was the *ubx2Δ* mutant (Copic et al., 2009).

Many of the deletions found through these screens were of genes that were poorly characterized, or were unsurprising finds given the known functions of the genes, and the *ubx2Δ* mutant initially appeared to be one of the latter. Since Ubx2p is important for the proper function of ERAD, whose role is to eliminate misfolded proteins and relieve ER stress, it stands to reason that the deletion of UBX2 could result in the buildup of misfolded proteins and upregulation of the UPR, which is known to cause elevated secretion of ER resident proteins.

This explanation fails to hold up, however, because ERAD has many other component proteins which are at least as vital to its function as Ubx2p, and mutants of the relevant genes were not identified in either screen (Copic et al., 2009). In other words, when ERAD is disrupted due to the removal of Ubx2p, chaperones Kar2p and Pdi1p are secreted in abnormal amounts, but the same cannot be said when ERAD is disrupted

through removal of its other components. This raises the question: what is unique about Ubx2p among ERAD's constituent parts that causes the *ubx2Δ* strain to secrete ER resident proteins? Attempting to answer this question has been the objective of my research.

Research Plan

After doing some initial work and developing a plan to investigate a link between lipid droplets and ER resident secretion, a new study was published that gave us a new and better starting point. According to this research, *ubx2Δ* cells, when compared to wild type cells, have reduced levels of the protein Ole1p, critical for the cells to produce unsaturated fatty acids. They also have different proportions of various classes of lipids in their membranes, and they show elevated levels of UPR induction. In addition, when oleate—an unsaturated fatty acid—was added to the media in which these cells were grown, UPR induction and membrane lipid composition appeared much more like those of wild type cells (Surma et al., 2013), presumably because the cells are able to use this newly-introduced oleate into their membranes. This indicated a previously unknown function for Ubx2p which could potentially explain the secretion phenotype.

Therefore, my goals were three-fold: to replicate the previous finding that *ubx2Δ* cells secrete ER resident proteins at elevated levels; to confirm my hypothesis that growing these cells in media containing oleate would reduce this secretion phenotype; and to introduce UBX2 constructs into *ubx2Δ* cells missing either the UBA or UBX domains to discover whether they are crucial for proper retention of ER resident proteins. Since Cdc48p is known to be involved in both ERAD and Ole1p regulation, my hypothesis was that Ubx2p's UBX domain, a Cdc48-recruiting sequence, would be

critical to proper retention and thus its deletion should mimic the full gene deletion. The significance of the UBA domain was uncertain, and its deletion was approached without a specific hypothesis in mind.

RESULTS

Both screens which had previously shown that *ubx2Δ* cells secrete ER resident proteins at elevated levels used similar methods, so the first step was to replicate this assay. Wild type (WT) and *ubx2Δ* cells were spotted on YPD plates using a pipetter and then overlaid with a nitrocellulose membrane soaked in YPD. The cells grew in contact with this membrane, meaning that any proteins secreted to the cell surface would bind to it. These membranes were then probed with antibodies to Pdi1p and Kar2p, both ER resident chaperones. In the case of both proteins, *ubx2Δ* cells demonstrated higher levels of secretion than WT cells (Figure 2A).

Next, we wanted to see if oleate in the media would reduce the secretion phenotype. This assay was performed identically, except that in addition to standard YPD plates, cells were also spotted and probed on plates which contained oleate. Using the Pdi1p primary antibody, this assay showed that while *ubx2Δ* cells grown on YPD secreted more Pdi1p, on plates with oleate added (YPDO) they did not secrete noticeably more (Figure 2B). Due to unresolved technical problems, this result could not be reproduced using the Kar2p antibody.

Therefore, more traditional Western blots were also performed. WT and *ubx2Δ* cells were grown in liquid YPD both with and without oleate, then cells were spun down and the media removed. The proteins were TCA precipitated from the media and compared to the proteins in the cell lysate. This allowed comparison of intracellular levels of Kar2p to extracellular levels. The subsequent Westerns (Figure 3) confirmed

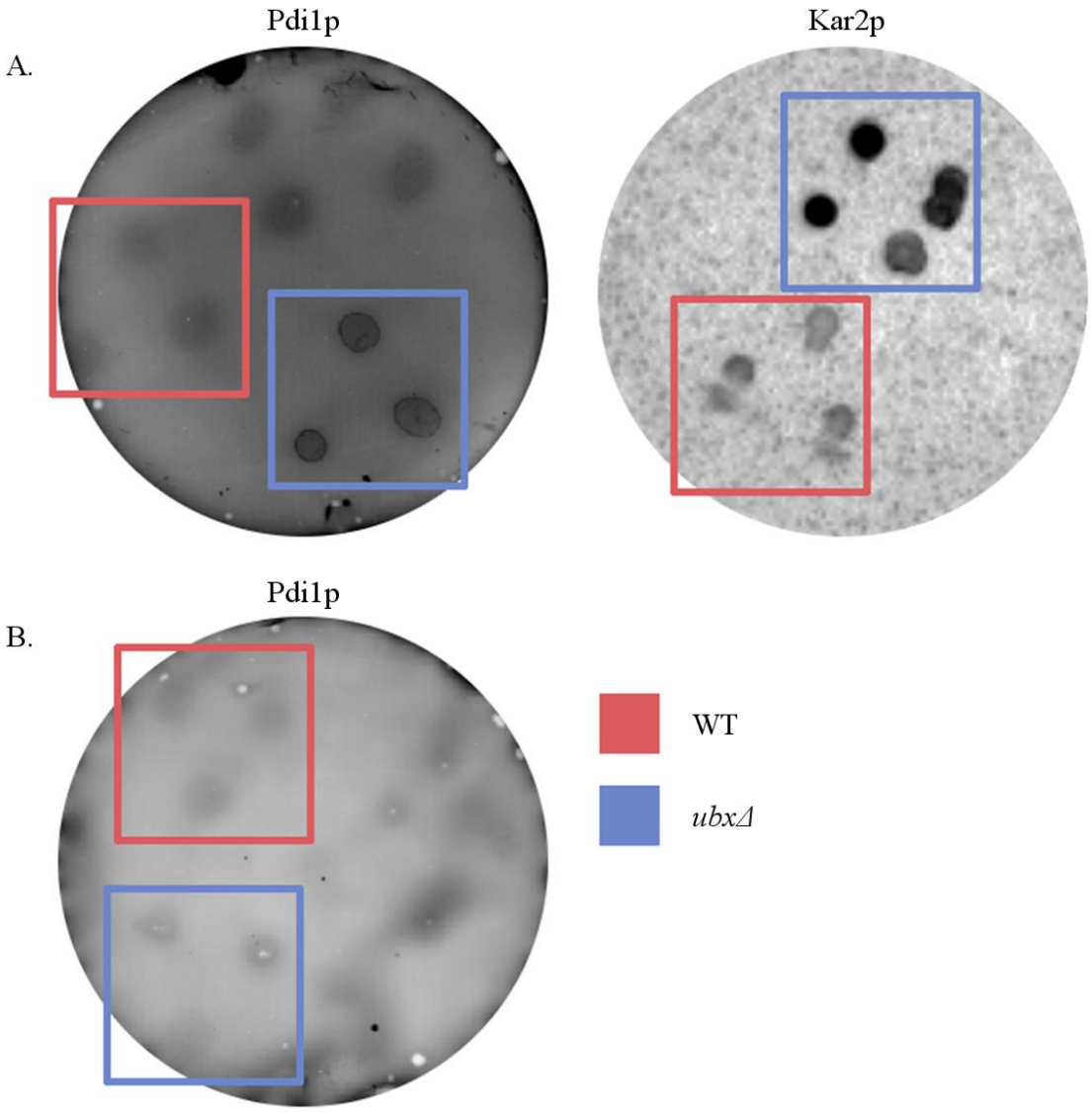

Figure 2. Colony Lift. Colony lift assays comparing WT and *ubx2Δ* cells grown on YPD (A) and YPDO (B). Both Pdi1p and Kar2p are secreted at higher levels in *ubx2Δ* cells than in WT cells, shown by the darker spots, but this difference was greatly reduced for Pdi1p when cells were grown on YPDO.

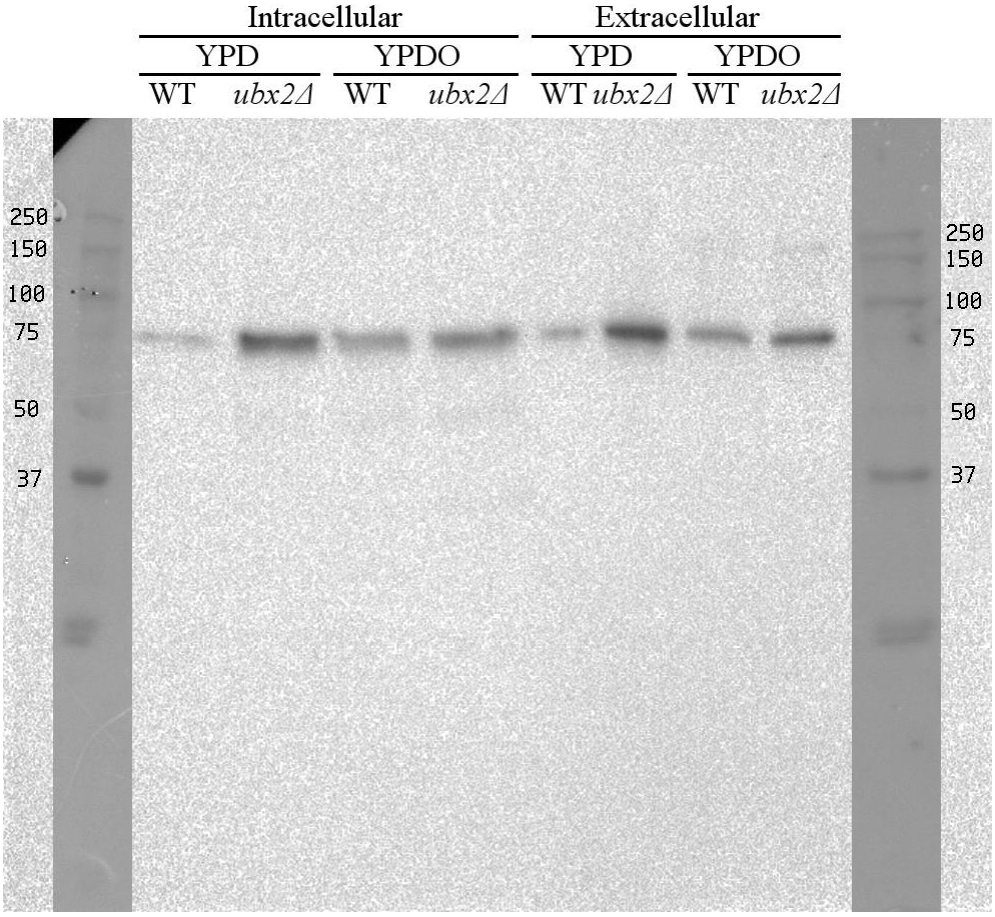

Figure 3. Full Kar2p Blot. This full blot is presented to show that the Kar2p antibody used was very specific for its target. The very faint banding seen below the primary bands in the intracellular lanes is greatly increased when protease inhibitors are not used with the lysis buffer, indicating that they represent various degraded forms of Kar2p.

that *ubx2Δ* cells secrete more Kar2p than WT and this phenotype is reduced when the cells are grown in the presence of oleate. However, it was also found that *ubx2Δ* showed elevated intracellular levels of Kar2p (Figure 4A).

Because the original question was one of secretion, and because *ubx2Δ* cells showed increased Kar2p both inside and outside the cell, it was unclear if the extracellular increase represented higher levels of secretion or merely reflected normal leakage of an abnormally large *total* Kar2p reservoir. To examine this, ImageJ was used to estimate, for each Kar2p band on a blot, what percentage of the total Kar2p on the blot that band represented. Data from seven replicates were combined and Kar2p levels compared among cell lysate bands, and separately among media precipitate bands (Figure 5). Pairwise comparisons were made using Tukey's HSD, which showed that for both intracellular and extracellular Kar2p, levels were significantly different between WT and *ubx2Δ* cells in YPD, but not in YPDO.

Previous research has looked at other deletions which produce a similar secretion phenotype, but in many of these instances, there was no apparent difference in intracellular levels. So to show that the observed intracellular difference was real and not an artifact of the methods used, WT and *ubx2Δ* cells were compared to one of these previously-studied mutants, *bst1Δ* (Elrod-Erickson and Kaiser, 1996). The *bst1Δ* cells did not show increased intracellular levels of Kar2 compared to WT, but they did still show increased extracellular levels. In addition, this increased secretion was not rescued by the addition of oleate (Figure 4B).

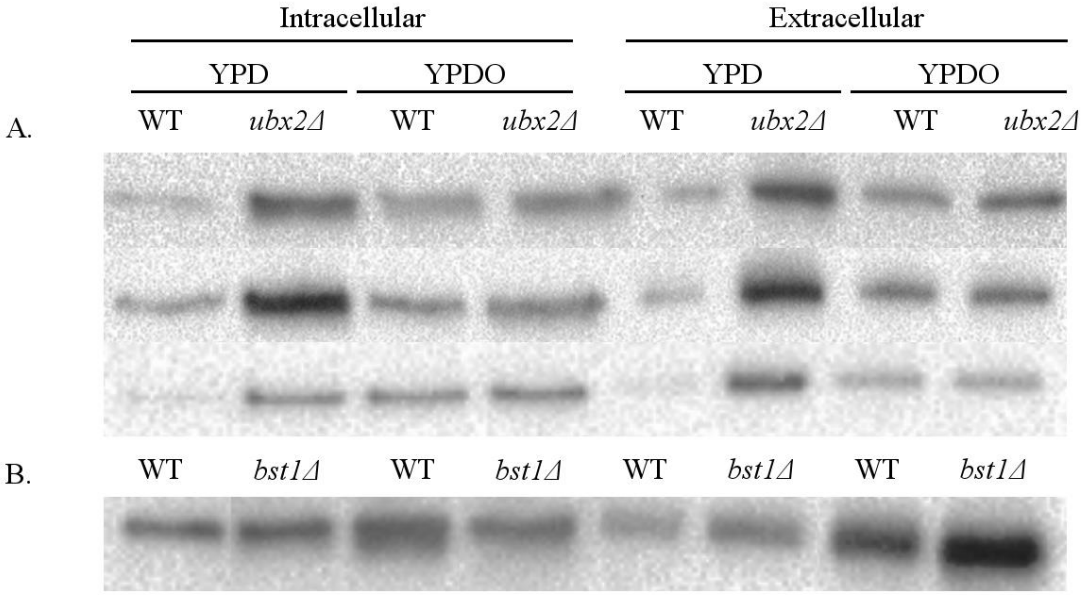

Figure 4. Intra- and Extracellular Kar2p. (A) Three representative examples of Western blots probing for Kar2p and comparing intracellular and extracellular samples of WT and *ubx2Δ* cells in YPD vs YPDO. While there was some variation in the darkness of the intracellular *ubx2Δ* YPD band from blot to blot, there was always more Kar2p than WT cells in YPD, and most commonly more than in either cell type in YPDO. This was true for the extracellular levels as well, but the *ubx2Δ* YPD condition always showed more Kar2p than either YPDO condition. (B) A blot using the same media conditions and preparation, but comparing WT to *bst1Δ* cells. Intracellular levels were similar between the two strains, and while *bst1Δ* showed more Kar2p in the media, oleate only served to increase Kar2p levels for both strains.

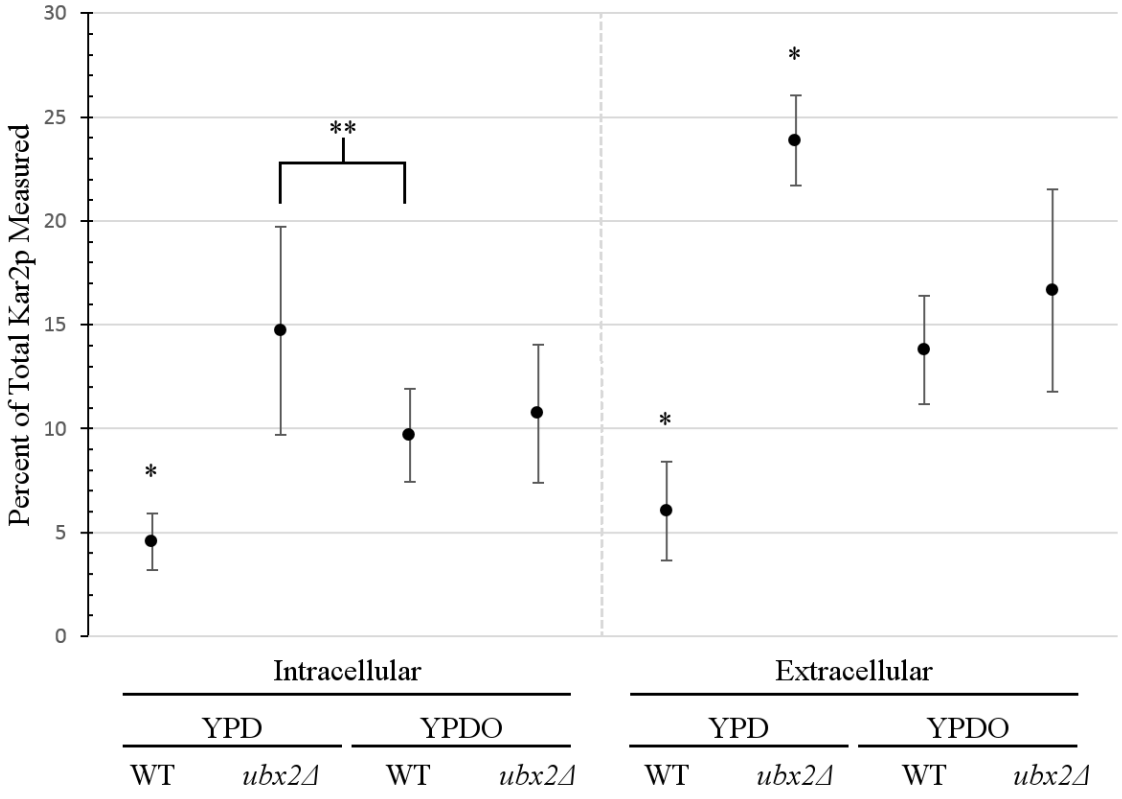

Figure 5. Kar2p Statistical Analysis. This graph compares the mean amounts of Kar2p present in each condition across seven blots as relative fractions of all Kar2p on the blot. Error bars represent one standard deviation. Intracellular conditions were compared using Tukey's HSD controlling for experimentwise error, as were extracellular conditions (intra- and extracellular conditions were not compared to one another). Conditions marked individually differed significantly from all three other conditions to which they were compared.

* $p < 0.01$

** $p < 0.05$

An initial goal had been to investigate whether deleting UBX2 resulted in the secretion defect due to Ubx2p's effect on lipid composition or ERAD, or through subsequent upregulation of the UPR. To do this, the original plan was to produce a double deletion, *hac1Δubx2Δ*, which would have no functional UPR. If this strain still demonstrated the secretion phenotype, it would indicate that the effect was not due to the UPR. Creating this mutant, however, proved difficult, and the study by Surma et al. indicated that they too had found it impossible, so this line of inquiry was abandoned.

Instead, to further characterize how Ubx2p interacted with the secretion phenotype, we decided to investigate whether either of the two domains (UBA and UBX) known to be critical for its ERAD functionality were also important for retention of ER resident proteins. Because the UBX domain acts to recruit Cdc48p, which is involved in both ERAD and in regulation Ole1p, the hypothesis was that deleting the UBX domain alone would produce a comparable secretion defect. Early attempts to delete these domains made use of a technique called overlap extension PCR (Wurch et al., 1998), but this proved much more difficult than the more traditional restriction enzyme approach that was finally adopted.

First, the full length UBX2 was PCR amplified from genomic DNA using primers with added restriction enzyme sites on their 5' ends. This product was then inserted into the centromere plasmid pRS316. Next, PCRs were used to amplify, from the UBX2-FL construct, the regions of UBX2 before and after each domain using primers with added restriction sites. These fragments were then ligated into unmodified pRS316. This produced two new constructs: one which contained UBX2 without its UBA domain (–UBA), and one without the UBX domain (–UBX).

After transformation of each of these constructs into *ubx2Δ* cells, the same Western blotting procedure was used to compare the transformants to WT and *ubx2Δ* cells. WT cell used in these blots were transformed with unmodified pRS316 and grown in the same media as the experimental strains. The UBX2-FL cells did not demonstrate consistently different Kar2p levels from *ubx2Δ* cells, either intra- or extracellular (Figure 6A). Similarly, neither domain deletion appeared to show clear differences from the *ubx2Δ* mutant, with the exception that the –UBA cells seemed to have slightly higher intracellular Kar2p levels (Figure 6B and C).

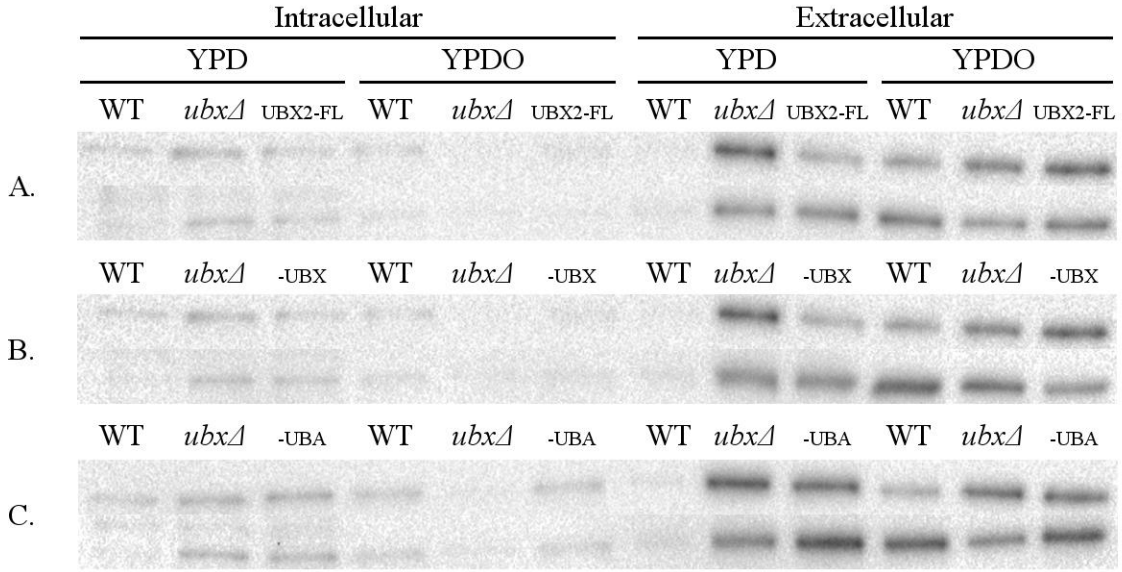

Figure 6. Transformant Blots. This image contains two representative blots from cells transformed with either the full-length UBX2-FL rescue construct (A), the –UBX construct (B), or the –UBA construct (C). While the intracellular bands did not resolve well on any of the transformation blots, there was no apparent difference in intracellular Kar2p levels between the *ubx2Δ* lanes and the construct lanes for any construct/media combination, except for the –UBA construct, where there appeared to be an increase in Kar2p in the YPDO media. In the extracellular samples, the UBX2-FL construct (A) did not clearly rescue the secretion phenotype relative to *ubx2Δ* cells. There were also no consistent differences in extracellular Kar2p between either the –UBX (B) or –UBA (C) transformants and *ubx2Δ* cells.

DISCUSSION

The research presented has shown that *ubx2Δ* cells secrete the two ER resident proteins Pdi1p and Kar2p at higher levels than do WT cells, and that at least for Kar2p, they have elevated intracellular levels as well. When these cells are grown on media containing oleate, however, these levels are reduced and are similar to WT cells. These data support our hypothesis that the increased secretion of ER resident proteins seen in *ubx2Δ* cells is closely linked to Ubx2p's regulation of Ole1p, rather than stemming directly from its role in ERAD.

My Western blots also showed that *ubx2Δ* cells have elevated *intracellular* levels of Kar2p. Previous to this finding, our hypothesis had been that the effect of the *ubx2Δ* mutation on Ole1p led to problems sorting proteins into vesicles, and that ER resident proteins were allowed to escape as a result. While this still might be the case, increased intracellular levels of Kar2p make it difficult to tease out. Even WT cells secrete a small fraction of their total Kar2p, so the increased extracellular levels seen in *ubx2Δ* cells may simply be a result of this normal leakage.

Both intra- and extracellular Kar2p levels increased for WT cells when oleate was added. This is not especially surprising since relative proportions of various classes of lipids in the cell's membranes are altered by the presence of oleate. This effect may cause any rescue in *ubx2Δ* cells to be harder to detect, or appear smaller. Likely this effect occurs because the mechanism by which cells take in lipids from the media for use in their own membranes is imperfect at excluding those it doesn't strictly need. If this

mechanism were more efficient, we might see WT Kar2p levels more similar between the YPD and YPDO conditions, and the rescue in *ubx2Δ* might be somewhat clearer.

The results of the Kar2p assays on cells transformed with our various UBX2 constructs failed to provide much insight. The only consistent result seemed to be a small increase in extracellular Kar2p in –UBA cells compared to *ubx2Δ* cells, which could suggest that a partially functional Ubx2p leads to greater ER stress than its complete absence. But there are reasons to suspect any results from these blots.

All blots using these transformants had poor resolution of the intracellular lanes, and seem to show less intracellular Kar2p in *ubx2Δ* compared to WT cells, which is inconsistent with all previous experiments. In addition, the UBX2-FL rescue construct should have shown at least some rescue of the secretion phenotype if it was present and usable by the cells. So while blue/white screening and PCR analysis indicated that we had successfully produced and transformed the appropriate constructs, perhaps more time and sequence analysis would have shown otherwise.

I attempted to make a case using data from ImageJ that oleate was better able to rescue the increased extracellular Kar2p than intracellular in *ubx2Δ* cells. But acquiring the numbers used in the statistical analysis required a small amount of subjective judgement, and the sample size was relatively small at seven. In addition, one group investigating the validity of methods used to quantify Western blots found that depending on a variety of variables, the same data can provide results which agree or disagree with actual protein levels in the samples (Gassmann et al., 2009). This study made several recommendations for achieving the best results, however, which I attempted to follow.

If taken at face value, this finding indicates that while oleate seems to somewhat rescue elevated intracellular Kar2p levels in *ubx2Δ* cells, this effect is greater and more definite for extracellular levels. This may mean that Ubx2p's role in the OLE1 regulatory pathway is more relevant to the secretion of ER resident proteins than its other functions are. That is to say, deregulation of OLE1 may affect secretion more directly than by simply inducing a UPR response.

When it proved impossible to produce a *hac1Δubx2Δ* strain of yeast to explore whether the high Kar2p levels resulted from UPR induction or some more direct effect, we considered making a *hac1Δmga2Δ* strain instead. Mga2p is another protein which was identified by both screens as a high secretor of ER resident proteins, and is involved in regulating Ole1p production. We reasoned that if the secretion defect was a result of Ubx2p's involvement in the same pathway, this alternate deletion might be informative. Unfortunately, attempts to assay *mga2Δ* cells for Kar2p secretion and PCR analysis revealed that our freezer stock was not the correct deletion, or was contaminated with other cells.

The question remains whether the secretion phenotype shown by *ubx2Δ* cells is simply a result of UPR induction or whether Ubx2p plays some more direct role in the retention of ER resident proteins. The elevated intracellular Kar2p observed in *ubx2Δ* cells is probably largely due to UPR upregulation. If the rescue in extracellular Kar2p levels by oleate truly is greater than that for intracellular levels, this may indicate that the UPR does not fully explain the secretin phenotype—the UPR induction was reduced by the same amount, after all, regardless of where we looked for Kar2p. The data presented here, however, is insufficient to make that claim with certainty. Future research might

investigate the behavior of *hac1Δmga2Δ*, though, even if it would only be rather indirect evidence about Ubx2p. Another possibility might be to use a mutated or temperature sensitive HAC1 allele, rather than deleting it, which might allow one to modulate UPR activity and determine if secretion of ER residents is dependent on it.

MATERIALS AND METHODS

Strains and Growth

All yeast strains used are listed in the Appendix. Cells were grown at 30°C in standard YPD or in CSM -URA (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.077% CSM -URA dropout mix) to select for successful transformants. For media containing oleate, sodium oleate was first dissolved in warm 10% Tergitol NP-40, which was then added to media before autoclaving with final concentrations of 1% Tergitol and 0.1% oleate. For oleate vs. non-oleate Western blots, non-oleate samples were grown in media containing 1% Tergitol.

Cloning was done using NEB 5-alpha High Efficiency Competent *E. coli* cells, grown at 37°C. *E. coli* were grown out in SOC medium following transformation, and transformants were grown in or on LB with 100µg/mL ampicillin. Selection plates for blue/white screening were top-spread with 8µL 0.5M IPTG and 40µL X-gal at 40µg/mL using glass beads.

Cloning and Transformation

Cloning and transformations used the pRS316 centromere plasmid. Restriction enzymes were heat-killed after each digest at appropriate temperatures, and DNA was purified using the Thermo Scientific GeneJET Gel Extraction Kit following PCR and restriction digests. Plasmid DNA was isolated from transformants using the Thermo Scientific GeneJET Plasmid Miniprep Kit. Ligations were performed using NEB T4 DNA Ligase overnight at 16°C. *E. coli* transformations followed the 5-alpha cell transformation protocol published on the NEB website.

For yeast transformations, dense overnight cultures of the strain to be transformed were diluted 10-fold and allowed to grow for 5-6h, then harvested by centrifugation at 3000g for 5m. For the following solutions, 1X TE refers to 10mM Tris, 2mM EDTA, pH 7.5. Cells were washed and resuspended in a LiOAc solution (0.1M LiOAc, 1X TE), then incubated on a roller at 30°C for 1h. Next, 1-5µg of plasmid DNA, 150µg Herring sperm DNA, and 700µL of a PEG/Te/LiOAc solution (40% PEG, 1X TE, 0.1M LiOAc). This was incubated on a roller at 30°C for 30m, then heat shocked at 42°C for 15m. Finally, cells were spun down briefly, resuspended in YPD, and spread on selection plates with glass beads.

To produce the UBX2-FL construct, the UBX2 gene was PCR amplified from yeast using primers with added restriction sites recognized by NotI and SalI from genomic DNA and inserted into the pRS316 MCS by digesting the PCR product and pRS316 with these enzymes and ligating them at a 5:1 insert:vector ratio.

Primers with added restriction sites were designed to amplify the regions of UBX2 precisely on either side of the UBA or UBX domain (Figure 7). After producing these fragments and cutting them and pRS316 with the appropriate enzymes, a 3-piece ligation was performed for each domain deletion which resulted in pRS316 with a copy of UBX2 missing either the UBA or UBX domain. All three constructs were then transformed into *ubx2Δ* cells and unmodified pRS316 into EEY2 cells, and these strains were compared using Western blots.

Colony Lift

For the colony lift secretion assay, dense overnight yeast cultures were spotted on plates (3-5µL). Once these spots dried, a nitrocellulose membrane soaked briefly in YPD

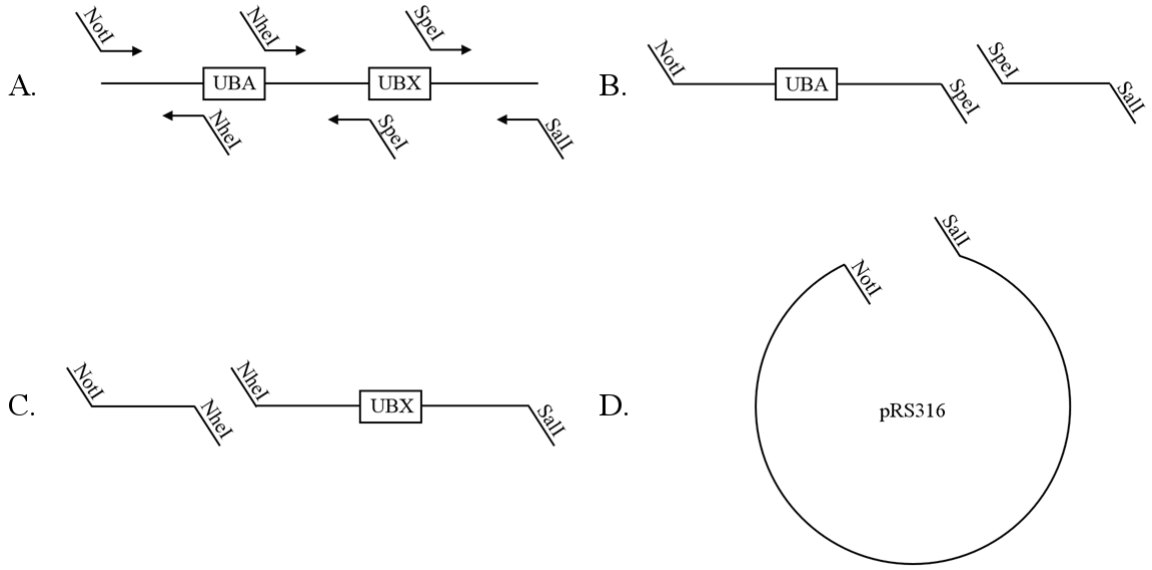

Figure 7. Domain Deletion Strategy. A diagram of the strategy used to create UBA and UBX domain deletions from the UBX2 gene. (A) Primers were designed with NheI sites (UBA) and SpeI sites (UBX) precisely on either side of each domain, and these were used in conjunction with the UBX2 end primers containing NotI and SalI sites to PCR amplify the regions on either side of the domain being deleted. This produced a fragmented copy of UBX2 with no UBX domain (B) and one with no UBA domain (C). For each deletion, the two fragments were then ligated simultaneously into pRS316 cut with NotI and SalI (D) and the final constructs were transformed into *ubx2Δ* cells. The UBX2-FL construct was created by using only the end primers and ligating the full-length gene into the vector.

liquid media was overlaid on top of the plate, which was then incubated at 30°C for 16-24 hours. Membranes used for this assay were Merck Millipore Immobilon Nitrocellulose Transfer Membranes, pore size 0.45µm, cut round to fit in a petri dish. This membrane was removed and rinsed of any cells still attached to it with a buffered saline solution (10mM Tris, 0.5M NaCl, pH 7.5). Detection of Pdi1p made use of the anti-PDI1 [38H8] mouse monoclonal antibody from Abcam (ab4644) diluted 1:5000, and the goat anti-mouse IgG1 heavy chain FITC-conjugated secondary antibody, also from Abcam (ab97239) diluted 1:250. Fluorescence was visualized using a GE Healthcare Typhoon TRIO+ Variable Mode Imager. Otherwise, probing and visualization procedures—and for Kar2p detection, antibodies and concentrations—were the same as for the standard Western blots.

Western Blot

The strains to be assayed were grown overnight in LC and then diluted to OD_{600} = 0.05 and then grown to OD_{600} ~= 0.5. Next, a volume of LC corresponding to 0.1 OD of cells was removed into a microcentrifuge tube for each sample, and these cells were spun down at 3000g for 5m. Extracellular protein samples were prepared using 75% of the supernatant. TCA was added to a final concentration of 10% and the samples were incubated on ice for 30m. Protein pellets were then spun down at 16060g for 5m in a centrifuge at 4°C, washed with acetone, and finally resuspended in 15µL 1x Laemmli sample buffer with Roche cOmplete Mini protease inhibitor cocktail added at the recommended concentration. Occasionally these samples turned yellow, indicating a low pH, and 0.5µL of 1M NaOH was added, returning them to the expected blue color.

Intracellular samples were prepared by removing the remaining media from the cells spun down previously and resuspending them in Thermo Scientific YPER Yeast Protein Extraction Reagent. Tubes were taped to a vortex which ran at its highest setting for 20m. Cell debris was spun down at 14000g for 10m, and 40µL of the supernatant containing extracted protein was mixed with 2x Laemmli buffer containing the protease inhibitor cocktail. The intracellular samples were thus at 1/5 the concentration of the extracellular samples because this meant that the normal low levels of secretion found in WT cells, for example, would produce a similar band to the intracellular protein, to make comparison easier.

All samples were loaded onto Bio-Rad 10% Mini-PROTEAN TGX Precast Gels and run at 200v for 35 minutes in standard running buffer (25mM Tris, 190mM glycine, 0.1% SDS, pH 8.3). Life Technologies NOVEX PVDF Membranes with 0.2 µm pores were activated by washing in MeOH for ~30s and rinsing in dH_2O for 2-5 minutes. Transfer was performed using the Bio-Rad Trans-Blot Turbo Blotting System at 25v for 30m after soaking the gel, membrane, and filter paper in Towbin transfer buffer (25mM Tris, 190mM glycine, 20% MeOH, pH 8.3). In a few instances, samples were loaded identically onto another gel which was stained with the Imperial Coomassie Stain from Fisher Scientific as a loading control (Figure 8).

Membranes were blocked with 5% milk in TBST for 1h at RT or overnight at 4°C. The Kar2 (y-115) rabbit polyclonal primary antibody from Santa Cruz Biotechnology (sc-33630) was used diluted 1:250 as a primary antibody, and the secondary, also from Santa Cruz, was a goat anti-rabbit IgG-HRP (sc-2004) used at 1:5000. Membranes were incubated with primary antibody for 1h at RT or overnight at

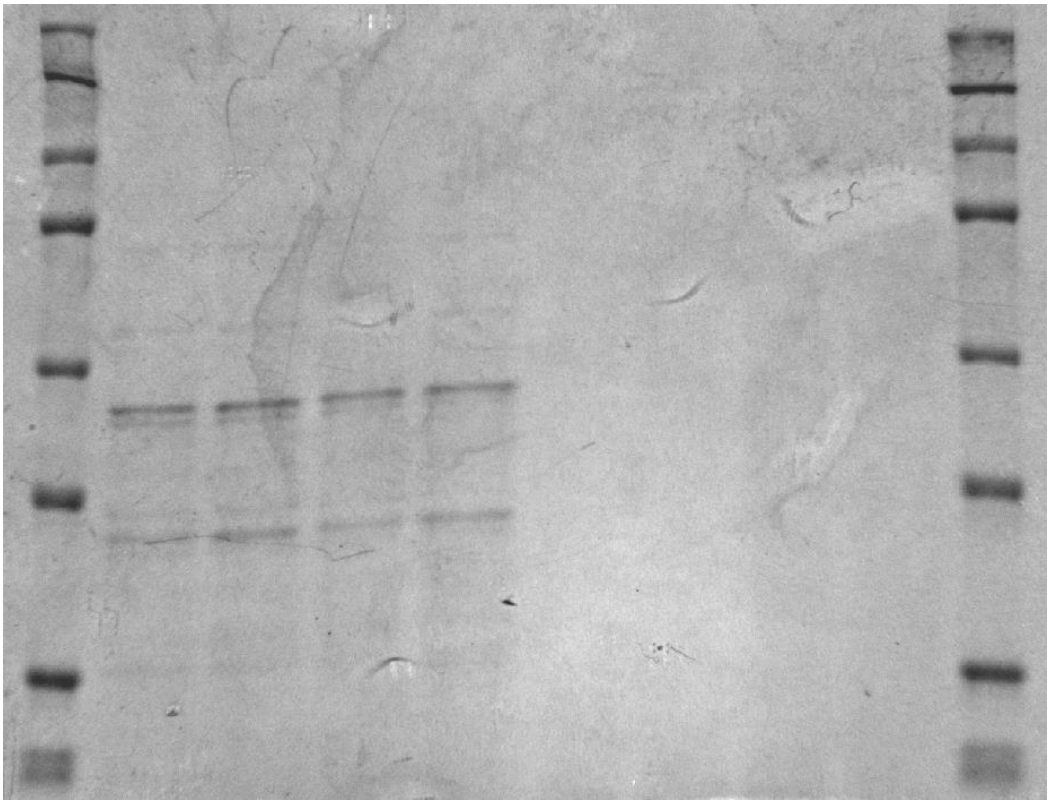

Figure 8. Coomassie Stain. An example of a Coomassie stain performed as a control against total protein to ensure that the differences between lanes were not due to more or fewer cells being loaded into them. There was not enough protein present in the media to show up with this stain.

4°C, washed 3 times for 5m with TBST, then incubated with secondary antibody for 1h at RT and washed 3 more times. Chemiluminescence was visualized using a Bio-Rad MP Imaging System.

ImageJ Analysis

Blot images were saved directly as digital images from the computers connected to the imaging devices in lossless PNG format. Each image was color-inverted before opening it in ImageJ. I then took the average pixel lightness of a region of each lane containing only background and subtracted that value from the entire lane (no whole-image background reduction was performed).

Next, I drew a selection rectangle roughly 1/3x as wide as the narrowest lane and 10x as tall as the thickest Kar2p band, and measured an area this size centered on the Kar2p band in each lane. I then used ImageJ's "Plot Lanes" feature to create a graph for each lane displaying average pixel lightness for each row of pixels from the top of the selection to the bottom, showing peaks at each band and smaller peaks representing background (Figure 9). I used the straight line tool to draw a line from the top of the highest background peak on one side of the band peak to the highest on the other side, closing off the main peak.

Then I measured the area under the Kar2p peak for each lane, and ImageJ calculated what percentage of the combined area of all peaks each one was. At this point, these values represented relative Kar2p values in each lane, with all lanes from a blot adding up to 100%. Each blot had the same eight conditions, and I used Microsoft Excel to calculate means and standard deviations for each condition across all seven blots and plotted these values with error bars representing one standard deviation on each side of

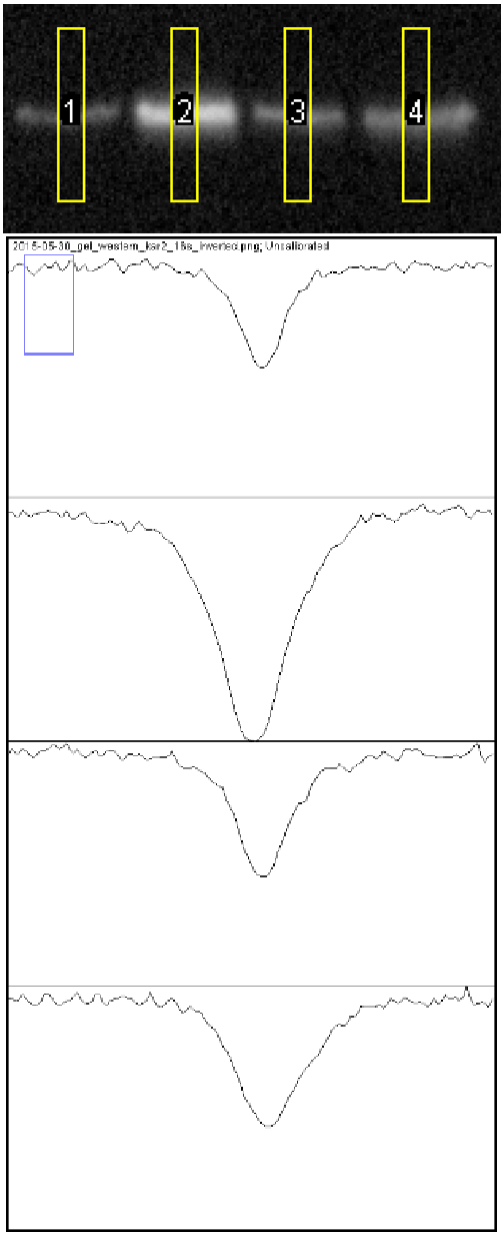

Figure 9. ImageJ Example. Blots were inverted before analyzing because this made it possible to subtract background signal from each lane before measuring. For each lane, a selection rectangle was centered on the lane at the Kar2p band. ImageJ then produced a graph of each lane (the top of the lane is on the left, with the leftmost lane first). The peaks represent the bands where the average pixel brightness across the selected region was highest. The area under each peak was taken as a percentage of the area of all peaks combined, and this data was gathered from each blot separately before calculating means and standard deviations as shown in Figure 5.

the mean.

Finally, I used SAS 9.4 to perform pairwise comparisons of intracellular conditions to one another, and extracellular conditions to one another, using Tukey's HSD. It did not make sense to compare intracellular to extracellular since I had artificially run the extracellular samples at 5x the concentration of the intracellular samples.

REFERENCES

Aguzzi, A (2008). Unraveling Prion Strains with Cell Biology and Organic Chemistry. Proceedings of the National Academy of Sciences *14*(1), 11-12.

Baek, G., Cheng, H., Choe, V., Bao, X., Shao, J., Luo, S., and Rao, H. (2013). Cdc48: A Swiss Army Knife of Cell Biology. Journal of Amino Acids *2013*.

Balasubramanian, M., Bi, E., and Glotzer, M. (2004). Comparative Analysis of Cytokinesis in Budding Yeast, Fission Yeast and Animal Cells. Current Biology *14*, R806–R818.

Barlowe, C. (2015). Membrane trafficking: ER export encounters dualism. Current Biology *25*(4), R151–R153.

Barlowe, C., and Miller, E. (2013). Secretory Protein Biogenesis and Traffic in the Early Secretory Pathway. Genetics *193*, 383–410.

Belden, and Barlowe (2001). Deletion of Yeast p24 Genes Activates the Unfolded Protein Response. Molecular Biology of the Cell *12*, 957–969.

Bernales, S., Papa, F., and Walter, P. (2006). Intracellular Signaling by the Unfolded Protein Response. Annual Review of Cell and Developmental Biology *22*, 487–508.

Buchberger, A., Howard, M., and Proctor, M. (2001). The UBX Domain: A Widespread Ubiquitin-like Module. Journal of Molecular Biology *307*, 17–24.

Copic, A., Dorrington, M., Pagant, S., Barry, J., Lee, M.C., Singh, I., Hartman, J.L., and Miller, E.A. (2009). Genomewide Analysis Reveals Novel Pathways Affecting Endoplasmic Reticulum Homeostasis, Protein Modification and Quality Control. Genetics *182*, 757–769.

Cox, J., and Walter, P. (1996). A Novel Mechanism for Regulating Activity of a Transcription Factor That Controls the Unfolded Protein Response. Cell *87*, 391–404.

Elrod-Erickson, M.J., and Kaiser, C.A. (1996). Genes that Control the Fidelity of Endoplasmic Reticulum to Golgi Transport Identified as Suppressors of Vesicle Budding Mutations. Molecular Biology of the Cell *7*, 1043–1058.

Gassmann, M., Grenacher, B., Rohde, B., and Vogel, J. (2009). Quantifying Western Blots: Pitfalls of Densitometry. Electrophoresis *30*, 1845–1855.

Hashimoto, M., Rockenstein, E., Crews, L., and Masliah, E. (2003). Role of Protein Aggregation in Mitochondrial Dysfunction and Neurodegeneration in Alzheimer's and Parkinson's Diseases. NeuroMolecular Medicine *4*, 21–35.

Heinemeyer, W., Ramos, P.C., and Dohmen, R.J. (2004). The Ultimate Nanoscale Mincer: Assembly, Structure and Active Sites of the 20S Proteasome Core. Cell and Molecular Life Sciences *61*, 1562–1578.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D., and Sommer, T. (2002). Protein Dislocation from the ER Requires Polyubiquitination and the AAA-ATPase Cdc48. Nature Cell Biology *4*, 134–139.

Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005). ERAD: The Long Road to Destruction. Nature Cell Biology *7*(8), 766–772.

Mouysset, J., Kähler, C., and Hoppe, T. (2006). A Conserved Role of *Caenorhabditis elegans* CDC-48 in ER-associated Protein Degradation. Journal of Structural Biology *156*, 41–49.

Neuber, O., Jarosch, E., Volkwein, C., Walter, J., and Sommer, T. (2005). Ubx2 Links the Cdc48 Complex to ER-associated Protein Degradation. Nature Cell Biology *7*(10), 993–998.

Nishikawa, S., Fewell, S.W., and Kato, Y. (2001). Molecular Chaperones in the Yeast Endoplasmic Reticulum Maintain the Solubility of Proteins for Retrotranslocation and Degradation. The Journal of Cell Biology *153*(5), 1061–1069.

Normington, K., Kohno, K., Kozutsumi, Y., Gething, M.J., and Sambrock, J. (1989). *S. cerevisiae* Encodes an Essential Protein Homologous in Sequence and Function to Mammalian BiP. Cell *57*, 1223–1236.

Römisch, K. (2005). Endoplasmic Reticulum-Associated Degradation. Annual Review of Cell and Developmental Biology *21*, 435–456.

Sandager, L., Gustavsson, M., Ståhl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., and Stymne, S. (2002). Storage Lipid Synthesis Is Non-essential in Yeast. Journal of Biological Chemistry *277*(8), 6478–6482.

Schuberth, C., and Buchberger, A. (2005). Membrane-bound Ubx2 Recruits Cdc48 to Ubiquitin Ligases and Their Substrates to Ensure Efficient ER-associated Protein Degradation. Nature Cell Biol *7*(10), 999–1006.

Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (2004). Shp1 and Ubx2 are Adaptors of Cdc48 Involved in Ubiquitin-dependent Protein Degradation. EMBO Reports *5*(8), 818–824.

Surma, M., Klose, C., Peng, D., Shales, M., Mrejen, C., Stefanko, A., Braberg, H., Gordon, D., Vorkel, D., Ejsing, C., Farese, R., Simons, K., Krogan, N., and Ernst, R. (2013). A Lipid E-MAP Identifies Ubx2 as a Critical Regulator of Lipid Saturation and Lipid Bilayer Stress. Molecular Cell *51*, 1–12.

Suzuki, M., Shinohara, Y., Ohsaki, Y., and Fujimoto, T. (2011). Lipid Droplets: Size Matters. Journal of Electron Microscopy *60*, S101–S116.

Travers, K., Patil, C., Wodicka, L., Lockhart, D., Weissman, J., and Walter, P. (2000). Functional and Genomic Analyses Reveal an Essential Coordination Between the Unfolded Protein Response and ER-Associated Degradation. Cell *101*, 249–258.

Trombetta, S.E., and Parodi, A.J. (2003). Quality Control and Protein Folding in the Secretory Pathway. Annual Review of Cell and Developmental Biology *19*, 649–676.

Tsai, B., Ye, Y., and Rapoport, T. (2002). Retro-translocation of Proteins from the Endoplasmic Reticulum into the Cytosol. Nature Reviews Molecular Cell Biology *3*, 246–255.

Wang, C., and Lee, S. (2012). The Ubiquitin-like (UBX)-domain-containing Protein Ubx2/Ubxd8 Regulates Lipid Droplet Homeostasis. J. Cell. Sci. *125*, 2930–2939.

Wilson, J., Liu, Y., Bentivoglio, C., and Barlowe, C. (2006). Sel1p/Ubx2p Participates in a Distinct Cdc48p-Dependent Endoplasmic Reticulum-Associated Degradation Pathway. Traffic *7*, 1213–1223.

Wurch, T., Lestienne, F., and Pauwels, P.J. (1998). A Modified Overlap Extension PCR Method to Create Chimeric Genes in the Absence of Restriction Enzymes. Biotechnology Techniques *12*(9), 653–657.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T.A. (2004). A Membrane Protein Complex Mediates Retro-translocation from the ER Lumen into the Cytosol. Nature *429*, 841–847.

APPENDICES

APPENDIX A: EXPERIMENTAL STRAINS

Strain	Genotype
EEY2	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3-52*
ubx2Δ	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ubx2Δ::Kan*
bst1Δ	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bst1Δ::Kan*
mga2Δ	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mga2Δ::Kan*

This table contains the experimental yeast strains used throughout the research.