Mutagenesis of a conserved cysteine in the Hsp70 molecular chaperone BiP: Impact of amino acid substitution on BiP’s ability to protect cells against endoplasmic reticulum-derived oxidative stress

Honors Thesis
Presented to the College of Arts and Sciences,
Cornell University
in Partial Fulfillment of the Requirements for the
Biological Sciences Honors Program

by
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January 2015

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ABSTRACT

Recently, it has been shown that oxidative protein folding in the endoplasmic reticulum (ER) produces potentially significant amounts of cellular reactive oxygen species (ROS). Although ROS accumulation can cause profound damage to the cell through a phenomenon called oxidative stress, ROS have also recently been shown to be fundamental components of many protective signaling pathways in the ER that respond and prevent oxidant-induced damage. However, many questions regarding how cells protect against ER-derived oxidative stress still remain. Here, we report further evidence of a new role for the well-characterized Hsp70 ER molecular chaperone BiP as a sensor of ROS imbalance in the ER. Previously, the lab has shown that BiP’s conserved cysteine is directly modified by ROS during stress and that this modification not only protects cells from oxidative stress, but also results in a loss of its ATPase activity. In order to better assess the impact of BiP cysteine modification on BiP function, we created and characterized mutant alleles that could possibly mimic the protecting feature of BiP’s modified cysteine by substituting BiP’s conserved cysteine with all 19 other amino acids. Our results show that when BiP’s conserved cysteine is substituted with charged or bulky residues, BiP not only protects cells under conditions of stress, but also triggers heightened unfolded protein response (UPR), decreased protein translocation into the ER, and prevents protein aggregation. We propose that under conditions of oxidative stress, BiP, modified by ROS, alters its activities in ways that protect cells from excess stress through loss of its ATPase and normal chaperone functions.
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INTRODUCTION

Although fundamental and necessary for almost all life on Earth, oxygen in high concentrations is toxic and corrosive, reacting with almost anything in its vicinity to produce unstable free radicals collectively called reactive oxygen species (ROS) (Turrens, 2003). When ROS strip electrons off neighboring atoms, a widespread domino effect known as oxidative stress results, which is a condition that occurs when cells accumulate an excess of ROS (Gupta et al., 2014). Because ROS are natural products of cell function, generated as byproducts of many cellular metabolic processes and oxidative protein folding, under normal conditions, cells are able to use various antioxidants and enzymes, like Vitamin C and E and superoxide dismutase, to limit ROS accumulation, prevent ROS from harming neighboring cellular components, and maintain an optimal redox environment (Peng et al., 2014). When cellular detoxification mechanisms fail and/or ROS production increases, however, profound damage to many cellular components can occur. Although not definitively proven as a causative agent, ROS has long been closely linked to many degenerative disorders, such as aging (Ziegler et al., 2014), Alzheimer’s disease (Lim et al. 2014), diabetes (Rochette et al., 2014) and atherosclerosis (Goncharov et al., 2014).

Despite all the detrimental effects that oxidative stress can have on cellular systems, recent evidence has suggested a new role for ROS as part of fundamental cellular signaling pathways that can help cells in the body sense elevated ROS levels and direct appropriate scavenging, protection and repair mechanisms (Reczek et al., 2014; Schieber and Chandler, 2014). Such evidence suggests that complete elimination of ROS may be as harmful as having an excess of them. Although such mechanisms involving ROS-dependent signaling pathways have begun to be elucidated, many questions still remain. How do cells protect themselves from
oxidant-induced damage? What key players are involved in protective redox signaling pathways?
What modifications are needed to ensure proper pathways are activated or shut down?

Although more is known about how cells respond to high levels of cellular ROS produced from the mitochondria, the energy-churning machinery of cells, little is known about how cells respond to ROS produced in the endoplasmic reticulum (ER), the cellular organelle responsible for the folding and assembly of many proteins and recently proven to be a significant source of ROS (Malinowski et al., 2011). A critical step of protein assembly in the ER is the formation of disulfide bonds between cysteine residues, which ensures stabilization of protein structure and proper function (Feige et al., 2011). An enzyme, Ero1, catalyzes these disulfide bond formations, in turn, generating significant amounts of peroxide byproducts likely to be a major source of ROS (Araki and Inaba, 2012; Sevier and Kaiser., 2008; Figure 1A). The activity of Ero1 is normally tightly regulated in the cell to prevent excess ROS from accumulating in the ER (Sevier et al., 2007; Figure 1A). When the ER starts accumulating damaging levels of oxidants, Ero1 shuts off, thus moderating the amount of ROS produced in the cell (Sevier et al., 2007; Figure 1A). Notably, Ero1 can be decoupled genetically from its regulatory feedback loop by mutating its regulatory cysteines into alanine residues (Ero1-C150A-C295A), resulting in a constitutively active, de-regulated mutant of Ero1, referred to in this paper as Ero1* (Sevier et al., 2007; Figure 1B). We have taken advantage of this hyperactive Ero1 to study how cells respond oxidative stress within the ER lumen, specifically the effect of ROS on a Hsp70 molecular chaperone in the ER, BiP.

Wild-type BiP is an ATPase that binds newly synthesized proteins in the ER and maintains them in a folding, competent state (Tokunaga et al., 1992). Comprised of a nucleotide binding domain that binds and hydrolyzes ATP and a substrate binding domain that binds
peptides, BiP is an essential protein involved in many other processes in the ER, such as facilitating protein translocation, preventing protein aggregation, and moderating the unfolded protein response (UPR) (Wang et al., 2014). As seen a strain made by the lab in which yeast BiP’s gene has been deleted (kar2A), removing BiP completely from cells results in cell death (Wang et al., 2014).

BiP’s nucleotide binding domain has a highly conserved cysteine residue (Kar2-C63 in yeast) that is involved in redox signaling pathways of the ER (Wang et al. 2014; Figure 2A). Currently, our lab has discovered that excess ROS in the ER (created by Ero1*) results in oxidation of BiP’s conserved cysteine (Wang et al. 2014; Figure 2B). Under hyper-oxidizing conditions, BiP’s cysteine is directly modified by peroxide, a modification that decouples BiP’s ATPase activities from its peptide-binding abilities (Wang et al., 2014; Figure 2B). Although BiP’s cysteine can be reversibly modified by ROS to protect cells during oxidative stress, it is not essential for normal BiP function, as replacement of yeast BiP’s cysteine (C63) with alanine in a delete strain does not affect cell survival (Wang et al., 2014; Figure 3A). Under conditions of oxidative stress created by overexpression of Ero1 (Ero1*) however, cells inserted with BiP-C63A become defective in their response to ROS in the ER (Figure 1B). Conversely, when BiP's cysteine is replaced by amino acids with "bulky" side chains, such as phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr), these alleles, behaving like phenotypic mimetics of oxidized BiP, are able to confer protection to the cell under stress conditions, yet strikingly are unable to support life under normal conditions (Wang et al., 2014). Purification of these “bulky” mutants and subsequent assays testing for their ATPase and protein folding capabilities show these mutant alleles to have decreased ATPase activities and enhanced abilities in preventing protein aggregation (Wang et al. 2014). Collectively, these results suggest that when BiP is activated and
modified by ROS, its folding abilities are enhanced to minimize protein misfolding caused by oxidative stress (Wang et al., 2014). BiP modification and subsequent loss of its ATPase activity, therefore, seem to be critical, necessary steps of redox signaling pathways in the ER that protect cells from oxidative stress.

Following these observations, here we report further evidence that oxidation of the BiP cysteine alters BiP’s activities in ways that protect cells from oxidant-induced damage. In this study, we show that substitution of BiP’s conserved cysteine with large, bulky, charged residues can protect cells against ER-derived oxidative stress by mimicking its modified form. In this “screen” of possible mutant BiP alleles, we have identified, in addition to the lab’s published BiP-C63F/W/Y alleles that protect cells from stress, 4 more mutant alleles (BiP-C63H/K/P/R) that display the similar phenotypes. Notably, we have also discovered mutants (BiP-C63L/M/Q) that do not significantly or only moderately protect cells from oxidative stress, yet show decreased ER translocation abilities and enhanced unfolded protein response (UPR). These results support recent data published by the lab that show that loss of critical ATPase activity contributes to but is insufficient in protecting cells from oxidative stress. Taken together with what the lab has discovered about BiP modification, our results on the functions of mutant BiPs provide compelling evidence that BiP, acting as a critical redox sensor in the ER, not only alters its own activities during times of severe stress by decreasing protein translocation into the ER and increasing peptide binding, but also alerts the cell to activate other fundamental response pathways, like the UPR. Elucidating the functions of these mutant BiP alleles thus, will help us better understand the link between BiP modification and protection under stress conditions and provide clues on how cells are able to cope with oxidative stress in the ER.
RESULTS

Substitution of BiP’s conserved cysteine with bulky amino acid adducts can protect against ER oxidative stress

Previously, the lab has shown that replacement of BiP’s conserved cysteine (C63) with alanine in yeast BiP (Kar2) renders cells hyper-sensitive to Ero1* overexpression, which generates oxidative stress in the ER lumen (Wang et al. 2014). In contrast, replacement of BiP’s conserved cysteine with “bulky” amino acid residues notably rescues cells from Ero1*-induced oxidative stress. Following previous work done in the lab that shows that oxidation of BiP’s cysteine alters BiP activity in a way that is beneficial to cells during stress, we sought to create an entire spectrum of BiP-cysteine mutant alleles that could possibly mimic BiP’s protective modified form (Figure 3B).

*KAR2* is an essential gene in yeast (Rose et al., 1989). Using Quikchange site-directed mutagenesis, we individually replaced Kar2’s conserved cysteine with all 19 other amino acid residues to see what mutating BiP’s cysteine would do to BiP’s protective capabilities during oxidative stress. To observe the effects of these cysteine mutations under the combined conditions of oxidative and heat stress, we inserted our mutant plasmids into CSY 278 (genotype *MATa GAL2 ura3-52 leu2-3,112 kar2-C63A can1::P*$_{GAL1}$*-ERO1*-myc), a yeast strain that has two notable features: 1) an endogenous Kar2 with its cysteine mutated to alanine (*kar2-C63A*), and 2) an additional inducible copy of galactose-promoted Ero1* (expressed with endogenous Ero1). Previous data have shown that when grown on galactose plates (which turns on the hyperactive Ero1*), CSY 278 alone without any additional copy of *KAR2* dies from the excess oxidative stress caused by activities of Ero1* (Wang et al. 2014), due to BiP’s conserved cysteine being replaced with alanine, which knocks out its protective functions. When inserted
with wild-type or mutant KAR2 plasmids kar2-C63F, kar2-C63W, kar2-C63Y, and kar2-C63D, cells are notably rescued and protected from stress (Wang et al. 2014). We have attributed the growth observed with these protective “bulky” alleles to reflect primarily the activities of the inserted mutant KAR2 plasmids, as insertions with an empty vector (pRS215) or an additional copy of kar2-C63A do not rescue cells from oxidative stress caused by Ero1* (Figure 3).

Notably, along with the three “bulky” (kar2-C63F/W/Y) and one acidic (kar2-C63D) amino acid substitutions previously tested that mimic BiP’s modified form, we found that Kar2 cysteine mutants with similarly bulky, charged residues like kar2-C63H, kar2-C63P, kar2-C63R, kar2-C63K, when expressed in CSY 278 under conditions of stress, consistently protected cells from oxidative stress (Figure 4). While other substitutions, mostly with hydrophobic, uncharged residues conferred little to no protection, alleles such as kar2-C63G and kar2-C63V displayed more variable levels of protection (Figure 4). kar2-C63M and kar2-C63Q interestingly display a phenotype similar to kar2-C63D and conferred only moderate protection (Figure 4).

Correlating with the spotting assays performed on galactose plates (which induce Ero1* hyperactivity), spotting assays on plates mixed with the chemical diamide, which provides an alternative source of cellular oxidative stress (Gasch et al., 2000), produced similar, although slightly altered responses, with the same mutant alleles showing the most protection (Figure 5). Some alleles that consistently protected cells on the galactose plates such as kar2-C63P and kar2-C63K, however, did not protect as well as some other protective alleles on the diamide plates (Figure 5). In contrast, kar2-C63Q, which showed only modest protection on the galactose plates conferred greater protection on the diamide plates than many of the other identified protective alleles (Figure 5).
Single-copy BiP-cysteine mutants show differing levels of function in vivo

Previously, the lab has shown that BiP mutants that protect against stress also show a loss of critical ATPase activity (Wang et al., 2014). Since BiP’s ATPase activity is essential for cell survival under normal conditions, we hypothesized that any new alleles we created that protect against stress would also result in decreased ATPase activity. To test the ATPase capabilities of our BiP-cysteine mutants in vivo, we inserted our mutant plasmids into CSY 214 (genotype MATα GAL2 ura3-52 leu2-3,112 kar2 Δ::KanMX [pCS623]), a yeast strain in which the original KAR2 gene has been deleted. Since KAR2 is an essential gene in yeast, CSY 214 is kept alive by an inserted copy of wild-type KAR2, which allows us to grow the strain in the lab. To insert our mutant plasmids and remove the wild-type KAR2 plasmid so that we could observe the effects of mutant KAR2 alone, we used 5-Fluoroorotic acid (5FOA), a drug commonly used in yeast genetics to remove uracil-marked plasmids (Boeke et al., 1987), to counterselect for our mutant plasmids.

Previously the lab has observed that although substitutions of BiP’s conserved cysteine with charged, bulky residues protect cells under hyper-oxidizing conditions, most of these alleles (kar2-C63D/F/W/Y) still lacked fundamental BiP activity. Following these results, we expressed all of our mutant alleles in CSY 214 and found that although most BiP-cysteine mutant strains were able to survive our 5FOA plasmid shuffle, kar2-C63H, kar2-C63P, kar2-C63R, kar2-C63W and surprisingly kar2-C63N, an allele that does not protect under stress when expressed in CSY 278, were all unable to support normal BiP function and thus, cellular growth (Figure 6A). Along with the lab’s previously published kar2-C63D/F/Y alleles, strains that were viable but were temperature sensitive for growth at 37°C when expressed in CSY 214, and able to protect cells from oxidative stress when expressed in CSY 278, we have also found kar2-C63K and kar2-
C63Q to exhibit similar phenotypes (Figure 6B). Interestingly, kar2-C63L, kar2-C63M, and kar2-C63Q, alleles that did not significantly protect in our CSY 278 strain, were also temperature sensitive when expressed as the sole copy of BiP, suggesting that BiP’s protective abilities are not necessarily always correlated with defects in cellular growth at high temperatures (Figure 6B).

Another one of BiP’s functions in the ER is to translocate and fold nascent polypeptides (Brodsky et al., 1995). Correlating with such inabilities to maintain growth at high temperatures, we found temperature-sensitive BiP-cysteine mutants (expressed in CSY 214) to also be defective in facilitating proper ER translocation and folding. In the secretory pathway, proteins are directed to their eventual destinations through a number of ways using physical adducts such as signal sequences (Kar2, Ero1), glycosylation (PDI, Gas1), and GPI glycolipid anchors (Gas1) (Barlowe and Miller, 2013). As part of post-translational processing in the ER lumen, when nascent polypeptide chains are translocated through channels in the ER, adducts such as signal sequences are usually cleaved off (Barlowe and Miller, 2013). Other polypeptides are modified through different ways through the addition of either glycosyl chains or GPI anchors (Barlowe and Miller, 2013). When ER translocation mechanisms are blocked or defective however, significant amounts of un-translocated, unprocessed precursor polypeptides accumulate in the cell, which we were able to detect through western blotting. As seen in our blots of all viable BiP-cysteine mutant strains, decreased polypeptide translocation into the ER was observed for all strains that were temperature sensitive, including the non-protective kar2-C63L and kar2-C63M alleles (Figure 7). Because precursor polypeptides are usually quickly degraded in the ER, it is surprising that we were able to detect such notable accumulations of precursor protein forms of Kar2, PDI, Gas1, and Ero1 (pre-Kar2, pre-PDI, Gas1 precursor, pre-Ero1) in whole-cell lysates.
after merely a 90-minute shift to 37°C. These results suggest that BiP’s ER translocation abilities are severely impacted when its cysteine is replaced with some other amino acid residues.

**Disrupting BiP’s essential functions impacts cells’ ability to respond to ER stress**

ER stress occurs when the flux of unfolded proteins entering the ER surpasses the capacity of the cell’s folding machinery, leading to the accumulation of unfolded proteins in the ER lumen (Pincus et al., 2012; Chambers et al., 2012). All cells respond to ER stress by activating a response pathway called the unfolded protein response (UPR). In yeast cells, this occurs through the formation of a Ire1 (inositol-requiring kinase 1) protein dimer that is phosphorylated when misfolded proteins accumulate, initiating RNA splicing of Hac1 mRNA (Cox et al., 2011). Active Hac1 is a nuclear transcription factor in yeast that initiates transcription of many UPR target genes, including a slew of molecular chaperones like BiP and PDI (Back et al., 2005). Following our observations that mutating BiP’s conserved cysteine affects its primary functions like ER translocation and dealing with heat stress, we wanted to see whether yeast strains expressing mutant BiP (in CSY 214) would also have similarly affected responses to misfolded proteins.

With an inserted UPR reporter pJC8, a plasmid with UPR promoter response elements inserted upstream of the lacZ gene that codes for the enzyme beta-galactosidase, we were able to assess levels of UPR in our mutant BiP-cysteine strains using a colometric assay correlating UPR activity with amounts of beta-galactosidase generated due to activation of the UPR promoter. In this assay, lysed cells (grown with or without a 90-minute shift to 37°C) were mixed with ONPG, a compound that reacts with the beta-galactosidase produced from activation of the UPR promoter in pJC8, generates a visible yellow color that can be measured by a plate reader. Wild-
type cells cultured at 24°C, 37°C, and at 37°C with 2mM dithiothreitol (DTT), a strong reducing agent that causes proteins to misfold, thus activating the UPR (Cox et al., 2011; Cleland, 1964), served as controls. Along with defects in ER translocation, all temperature-sensitive BiP-cysteine mutants at 37°C had significantly higher UPR induction levels comparable to or even higher than in wild-type cells treated with DTT at 37°C (Figure 8B). Notably, although many alleles that showed no translocation defects showed little or only moderately enhanced UPR induction (Figure 8A), \textit{kar2-C63D, kar2-C63E, kar2-C63F, kar2-C63K, kar2-C63Y, kar2-C63Q, kar2-C63L}, and \textit{kar2-C63M} alleles exhibited high levels of UPR induction comparable to the positive control, wild-type treated with DTT (Figure 8B). Interestingly, \textit{kar2-C63S}, an allele that is not temperature sensitive and does not exhibit any ER translocation defects, was observed to exhibit high levels of UPR comparable to the positive control (Figure 8A). Conversely, \textit{kar2-C63V} had markedly lower levels of UPR activity similar to wild-type or even lower than other mutants with similar phenotypes in ER translocation defects, like \textit{kar2-C63A, kar2-C63G, kar2-C63I,} and \textit{kar2-C63T} (Figure 8A).

\textbf{Modified BiP-Cysteine mimetic mutants are able to prevent protein aggregation}

Normally, BiP is an ATP-dependent molecular chaperone that is able to bind and release ATP in order to fold substrate polypeptides. Under conditions of oxidative stress however, as shown by recent data produced by the lab, oxidized BiP treated with peroxide and mimetics of modified BiP (Kar2-C63F/W/Y) exhibit minimal ATPase activity (Wang et al. 2014), yet are still able to prevent protein aggregation. To test whether modified-BiP mimetics identified from previous \textit{in vivo} assays show the same capacity to prevent aggregation, we used an \textit{in vitro} IgY aggregation assay with purified IgY, denatured with guanidine chloride and DTT, as our model
substrate and purified mutant Kar2 proteins. Although native IgY is not normally prone to aggregation, once denatured, it is able to aggregate, creating a useful system in which to look at the peptide binding activities of our BiP-cysteine mutants. Because we wanted to focus on mutants that exhibited the strongest protective phenotypes, we chose only 4 out of our 19 BiP-cysteine mutant alleles to purify and test, *kar2-C63H, kar2-C63P, kar2-C63R, kar2-C63K.*

Mutant BiPs were incubated with denatured IgY and measured for their relative light scattering over the course of 1 hour at OD$_{360\text{nm}}$ in a spectrophotometer. Although we have not yet fully verified our data from these assays, preliminary results show similar trends to our published modified BiP mimetic alleles, that Kar2-C63K/P/R have enhanced abilities to prevent protein aggregation (Figure 9). Kar2-C63H, notably, seemed prone to aggregation on its own, which would explain data showing increased light scattering when Kar2-C63H is incubated with denatured IgY (Figure 9).
DISCUSSION

**BiP’s role as a ROS sensor in ER redox-signaling pathways**

As mentioned previously, our lab’s work on the redox sensing capabilities of the Hsp70 molecular chaperone BiP essentially began with studies using a unique *Ero1* yeast strain that allowed us to selectively induce oxidative stress in the ER lumen. Taking advantage of this novel tool, our lab was able to uncover an unexpected link between BiP and redox signaling pathways that help cells respond and cope with oxidative stress. Under normal conditions, BiP is an essential ER chaperone, driven by ATP hydrolysis, that facilitates the folding and release of nascent polypeptides (a “foldase”). Under conditions of stress, however, BiP’s activity is altered and moderated to protect cells from the detrimental effects of ROS and oxidative damage.

BiP contains a sole, conserved cysteine within its nucleotide binding domain. Following data recently published by the lab that establishes that BiP’s cysteine is directly modified by ROS and that this modification alters BiP activities in ways that protect cells against oxidative stress, our lab was able to propose a model for how BiP functions during highly-oxidizing conditions in the ER. Because we have evidence that BiP oxidation results in both markedly decreased ATPase activities and enhanced protein folding capabilities, we think that during conditions of stress, BiP acts as a critical redox sensor in the ER, alerting the cell that increased levels of ROS are present. As ER oxidative stress can cause the formation of non-native disulfide bonds that lead to protein aggregation, when ROS levels accumulate, we believe that a small portion of BiP converts itself from a ATP-dependent “foldase” into a “high-avidity polypeptide holdase” (Wang et al., 2014), a BiP that only has high affinity for misfolded polypeptides and little ATPase activity. In this situation, it is disadvantageous for BiP to assume its normal chaperone activity in folding polypeptides with mispaired disulfide bonds, as eventually...
misfolded proteins would rapidly accumulate in the ER, causing ER stress. We, therefore, hypothesize BiP’s “holdase” state to be beneficial to cells during conditions of stress because of its enhanced abilities to prevent harmful protein aggregation.

**BiP-cysteine mutant alleles can be separated into four distinct classes**

Because the lab has observed similar results for directly modified BiP and also BiP-cysteine-substitution alleles that are phenotypic mimetics of BiP’s protective oxidized form, we reasoned that creating more BiP-cysteine mutant alleles could provide useful clues to elucidating more roles of BiP in the ER during oxidative stress. The purpose of our “screen” of all possible BiP-cysteine mutants, therefore, was to not only identify more protective alleles of BiP but also characterize the activities and functions of BiP-cysteine mutants that did not mimic BiP’s protective form.

Following our hypothesis that similarly bulky or charged substitutions would protect against oxidative stress, we identified, through spotting assays with CSY 278 Ero1* background strain, 4 more alleles of BiP (kar2-C63H/P/R/W) that behaved similarly to the lab’s established kar2-C63F/W/Y alleles (Figure 4). Using results gained from these assays, we were able to classify these 7 protective alleles based on two features: 1) whether they were able to function effectively as the sole copy of BiP in cells under standard growth conditions, and 2) whether they exhibited any temperature sensitivities and defects in ER translocation. Of these 7 alleles, 4 alleles, kar2-C63H/P/R/W, could not sustain normal BiP function on their own while the remaining 3, kar2-C63D/F/K/Y, exhibited temperature sensitivity and defects ER translocation.

Furthermore, we were able to categorize the remaining non-protective or moderately-protective mutants into two classes, mutants that exhibited defects in ER translocation and
mutants that did not. On one hand, substitutions with negatively charged residues (kar2-C63D/E) as well as some hydrophobic residues (kar2-C63L/M/Q) that did not protect or only moderately protected against oxidative stress in CSY 278, were temperature sensitive and exhibited moderate to severe defects in ER translocation when expressed in our CSY 214 kar2Δ strain. Substitutions with other charged and uncharged hydrophobic residues like kar2-C63A/G/I/S/T/V, on the other hand, did not significantly impact normal BiP function, did not protect cells from stress when expressed in CSY 278 (although the protective abilities of kar2-C63G and kar2-C63V were observed to be more inconsistent and varied) and did not result in ER translocation defects in CSY 214.

Based on the lab’s acquired lab data on kar2-C63F/W/Y, it is not surprising that the additional alleles we have identified to protect against oxidative stress are larger, more bulky residues like our published “bulky” strains. What is harder to tease apart is how the rest of the mutants fit into our BiP model, particularly those that do not fall neatly into their respective class of mutants, such as kar2-C63D/E and kar2-C63Q, alleles that moderately or strongly protect expressed in CSY 278 (although their phenotypes are variable depending on the type of stress used during experimentation), yet exhibit modest to severe ER translocation defects and moderately enhanced UPR when inserted into CSY 214. Looking at the spotting assays and western blots used to determine for ER translocation defects, kar2-C63D/E show slightly different phenotypes, although they are both acidic amino acid residues and are fairly similar in structure. While kar2-C63D moderately protects cells from oxidative stress and is temperature sensitive, kar2-C63E does not protect at all and is only mildly sick when grown at high temperatures. Although they have similar ER translocation defects, it would be interesting to pursue more assays that can differentiate between the two mutant alleles. kar2-C63Q, on the
other hand, is more problematic to characterize, as one can easily argue that it is a protective mimetic allele, given its unexpectedly strong response to cellular stress caused by diamide. Because lack of the same response to galactose-induced ROS caused by Ero1* puts it in an ambiguous gray area between the two classes (between ones that protect and ones that do not), I think, again, that more assays may be necessary to fully assess kar2-C63Q’s capabilities.

The last unaddressed mutant, kar2-C63N, falls into its own category as a BiP-mutant allele unable to protect and unable to support normal cellular growth. Due to its inability to support cellular growth on its own or protect the cell from stress, further assays were not pursued to test the functions of this mutant.

**Temperature-sensitive BiP-cysteine mutant alleles have enhanced UPR**

With four classes of BiP mutants now roughly categorized (summary shown in Table 1), we next sought to assess surviving BiP-cysteine mutants (expressed in CSY 214) for their UPR activities, triggered by heat stress. Previous data published by the lab suggests that oxidized BiP triggers enhanced UPR induction, allowing cells to sustain growth under conditions of stress (Wang et al., 2014). Although it was eventually concluded that UPR induction is insufficient in managing oxidative stress, given that UPR induction seems to do moderately little in overly-oxidizing ER environments created by Ero1* (Wang et al., 2014), we nevertheless were still interested in looking at the UPR activities of these mutants to see if enhanced UPR induction was correlated with increased temperature sensitivity and/or defects in ER translocation. Predictably, we were able to see that mutant alleles that exhibited these phenotypes had enhanced UPR levels comparable to WT treated with DTT, a reagent that causes large amounts of proteins to misfold. These data suggest that cells with defective BiP may indeed have enhanced abilities to trigger
different elements of the UPR precisely because BiP, as a key UPR chaperone protein, is unable to carry out most of its normal functions. Further assays may be necessary, however, to fully demonstrate this hypothesis.

**Protective BiP-Cystiene Mutant Alleles may have enhanced abilities to prevent protein aggregation**

As established before, protein aggregation is a significant source of ER stress. Although one of BiP’s fundamental functions in the ER, under normal conditions, is to fold nascent polypeptides, it can easily get overwhelmed during times of ER stress and exacerbate the levels of misfolded proteins in the cells helping fold proteins with non-native disulfide bonds. Because recently the lab has acquired data that support the hypothesis that BiP is modified by ROS to convert itself to a “holdase”, a state that the lab believes to be beneficial to cells during stress, we wanted to see whether our newly-identified BiP-cysteine mutant alleles had enhanced abilities in preventing protein aggregation.

To look BiP’s peptide-binding activities *in vitro*, we used IgY as a model substrate with purified BiP mutants in an IgY protein aggregation assay that measured for light scattering, showing IgY aggregation over the course of 1 hour. Preliminary results show that BiP-C63K/P/R have enhanced abilities to bind polypeptides and prevent protein aggregation while BiP-C63H aggregates on its own, causing what seems to be increased IgY aggregation in the assay. Although the success of this experiment is dependent on many factors, including the sample of IgY used that day as well as the concentrations of proteins used, we have tried to mitigate these variable factors by performing the assay with all mutants in one experiment in one day. Denatured IgY, unfortunately however, has a finite “life-span” of 5 hours, after which it does not
aggregate well. Although a new denatured IgY stock can always be made, there is always the possibility that differences among the different batches can cause some variability in final results. Because we have not yet fully troubleshooting on this experiment, the results shown in Figure 9 are therefore only an indication that the BiP-cysteine mutants we purified may have enhanced capabilities to prevent aggregation, which fits with previous data acquired by the lab. Further studies with possibly a different model substrate, like rhodanese, would probably be more useful if we wanted to follow up on these results.

The connection between BiP modification and protection against oxidative stress

Separation into these four classes of mutant BiPs reveals several telling features about BiP-modification and protection from oxidative stress. Fitting with the lab’s model that upon cysteine modification, BiP is converted from a ATP-dependent chaperone into a ATPase-dead but competent “holdase”, we observed that BiP mutant alleles that mimicked BiP’s protective modified form in CSY 278 but were unable to sustain cellular life on their own in CSY 214 exhibited not only enhanced abilities to prevent protein aggregation, but also decreased translocation of polypeptides into the ER (if they were still functional) and enhanced UPR. From our spotting assays that investigated the functionalities of mutant BiP in vivo under normal conditions, we have also identified BiP-cysteine mutants that do not significantly protect from oxidative stress yet exhibit considerable temperature sensitivities, ER translocation defects, and enhanced UPR. Although it is quite possible that in this class of mutants, BiP is functionally so compromised that it is unable to properly to respond to stress, there may be other reasons to why replacing BiP’s cysteine with hydrophobic residues creates such strong defects in its normal chaperone activities. Nevertheless, these results support previous data published by the lab, in
which another mutant BiP allele, *kar2-T249G*, was created to directly assess the impact of loss of ATPase activity on cell protection during oxidative stress, showing that loss of ATPase activity is necessary but insufficient for protecting the cell from stress. Together, these data indicate that there may be other mechanisms and pathways in the cell involving modified BiP that we have not yet uncovered.

Although we have roughly characterized four classes of BiP-cysteine mutants based on their different functional capabilities in the ER, many questions still remain about the nature of these mutants and why bulky, charged adducts in BiP’s nucleotide binding domain enhance its protective functions *in vivo*. What I want to especially stress about these mutants is that assaying their functions is *not* the same as looking at BiP modification directly. BiP-cysteine substitution alleles are merely suggestive of what modified BiP is capable of doing *in vivo*; however, based on these assays, we do not yet know what the modification actually is or how it acts *in vivo*. Although we know that BiP can be reversibly sulfenylated and more recently glutathionylated (unpublished data), these are only two of many possible modifications to BiP’s conserved cysteine that could trigger different redox signaling pathways in the ER during oxidative stress. What is promising about these mutants is that we now have the tools to better understand BiP’s fundamental functions in the cell during ER oxidative stress and specifically what modification of its cysteine can do to trigger protective mechanisms that help cells cope with that stress.
FUTURE DIRECTIONS

Because the purpose of this project was to assay all possible BiP-cysteine mutants for their various functions, ideally we would have liked looked more comprehensively at all of these mutants in the same way we looked at kar2-C63H/P/R/K. First, we would have liked to further verify the results of our IgY assay using our already purified mutants. Second, since we have identified mutants (kar2-C63L/M/Q and kar2-C63E) that just seem to have a defective BiP, we would also like to see what functions they are capable or not capable of carrying out. To do this, we would need to create more pET28-derived plasmids for recombinant protein purification and then test all of our mutants for ATPase activity and ability to prevent protein aggregation. Since the IgY aggregation assay is difficult to manage with more than 4 or 5 mutants, another assay we have yet to try is another rhodanese aggregation assay, which uses rhodanese instead of IgY as a model substrate. The rhodanese assay has several advantages over IgY in that it takes a much shorter time for rhodanese to aggregate and that it seems to show more consistent and reliable aggregation curves. Third, we would like to test for ATPase activity of our mutants in vitro, using purified proteins and radiolabeled ATP to determine rate of ATP hydrolysis to ADP and thus determine whether all of our mutants have decreased ATPase activity or not.

BiP, on its own, is known to be a poor ATPase and requires other proteins to help with its chaperone activities, such as co-chaperones like J-proteins or nucleotide exchange factors like Sil1 (Misselwitz et al., 1998; Guo et al., 2013; Chung et al., 2002). If given more time, we would also probably want to further assess our BiP-cysteine mutants to see how BiP modification could impact its interactions with other proteins that help protect cells from oxidative stress.
CONCLUDING REMARKS

Using a unique genetically manipulated cellular system to create oxidative stress in the ER by deregulating the activities of Ero1, we have uncovered new roles for the Hsp70 molecular chaperone BiP as an essential redox sensor in redox signaling pathways of the ER. BiP has a conserved cysteine in its nucleotide binding domain that can be reversibly modified by ROS created during conditions of oxidative stress (Wang et al., 2014). Because BiP’s cysteine is so critical to its protective functions during oxidative stress, in this study, we have created an entire range of BiP-cysteine mutant alleles to identify protective alleles of BiP that could mimic its modified form and also other mutants that do not protect from stress yet still have altered BiP functions \textit{in vivo}. We have found that substitutions with charged, bulky residues significantly enhances BiP’s protective capabilities in cells during oxidative stress, although not all of these substitutions are functional under normal conditions. We have also found these mutants to not only have increased ER translocation defects but also exhibit increased UPR activity, triggered by heat stress, and enhanced capacities to prevent protein aggregation. With these assays, we hope to not only uncover more protective alleles of BiP in the future, but also use our work to prompt future studies in drug design, as small molecule drugs that directly target BiP to turn on its protective functions could be beneficial to patients in slowing the progression of disease.

Before we can move on to therapeutic design however, we need to first enhance our current understanding of how cells cope with oxidative stress, so we can identify cellular pathways that can promote cell survival in the wake of cellular damage caused by oxidative stress.
EXPERIMENTAL PROCEDURES

Plasmid construction

Construction of LEU2-marked plasmids containing KAR2 and several kar2-C63 mutants has been described previously in Wang et al. 2014. Additional BiP-cysteine mutant plasmids were made using QuikChange site-directed mutagenesis with pCS681 as the template (Stratagene, Santa Clara CA). QuikChange reactions were digested with 1 µL DpnI for 2 hours at 37°C and transformed into competent XL1-Blue bacteria cells using standard transformation procedures. After an overnight incubation at 37°C, colonies generated from bacterial transformations were inoculated into 5 mL of Luria Broth (LB) plus ampicillin to procure cell cultures for DNA minipreps. Using standard alkaline-lysis miniprep procedures, DNA pellets were harvested, washed with ethanol, dried, and resuspended in 50 µL 10 mM Tris-HCl, pH 8 for long-term storage. Additionally, 2 µL of plasmid DNA was reserved for a diagnostic restriction-enzyme digest using 0.5 µL each of NdeI and XbaI, to determine whether plasmid rearrangement had occurred. All mutations were confirmed by sequencing.

Similar procedures were used to prepare pET28-derived BiP-cysteine mutant vectors for recombinant protein purification. The pET28 KAR2 plasmid pCS817, described previously was used as the template for mutagenesis (Wang et al. 2014). All plasmids were digested with 0.5 µL EcoRV to determine whether plasmid rearrangement had occurred. All mutations were confirmed by sequencing.

Plasmids used in this study are listed in Table 1.
Yeast strains

Plasmids were transformed into *Saccharomyces cerevisiae* strains CSY 214 and CSY 278 (genotypes listed in Table 2), plated onto SMM-leu plates, and grown at 24°C for 2-3 days (SMM is a synthetic minimal medium supplemented with 2% glucose and specified amino acid mixtures). Single colonies were picked, struck out onto fresh SMM-leu plates, and allowed to incubate at 24°C for 2-3 additional days in preparation for future spotting assays. A minimum of three colonies per yeast strain were picked and tested.

To isolate cells with the mutant *KAR2* plasmid as the sole copy of *KAR2*, individually struck out CSY 214 transformants grown on SMM-leu plates were then re-striked onto 5-Fluoroorotic Acid (5-FOA) plates to counter-select for the original wild-type *URA3* plasmid retained by the strain (Boeke et al., 1987). To ensure full counter-selection, mutant strains were struck onto 5-FOA plates twice before a final transfer to YPD plates (rich yeast medium plates with 2% glucose). Since not all single-copy BiP-cysteine mutants were viable, CSY 214 strains observed to be growing poorly on 5FOA plates were not used in further assays testing for ER translocation defects and endogenous UPR activity.

Spotting assays

To test BiP-cysteine mutants under conditions of oxidative stress, all CSY 278 strains grown on SMM-leu plates were grown to saturation overnight at 30°C in 2 mL SRaf-leu (a SMM medium where 2% glucose is replaced with 2% raffinose) at 30°C. Samples were then diluted to 1 OD<sub>600</sub> and spotted in serial dilutions onto fresh SMM-leu and SGal-leu plates (SMM-leu plates with 2% galactose). A wildtype strain containing pCS681 and a strain containing the empty vector pRS315 were provided as controls. To provide maximum stress, plates were incubated at
37°C for 2-3 days, depending on rate of growth, and imaged for direct visual comparison. Figures shown in this paper are representative of several independent experiments, in which all three colonies picked per transformation showed the same phenotype.

To test BiP-cysteine mutants under conditions of diamide stress, transformed CSY 278 strains were grown overnight in 2 mL SMM-leu media and similarly spotted onto SMM-leu plates containing various concentrations of diamide (0 mM, 0.5 mM, 1.0 mM, 1.5 mM, and 2 mM). A wildtype strain containing pCS681 and a strain containing the empty vector pRS315 were provided as controls. Separate sets of plates were incubated at 30°C and 37°C for 2 days before imaging. Figures shown are representative of two independent experiments.

To determine whether BiP-cysteine mutants exhibited growth defects at non-permissive temperatures, transformed CSY 214 strains (post 5-FOA counter-selection) were grown in 2 mL of YPD media overnight and then spotted in serial dilutions onto YPD plates. Separate sets of plates were incubated at 24°C, 30°C, and 37°C for 2-3 days before imaging. Figures shown are representative of several independent experiments, in which all three colonies picked per transformation showed the same phenotype.

**ER-translocation westerns**

After 5FOA counter-selection, all surviving CSY 214-derived yeast strains with (single copy) mutant BiP were cultured in 5 ml of YPD at 24°C overnight. Saturated cultures were then sub-cultured to an OD$_{600}$ of 0.2 in 30 mL of fresh YPD and incubated at 24°C for 3 hours or until mid-log phase. Duplicate cultures were made for each yeast strain. After 3 hours, half of the cultures were moved to a 37°C water bath to grow for 1.5 hours. 5 OD$_{600}$ units of cells per 30 mL culture were harvested for lysate preparation, using a standard alkaline-treatment procedure.
Lysate samples were then re-suspended in 50 µL sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) and 2% beta-mercaptoethanol (BME), and boiled for 3 min at 100°C. Proteins were separated by SDS-PAGE (8%) and detected using 4 rabbit antibodies, anti-Kar2, anti-Gas1, anti-PDI, and anti-Ero1 after a semi-dry transfer to a nitrocellulose membrane.

Detection of unfolded protein response (UPR) activity after heat stress

All CSY 214-derived yeast strains with (single copy) mutant BiP were transformed with the UPRE-lacZ reporter plasmid pJC8 and streaked onto SMM-ura-leu plates for 2-3 days at 24°C. Streaked out colonies were then grown in SMM-ura-leu media overnight and then sub-cultured in the morning to an OD$_{600}$ of 0.2 in 1.5 mL of fresh SMM-ura-leu media. Two transformants were assayed per strain and duplicate cultures were made for each transformant. Cultures were grown for 4 hours at 24°C before half the samples were moved to a 37°C water bath for 1.5 hours. Additional wild-type cultures treated with 2 mM DTT at 37°C served as positive controls for the experiment. After 5.5 hours, 70 µL cells per culture were permeabilized with 70 µL of 1:1 solution of 2x Z-Buffer (120 mM Na$_2$HPO$_4$·7H$_2$O, 80 mM NaH$_2$PO$_4$·2H$_2$O, 20 mM KCl, 2 mM MgSO$_4$·7H$_2$O, pH 7) with 1.33 mg/mL ONPG, and Yeast Protein Extraction Reagent (Y-PER; Thermo Scientific) with 4 µL BME/mL working solution in a microplate (in duplicates) and timed in minutes until 56 µL of stop solution (2M Na$_2$CO$_3$) per well was added. The OD$_{600}$ of every 1.5 mL culture was also measured. Beta-galactosidase activity was finally quantified using the equation: (1000 x OD$_{420}$)/(time x cell volume x OD$_{600}$) and plotted onto bar graphs.
Recombinant protein purification

To grow up bacterial cell cultures for protein purification, select pET28-derived BiP-cysteine mutant vectors were transformed into BL21 (DE3) pLysS cells and then grown overnight in 37°C in 5 ml LB media with 34 µg/ml chloramphenicol and 15 µg/ml kanamycin. The overnight culture was then separated into 10 x 1 ml “starter cultures”, made with equal volumes of cell culture and 40% glycerol, flash-frozen in liquid nitrogen, and stored at -80°C. Each 1 ml starter culture was then thawed and added to 50 mL fresh LB with antibiotics and grown overnight at 37°C. Each 50 mL “super-culture” was added into 1 L of fresh LB with antibiotics and grown for 2 hours at 37°C, and then moved to a 24°C incubator. Protein expression was then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 hours of IPTG induction, bacterial cells were harvested and frozen down with liquid nitrogen and stored at -80°C.

To purify N-terminally tagged Kar2 and mutant Kar2 proteins, cell pellets were thawed and lysed with 20 mL sonication buffer (50 mM HEPES, pH 7.4, 0.3 M NaCl, 10 mM imidazole) with 1 mM PMSF, 1 µM pepstatin A, 5 mM BME, and 20 mg lysozyme. After 4 x 30 sec of sonication (with 2 min rests on ice in between), cells were centrifuged at 16,000 x g for 20 min to separate out cellular debris. The supernatant was then loaded onto a HiTrap chelating column (GE Healthcare) charged with nickel before undergoing several buffer washes. To wash out contaminating DnaK proteins, the nickel column, loaded with lysate, was washed with 10 column volumes (cv) sonication buffer, 10 cv sonication buffer with 5% glycerol, 1% Triton-X-100, 10 cv sonication butter with 5% glycerol, 1 M NaCl, 10 cv sonication butter with 5% glycerol, 5 mM ATP, 10mM MgCl2, 10 cv sonication buffer with 5% glycerol, 0.5 M Tris-HCl, pH 7.4, and 10 cv sonication buffer with 5% glycerol, 25 mM imidazole. Purified Kar2 proteins
were then eluted with 3 cv sonication buffer with 5% glycerol, 0.25 M imidazole. 2.5 mL of purified fractions were next loaded onto a PD-10 desalting column and exchanged into 40 mM Tris-HCl, pH 7.4, 80 mM NaCl, 10% glycerol. All purified proteins were then flash frozen in liquid nitrogen and stored at -80°C.

Protein concentrations were determined using BCA protein assays (Thermo Fisher Scientific, Waltham, MA) with bovine serum albumin standards.

**IgY aggregation assays**

IgY aggregation assays were performed following the methods of Stronge et al., 2001. IgY extracted from chicken eggs was prepared by the lab using a Chicken IgY Purification kit (Thermo Fisher Scientific) and resuspended in 0.1 M Tris-HCl, pH 8, for storage at 4°C. To prepare 33.3 µM of denatured IgY for the aggregation assay, native IgY was treated with 6 M Guanidine-Hydrochloride (GdnHCl) in 0.1 M Tris, pH 8, and 40 mM DTT for 2 hours minimum (rotating). To prepare the protein samples, Kar2 and Kar2 mutant proteins were diluted to a final concentration of 0.7 µM and mixed with 940 µL TSC Buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 5mM CaCl₂), 10 µL Desalting Buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% glycerol), and 30 µL 0.1 M ATP, pH 8, in a quartz cuvette. After a 1 min warm-up in the spectrophotometer, 20 µL of denatured IgY was added, giving a final IgY concentration of 0.67 µM. The sample was then read at an absorbance of 360 nm for 60 min. Protein aggregation curves were normalized to initial OD₃₆₀ readings at 0 min.

BCA protein assays (Thermo Fisher Scientific, Waltham, MA) with bovine serum albumin standards were used to determine all protein concentrations for the IgY aggregation assay.
**Figure 1:** Ero1 maintains redox homeostasis in the ER. **A)** Ero1 catalyzes the formation of disulfide bonds in the ER, generating peroxide as a by product. Through tightly controlled regulatory mechanisms, Ero1 is post-translationally buffered against changes in the ER redox environment, switching off when ER becomes highly oxidizing. **B)** When Ero1 is decoupled from its regulatory feedback loop, Ero1 becomes constitutively active (Ero1*) and generates excess amounts of oxidants in the ER, causing ER oxidative stress (Sevier et al., 2007).
Figure 2: The Hsp70 molecular chaperone BiP is an essential component of redox signaling pathways in the ER. 

A) BiP is an ATPase that couples ATP hydrolysis to protein folding (crystal structure with conserved cysteine residue shown). It has two domains, a nucleotide-binding ATPase domain with a highly-conserved cysteine residue that binds and releases ATP and a substrate-binding domain that binds peptides. 

B) During oxidative stress, BiP senses ROS and responds to redox imbalances in the ER by activating critical protection mechanisms. 

C) BiP’s conserved cysteine (-SH) is modified by peroxide (one form of ROS) to form a sulfenic acid (-SOH) adduct modification that protects cells from the overly-oxidizing ER environments (Wang et al., 2014). Additionally, the lab has shown that unmodified BiP and sulfenylated BiP could also be further modified by the tripeptide glutathione (GSH), forming an oxidized glutathione adduct (-SSG) that possibly modulates BiP activity during oxidative stress.
Substituting BiP’s conserved cysteine with other amino acid residues affects BiP’s function in vivo. A) Substitution of BiP-Cysteine with alanine renders the cell hypersensitive to the overexpression of Ero1* (Wang et al., 2014). B) Substitutions of BiP-Cysteine with phenylalanine (Phe), tyrosine (Tyr), or tryptophan (TrP) are mimetics of BiP’s modified form and trigger protective mechanisms during oxidative stress (Wang et al., 2014). C) Since BiP’s cysteine is modified by ROS during highly oxidizing conditions, disrupting BiP’s functions by replacing its conserved cysteine with other amino acid residues may uncover clues about BiP’s many functions triggered during ER oxidative stress.
Figure 4: Substitution of yeast BiP’s cysteine with all 19 other amino acid residues, expressed in CSY 278, results in varied protective phenotypes under stress conditions, with substitutions with bulky, charged residues (kar2-C63F/Y/W/H/P/R/K) conferring stronger protection from stress than other amino acid replacements. Cells containing mutant plasmids were grown overnight in 30°C raffinose media, diluted to 1 OD-600nm, and spotted in serial dilutions on glucose and galactose plates. Spotting on galactose plates induces overexpression of Ero1*, which generates ER oxidative stress. Growth on glucose plates show that Ero1* is not induced and that even spotting was performed during the assay. All plates were incubated at 37°C for either 2 days or 3 days. Plates shown are representative of at least three independent spotting experiments.
Figure 5: Yeast BiP-cysteine mutants that protected against Ero1*-induced oxidative stress similarly protected cells from diamide-induced cellular stress. *kar2-C63H/P/R/K/F/Y/W showed the most protection, although the responses of kar2-C63K and kar2-C63P to diamide were not as robust as other protective alleles. Unexpectedly, kar2-C63Q also showed a strong protective phenotype. CSY278-derived mutants (Ero1* strain background) were grown overnight in glucose media, diluted to 1 OD-600nm, and spotted in serial dilutions on different concentrations of diamide (0mM, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM) and incubated at 30°C and 37°C overnight. Plates incubated at 30°C show the effects of diamide only while plates incubated at 37°C show the cumulative effects of both heat and diamide stress.
Figure 5: BiP is essential for normal cell function and substituting BiP’s conserved cysteine disrupts its essential functions in vivo. A) Replacement of yeast BiP’s cysteine with some residues (kar2-C63H/N/P/R/W) results in cell mortality. Cells containing mutant plasmids were spotted onto glucose and 5FOA plates to counter-select for mutant plasmid and observed for growth after 2 days at 24°C. B) Replacement of yeast BiP-cysteine with some amino acid residues (kar2-C63D/F/K/L/M/Q/Y) results in increased sensitivity to heat stress (37°C). BiP-cysteine mutants that survived the 5FOA plasmid shuffle shown in A) were grown overnight in YPD and spotted onto YPD agar media plates at 24°C, 30°C, and 37°C.
**Figure 7:** Temperature-sensitive yeast BiP-cysteine mutants exhibit ER translocation defects after a 37°C heat shock for 90 minutes. Cells were grown in YPD, harvested at mid-log with or without heat shock, lysed, and then run through a SDS-Page gel and transferred to nitrocellulose membranes. Membranes were stained with anti-Kar2, anti-PDI, anti-Ero1, and anti-Gas1 antibodies to detect for untranslocated, unprocessed polypeptides.
The figure shows the temperature sensitivity of BiP-cysteine mutants in yeast, with a 90-minute shift to a 37°C water bath. In these colorimetric beta-galactosidase assays, BiP-cysteine mutants inserted with a UPR reporter plasmid pJC8 were cultured to mid-log phase, subjected to heat shock, and then permeabilized using lysing reagents. ONPG, a compound that reacts with the beta-galactosidase produced from the UPR reporter, was mixed in the lysed cells, producing a visible yellow color that can be measured by plate reader at an absorbance of 420 nm. Wild-type cells grown at 24°C, 37°C, and 37°C plus DTT treatment served as controls. Data shown are representative of at least 3 independent experiments, with beta-galactosidase levels normalized to levels measured in wild-type cells grown at 24°C.

**A)** Yeast BiP-cysteine mutants *kar2-C63A/G/I/S/T/V*, alleles that do not significantly protect against stress show wild-type like UPR induction or only modest UPR induction. Of these alleles, *kar2-C63I* and *kar2-C63S* show the most UPR induction while *kar2-C63V* shows markedly depressed UPR activity.

**B)** Yeast BiP-cysteine mutants *kar2-C63L/M/Q/D/E/F/K/Y* that exhibited modest-severe ER translocation defects also how enhanced UPR induction triggered by heat stress.
Table 1: Summary of BiP-cysteine mutants

<table>
<thead>
<tr>
<th>BiP-cysteine Mutant</th>
<th>Level of Protection from Stress? (in ER01* strain)</th>
<th>Functional as sole copy of BiP (in kar2Δ strain)?</th>
<th>Temperature Sensitive at 37°C?</th>
<th>ER Translocation Block at 37°C?</th>
<th>Level of UPR induction (compared to WT at 37°C)</th>
<th>Prevents protein aggregation (compared to WT)?</th>
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<td>no</td>
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x = did not test in assay

* + = slightly enhanced UPR induction compared to WT at 37°C
** + = moderately enhanced UPR induction
*** + = enhanced UPR induction comparable to WT treated with DTT
- = no UPR induction compared to WT at 37°C

Figure 9: Mutant BiPs that strongly protect against stress in vivo are able to prevent protein aggregation in vitro (preliminary data). Mutant BiPs collected and purified from BL21 bacterial cells were incubated with denatured IgY and measured in a spectrophotometer for light scattering over 1 hour. Data shown shows IgY aggregation curves for mutant BiPs vs. wild-type BiP, with light scattering values normalized to IgY alone without any protein. Mutant BiPs BiP-C63P/R/K show enhanced abilities to prevent protein aggregation in vitro.
Table 2. Plasmids

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<th>Description</th>
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Table 3. Yeast Strains

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<th>Yeast Strains</th>
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<tr>
<td>CSY 214</td>
<td>MATa GAL2 ura3-52 leu2-3,112 kar2Δ::KanMX [pCS623]</td>
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<td>CSY 278</td>
<td>MATa GAL2 ura3-52 leu2-3,112 kar2-C63A can1::P$_{GAL1}$-ERO1*-myc</td>
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</table>
ACKNOWLEDGEMENTS

This work would not have been possible without the support and guidance of many extraordinary individuals.

First and foremost, I would like to thank my P.I. and mentor, Dr. Carolyn Sevier, for all her time and patience, as I endeavored on this long, difficult project. Dr. Sevier, thank you so much for everything, for taking me into your lab freshman year, for teaching me all there is to know (and more) to being a good scientist (and keeping a good lab notebook, of course), for helping me whenever I felt really lost and confused, for reading and correcting anything I sent your way, and for especially, giving me a home and a family these past three years.

I would also like to thank my lab mates and dear friends, graduate students Kristeen Pareja, Jie (Gia) Wang, and Kevin Siegenthaler, my favorite lab technician Heather Sickles, and long-departed fellow undergraduates Dongho (Chris) Shin and Andrew Sawires, as well as all the rotation students who have ever worked with me, for not only keeping me sane these past few years, but also making lab the happiest (and craziest) place on Earth. Kristeen, Gia, Chris, thank you so much for being the most wonderful, supportive friends a lonely little undergrad could ever ask for. Heather, thank you for always keeping us in line at lab and always putting up with my stupid questions. I wish you and your kids all the very best!

I would also like to take a moment to thank all the non-lab friends in my life, both at Cornell and at home, Torrey, John, Amy, Zack, Jenny, Connor, Sabrina, Angela, Colleen, Jessie, Mandy, Steffi, Keyi, and Alyssa, who have always supported and encouraged me no matter how much (or how little) they understood (or cared) about my research.

Finally, I would like to thank my family. Thanks Mom and Dad for letting me go to college so far away, for never blaming me for my failures, and especially for never, ever giving up on me. I hope I make you guys proud someday.
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