Thiol-based Misfolding: Linking Redox Balance to Cytosolic Proteostasis

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THIOL-BASED MISFOLDING: LINKING REDOX BALANCE TO CYTOSOLIC PROTEOSTASIS

by

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Thiol-based Misfolding: Linking Redox Balance to Cytosolic Proteostasis

A

DISSERTATION

presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Science
in Partial Fulfillment
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for the Degree of
DOCTOR OF PHILOSOPHY

by
Amy Eileen Ford, B.S.
Houston, TX
May, 2019
Thiol-based Misfolding: Linking Redox Balance to Cytosolic Proteostasis

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The eukaryotic cytosolic proteome is vulnerable to changes in proteostatic and redox balance caused by temperature, pH, oxidants and xenobiotics. Cysteine-containing proteins are especially at risk as the thiol side chain is subject to oxidation, adduction and chelation by thiol-reactive compounds. All of these thiol-modifiers have been demonstrated to induce the heat shock response and recruit protein chaperones to sites of presumed protein aggregation in the budding yeast *Saccharomyces cerevisiae*. However, endogenous targets of thiol stress toxicity responsible for these outcomes are largely unknown. Furthermore, I hypothesize proteins identified as redox-active are prone to misfolding and aggregation by thiol-specific stress. Perhaps these redox-sensitive proteins are those unknown targets. My work has determined that changes in cytosolic redox balance via thiol-specific stresses including cadmium, diamide, and glucose starvation are proteotoxic by monitoring aggregation of an endogenous redox-sensitive model protein. Emerging from the ribosome, newly synthesized cysteine-containing peptides are in a reduced state. Exposure to thiol-specific stress results in the modification of protein thiols by covalent interactions and oxidation. These non-native modifications to proteins result in an inability to properly fold, leading to the formation of protein aggregates. The thiol-reactive stress and subsequent aggregation is sensed by the cytoplasmic protein quality control network that activates stress responses and recruits chaperones such as Hsp104 and Tsa1. These chaperones assist in clearing existing aggregates and preventing further damage. Above all, protein aggregation as a result of exposure to thiol-specific stress extends to human cells, thus establishing a conserved mechanism and model for redox-associated proteostasis.
**Acknowledgements**

I would like to thank my PI and mentor Dr. Kevin A. Morano for investing in my training. I am appreciative for all of your patience, criticisms, and encouragements. I would like to thank Drs. Theresa M. Koehler, Vasanthi Jayaraman, Ambro van Hoof, and Jiqiang (Lanny) Ling for serving on my advisory committee and providing input in my research. I especially would like to thank Dr. Catherine Denicourt for collaborating with me on the human cell culture experiments presented in this dissertation. Thank you for training me on techniques and providing expert recommendations on experimental directions and data analysis.

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Chapter I: Introduction

The Protein Quality Control System

Protein homeostasis and proper protein folding are essential for cellular function. Molecular chaperones assist proteins in folding and prevent non-native conformations that lead to misfolding and aggregation (Verghese et al. 2012). Protein folding and stability are influenced by the local microenvironment, which is in turn impacted by extracellular conditions such as temperature, pH, nutrient status or the presence of proteotoxic xenobiotics. Molecular chaperones, protein degradation systems, and response pathways are all components of the protein quality control system (PQC) that maintains protein homeostasis (“proteostasis”).

The predominant protein folding machines consist of Hsp70-associated and Hsp90-associated chaperones. Hsp70 class chaperones utilize ATP and co-chaperones to carry protein substrates from “cradle to grave” through a cycle of substrate binding and release. Co-chaperones consist of J-domain-containing chaperones such as the yeast Ydj1 and nucleotide exchange factors (NEF) such as the Hsp110 family of proteins. J-domain containing chaperones bind protein substrates and facilitate their transfer to Hsp70. NEFs enhance the intrinsic ATPase activity of Hsp70 and exchange ATP for ADP allowing rapid cycling. Additionally, Hsp110 can function as a “holdase” by binding protein substrates alone or in concert with Hsp70 (Polier et al., 2010). This added function is potentially important during cellular stress or human disease states (Hahn et al., 2004; Garcia et al., 2017). Together, not only does the Hsp70/Hsp40/Hsp110 system aid in nascent protein folding, but acts as a disaggregase to unfold insoluble protein aggregates (Kaimal et al., 2017). The Hsp90 system functions similarly to Hsp70 by aiding in protein folding in concert with co-chaperones; however, Hsp90 interacts with specific “client” proteins, including kinases, receptors, and transcription factors, to aid in protein maturation and assembly of macromolecular complexes (Röhl et al. 2013). In addition to the Hsp70 and Hsp90 folding systems, cells employ small heat shock proteins (sHSP) that function as multimers to bind and intercalate within protein aggregates. This class includes the yeast sHSPs Hsp42 and Hsp26 as well as an atypical chaperone Tsa1. The yeast ATP-dependent disaggregase Hsp104 also plays a role in aggregation. Hsp104 functions as a homo-decamer to disaggregate protein
aggregates by threading peptide chains through its central channel to remove from the insoluble aggregate (Verghese et al., 2012). The newly disaggregated peptide is either degraded or re-solubilized. In mammalian cells, the Hsp70/Hsp110/Hsp40 system acts as the sole disaggregate as there is no Hsp104 homolog (Torrente and Shorter, 2013).

Chaperones and other stress-related proteins have been shown to localize into distinct stress foci in response to different stimuli. These non-membranous compartments include the general quality control bodies cytosolic Q-body (QB), juxtanuclear/intranuclear body (JUNQ/INQ), stress granules (SG), processing bodies (P-body, PB), and the insoluble protein deposit (IPOD) (Chen et al., 2011). All have been studied primarily in yeast and have been implicated to include protein aggregates either through recruitment or nucleation. The QB and JUNQ/INQ are dynamic compartments that shuttle general toxic misfolded protein between the cytosol and nucleus for protective sequestration and degradation by the proteosome (Escusa-Toret et al., 2013; Miller et al., 2015). Chaperones associated with these compartments are the disaggregases Hsp104 and Hsp70/Hsp40/Hsp110 system as well as the sHSPs Hsp42 and Btn2. QB are assembled and dissolved by these chaperones. Coordinated movement throughout the cytosol and integration into the JUNQ/INQ is achieved through association with the ER and chaperone activity. The IPOD contrasts morphologically as it forms a large structure adjacent to the vacuole (Chen et al., 2011). Unlike the other quality control bodies, the IPOD specifically houses insoluble protein such as amyloid-like and structurally-ordered aggregates that are not readily dissolved by the coordinated action of Hsp104 and the proteasome.

SG and PB are induced by specific stress stimuli that inhibit translation, including nutrient starvation and pH changes, and are composed of protein and RNA (Kshirsagar and Parker, 2004; Buchan et al., 2008). Unlike the QB and JUNQ/INQ, protein aggregates are not the main constituents, but rather soluble protein sequestered as a protection mechanism (Wallace et al., 2015a). Namely, these bodies allow controlled mRNA processing, including degradation, and sequestration of translation-associated proteins (Decker and Parker, 2012). One mechanism for formation of these assemblies is through pH-dependent phase transitioning from liquid to gel-like
droplets via negatively charged amino acid clusters and amyloid-like domains such as in the prion Sup35 (Munder et al., 2016; Franzmann et al., 2018).

With the aid of chaperones, misfolded and aggregated proteins are either degraded or re-introduced into the soluble protein pool. The eukaryotic proteasome is a specialized protein degradation machine that has ubiquitin (Ub)-dependent and Ub-independent conformations (reviewed by Bhattacharyya et al., 2014). Both conformations function as part of the protein quality control network to maintain proteostasis by degrading normal and aberrant proteins. The Ub-dependent 26S proteasome contains the evolutionarily conserved 20S proteolytic core and a 19S regulatory subunit that utilizes ATP and the Ub-machinery to degrade protein substrates. In contrast, the Ub-independent 20S proteasome lacks both the 19S regulatory subunit and requirement for ATP. The core particle consists of two outer alpha rings that regulate proteasome activity and two inner beta rings that utilize caspase-like, trypsin-like, and chymotrypsin-like activity to degrade substrates (Coux et al., 1996; Groll et al., 1997). The regulatory particle acts as an ATPase that recognizes, unfolds and translocates ubiquitinated substrates into the core for degradation. At sites such as QB and JUNQ/INQ, chaperones and Ub machinery are recruited to mark and transport misfolded protein to the proteasome for degradation (Miller et al., 2015).

In the eukaryotic cytosol, there are multiple transcriptional responses that are activated by a multitude of stressors. For example, the general environmental stress response is regulated by the transcription factor (TF) Msn2/4 and is activated by a variety of stresses such as heat and starvation (Verghese et al., 2012). In contrast, the oxidative stress response (OSR) controlled by Yap1 is primarily activated by oxidative stress (OS). The relationship between OS and protein folding is well established in the endoplasmic reticulum (ER) and imbalance activates the unfolded protein response (UPR). On the other hand, this relationship in the cytosol, specifically, is unclear and remains to be further investigated. The response to misfolded proteins in the cytosol is known as the Heat Shock Response (HSR) and, as the name suggests, has been studied extensively in response to heat shock (HS). The HSR is primarily modulated by the TF Hsf1. In yeast, Hsf1 exists as a trimer bound to heat shock elements (HSE) located upstream of
heat shock induced genes and is repressed directly by molecular chaperones including Hsp70 via regions within the amino and carboxy termini (Zheng et al., 2016). Upon cellular stress such as heat, proteins misfold and begin to aggregate. Protein misfolding is sensed by chaperones including Hsp70, resulting in titration away from Hsf1 and transcription of heat shock genes. In contrast to yeast, mammalian Hsf1 exists primarily as a Hsp90-repressed monomer that trimerizes and localizes to the nucleus to activate stress genes following proteotoxic stress; though, Hsp70 has been shown to interact with Hsf1 in a potentially regulatory manner (Abravaya et al., 1992; Bharadwaj et al., 1999; Grunwald et al., 2014). Although these compartmentalized responses control distinct transcriptional programs, the different stress response pathways occasionally converge to co-regulate expression of the same stress genes, indicating crosstalk between different cellular stresses and the responses they elicit (Liu and Thiele, 1996; Sugiyama et al., 2000; Chen et al., 2009; Solís et al., 2016).

Redox Balance and Thiol Reactivity

A distinguishing feature of eukaryotes is the presence of membrane-bound organelles that allow for the compartmentalization of protein folding into two distinct redox environments: oxidizing in the ER and mitochondrial inner membrane space and reducing in the cytosol (López-Mirabal and Winther, 2008). Mechanisms to preserve redox balance include the thioredoxin and glutathione systems and other antioxidant enzymes such as superoxide dismutase (Sod1). The primary components consist of thioredoxin and glutaredoxin and their cognate reductases that either function in a distinct compartment or are shared between redox compartments. Thioredoxin-dependent peroxiredoxins including Tsa1/2 and Ahp1 provide additional antioxidant function in maintaining a reduced cytosol. Both redox systems utilize reducing equivalents NADH+ and glutathione to detoxify ROS and isomerize or reduce protein disulfides important for protein folding via dithiol CxxC motifs.

Cysteine-containing proteins are especially vulnerable to alterations in redox chemistry as they are maintained in an oxidized state in the ER and reduced in the cytosol. Cysteines are
one of the least abundant residues, but is highly conserved among protein homologues across species due to its irreplaceable functionality as the only amino acid with a highly reactive thiol group (Poole, 2015). Cysteine residues are most often buried and clustered within the protein structure, which is characteristic of metal binding and redox centers. Although rare, solvent exposed cysteines often indicate a role in redox sensing (Wang et al., 2012, 2014). Reactivity of the thiol group depends on the accessibility and the protonation state (pKₐ), which is influenced by microenvironmental conditions such as pH and neighboring residues (Poole, 2015). For example, distribution of cysteines at amino-termini of α-helices and hydrogen bonding lower pKₐ values and increase the chance of deprotonation, thus, enhancing reactivity. These chemical and functional properties allow for rapid and reversible redox regulation of protein activity to sense and control diverse cellular states and processes.

Reactive oxygen species (ROS) produced as a byproduct of aerobic metabolism and exposure to oxidants and highly toxic xenobiotics have the potential to modify reactive thiols (Figure 1.1, modified from West et al., 2012). Oxidants such as hydrogen peroxide (H₂O₂) and diamide can react with a number of amino acids including methionine, histidine, phenylalanine, and tyrosine; however, cysteine residues are the most susceptible to oxidation due to their lower pKₐ value (West et al., 2012; Poole, 2015). Following initial formation of sulfenic acid, the modified thiol can either be further oxidized into sulfinic or sulfonic acid or form a disulfide bond with a nearby free thiol (e.g. intramolecular Cys or glutathione). Like oxidants, cysteines are also susceptible to modification by organic electrophiles such as diethyl maleate (DEM) and diethyl acetylenedicarboxylate (DAD), which form thiol adducts and induce intermolecular cross-links between proteins (Zhang et al., 1995; Sánchez-Gómez et al., 2010).

The highly toxic heavy metal cadmium ion (Cd²⁺) and metalloid-anion arsenite are widespread naturally-occurring and industrially-produced environmental contaminants (Mouliès, 2010). The toxic metals enter cells through non-selective or promiscuous cation transporters such as DMT1, which are biologically used for the import of metal ions. Following uptake, these transition metals can target proteins in multiple ways by: covalently binding free thiols, displacing
essential metal ions such as zinc, iron, and copper, and catalyzing oxidation through the generation of ROS, all of which interfere with the biological activity of proteins resulting in cell damage and death (Tamás et al., 2014). For cadmium in particular, a minimum concentration of 0.1 µM CdCl₂ is cytotoxic (Limaye and Shaikh, 1999). Furthermore, cadmium interference impacts a wide variety of signaling pathways that utilize ion sensing to transduce signals from the cell membrane and throughout the cell (Moulis, 2010). Acute resistance to cadmium exposure is primarily through downregulation of cation transporters, though cells eventually undergo apoptosis. Adaptation following long term exposure has been observed through the down regulation of apoptosis resulting in a quiescent state.
Figure 1.1 Conditions affecting protein thiols. Protein thiols can be modified through oxidation, metal chelation, and electrophilic adduction. Examples of thiol compounds that induce each modification are boxed.
Multiple studies have investigated the *in vivo* redox state of thiol-containing proteins. In 2006, Le Moan and colleagues used thiol-trapping and mass spectrometry techniques with two thiol-specific reagents to purify and quantify oxidized protein thiols (Le Moan *et al.*, 2006). The majority of oxidized protein thiols were cytoplasmic and functioned in carbohydrate and amino acid metabolism. Another study by Brandes and colleagues applied a mass spectrometry-based redox technique called OxICAT to quantify the steady state oxidation levels of approximately 400 protein thiols in *S. cerevisiae* and determine peroxide sensitivity (Brandes *et al.*, 2011). Analysis revealed that the majority of the proteome is reduced confirming that the yeast cytosol is maintained as a reducing environment. Proteins found to be significantly oxidized include those with known disulfide bonds (Sod1), those involved in disulfide exchange (thioredoxin reductase), glycolytic enzymes (GAPDH), and many not identified previously to contain oxidative thiols. Analysis of peroxide-treated cells identified 41 different proteins with stable hydrogen peroxide-mediated oxidation (disulfides or sulfenic). In agreement with the study conducted by Le Moan and coworkers, the majority are located and function within the cytoplasm. This study went even further and analyzed the oxidized thiol location within the protein secondary structure and its accessibility revealing the majority of cysteines are buried within the protein tertiary structure (Brandes *et al.*, 2011). In a more recent study, Brandes and colleagues determined the redox status of cysteine-containing proteins and why oxidation occurred in postmitotic aging cells using the same redox technique (Brandes *et al.*, 2013). Chronologically aged yeast undergo redox collapse in part due to a drop in reducing equivalents, resulting in oxidation of the proteome. Together, these studies found that cytoplasmic protein thiols are sensitive to oxidative stress and function in a variety of essential cellular processes—redox systems, energy metabolism, protein folding, and translation.
Redox Regulation of PQC

The chemical and functional properties of cysteines allow for rapid and reversible redox regulation of protein activity to sense and control diverse cellular states and processes. Furthermore, modifications by ROS and oxidants are known to play biological roles in sensing and regulation of activity of redox enzymes and transcriptional programs. Although xenobiotics are not involved in normal, steady state redox regulation, exposure could mimic endogenously found modifications and induce similar downstream signaling. It is well established that in the cytosol, oxidative stress (OS) is sensed through the Yap1 TF identified in yeast (Figure 1.2A) (Harshman et al., 1988; Morano et al., 2012). Yap1 contains six cysteine residues and under normal growth conditions these residues are in a reduced state which allows binding to trans-regulator Crm1 and nuclear export (Kuge et al. 1998, Yan et al. 1998). In oxidizing conditions via oxidants or loss of key thioredoxin system components, Yap1 undergoes oxidative protein folding with the help of glutathione peroxidase Gpx3 and the Yap1 binding protein Ypb1 preventing Crm1 binding and nuclear export (Delaunay et al., 2002; Veal et al., 2003; Wood et al., 2004). Yap1 can then activate the oxidative stress response (OSR) by inducing transcription of OS response genes.
Figure 1.2 Redox regulation of stress responses in the eukaryotic model budding yeast.

(A) Yap1-mediated redox regulation of the Oxidative Stress Response (OSR). Following oxidative or thiol-reactive stress, Yap1 undergoes oxidative re-arrangement through interactions with Gpx3 and Ypb1 allowing translocation into the nucleus and transcription of stress-protective genes.

(B) Redox regulation of the ER Unfolded Protein Response (UPR). Redox sensing through the Hsp70 BiP and its co-chaperone Sil1 as well as unfolded peptides triggers the UPR through Ire1-mediated activation of Hac1.

(C) Proposed mechanism of redox regulation of the Heat Shock Response (HSR) following oxidative or thiol-reactive stress. Modification of the Hsp70 Ssa1 cysteines in the nucleotide-binding domain (NBD) decreases Ssa1 interaction with Hsf1 activating domains leading to de-repression of the HSR and protection of redox sensitive substrates.
Unlike the OSR, the mechanism of Hsf1 activation following oxidation of the reducing environment is not entirely clear. Human Hsf1 contains two conserved cysteines in the DNA binding domain that form a reversible disulfide bond upon exposure to HS and OS (Ahn and Thiele, 2003). The authors found that C35 and C105 disulfide bond formation activates Hsf1 trimerization, nuclear localization, and target gene expression, indicating a direct sensing mechanism. This redox regulation of nuclear import is similar to what has been shown for the OSR TF Yap1. No known direct redox regulation has been identified for the yeast Hsf1 homolog as it lacks any cysteines, necessitating a partner protein(s) capable of sensing OS, possibly Ssa1 (see below). Transcript profiling experiments conducted in yeast have demonstrated that thiol-reactive compounds induce both the HSR and OSR, implying evolutionary pressure to mobilize the PQC network in response to these environmental insults (Trott et al., 2008; Wang et al., 2012).

Redox regulation of molecular chaperones has also been implicated in protein quality control and stress responses. Within the ER, the response to redox imbalance is well characterized and known as the unfolded protein response (UPR) (Figure 1.2B), which consists of multiple pathways extensively studied in yeast and mammalian cells (Walter and Ron, 2011). As proteins destined for secretion are synthesized across the ER membrane, the oxidative environment along with oxidoreductases and chaperones enable the formation of disulfide bonds necessary for proper protein folding. A switch to a reducing environment prevents disulfide bond formation resulting in protein misfolding and activation of the UPR. The ER-resident Hsp70 regulates UPR activation by binding directly to misfolded or unfolded polypeptides and downstream activators. Recent studies have found the Hsp70 Bip/Hsp78 and a nucleotide exchange factor Sil1 as a redox regulated pair to rapidly sense, respond, and recover from changes in the ER redox state (Xu et al., 2016; Siegenthaler et al., 2017). Following redox stress, BiP is oxidized in the nucleotide binding pocket resulting in loss of ATPase activity and a
functional switch to aggregation prevention exclusively. BiP oxidation was found to be reversible by Sil1 via an oxidoreductase-like motif.

A study from the Morano lab found that two of the three cysteine residues of the yeast cytosolic Hsp70 Ssa1 can be modified by an organic electrophile in vitro and are required for Hsf1 activation of the HSR in response to thiol compounds (Wang et al., 2012). Interestingly, the cysteines are not required for activation of the HSR when exposed to HS suggesting alternative mechanisms to regulate Ssa1 function in response to different stresses. The precise role the cysteines play in modulating the Ssa1-Hsf1 regulatory circuit remains to be established. Taken with what has been recently shown of the role of Ssa1 in Hsf1 regulation, one could speculate oxidation of the cysteine residues may result in Hsf1 de-repression due inactivation of one of Hsp70 functions (addressed above, Figure 1.2C). Thiol-mediated regulation of chaperones and the HSR is further supported by redox inactivation of Hsp90 chaperone activity suggesting a potential role for Hsf1 de-repression (Shen, et al. 2008, Beck et al. 2009). All in all, cytosolic Hsp70 may be a sensor for redox stress similar to the ER-specific Hsp70 BiP/Grp and mammalian Hsp90. Additionally, the novel antioxidant Tsa1 functionally switches to a molecular chaperone under hyperoxidative conditions. The peroxiredoxin is part of the Trx-fold superfamily of antioxidant proteins and forms dimers and small multimers localized throughout the cytosol (Jang et al. 2004). In hyperoxidative conditions, Tsa1 Cys residues are oxidized resulting in multimerization and a functional switch to a molecular chaperone. These large multimers localize to sites of aggregation in vivo in response to both HS and OS conditions and potentially prevent further protein aggregation (Weids and Grant 2014).

In addition to chaperones, studies have shown redox regulation of the proteasome and its two conformations. During OS, there is a preferential switch to primarily utilize Ub-independent proteasomal degradation of damaged proteins. Evidence suggests this is due to the increased sensitivity of the 19S regulatory subunit and Ub machinery to OS-induced modifications, such as glutathionylation, as compared to the 20S core (Jahngen-Hodge et al., 1997; Reinheckel et al., 1998). The majority of modifications lead to decreased activity. For example, the regulatory
subunits Rpn1 and Rpn2, which allow translocation of Ub-substrates into the 20S core, undergo glutathionylation following exposure to OS resulting in inhibition of 26S proteasome function (Zmijewski et al., 2009). On the other hand, a study has shown that glutathionylation of C76 and C221 of the 20S α5 subunit increased proteasomal activity by triggering and maintaining the core in an open conformation (Silva et al., 2012). Again, this evidence of redox regulation suggests that the Ub-independent machine is preferentially activated for degradation of oxidized proteins whereas the Ub-dependent iteration is inactivated. Furthermore, in human cells, the majority of oxidized proteins are not ubiquitinated with the exception of the molecular chaperones Hsp70, Hsp90, and Hsp60, which is a late consequence of OS; while the purpose of chaperone ubiquitination is unclear, it may help maintain appropriate chaperone levels and facilitate recovery from OS (Kästle et al., 2012).

Although there is some evidence regarding the impact of thiol stress on the PCQ machinery, little is known about how redox buffering contributes to protein folding in the cytosol. Previous studies from our lab began investigating the connection between OS and protein folding in the cytosol using S. cerevisiae as a model (Trott et al., 2008; Wang et al., 2012). Using an HSE-lacZ reporter fusion as a proxy for the activation of the HSR by Hsf1 (Morano and Thiele 1999), thiol-reactive compounds were found to induce the HSR suggesting potential endogenous protein misfolding in response to the unfavorable oxidative environment in addition to direct effects on Ssa1. Other studies focused specifically on the metals arsenic and cadmium show both to inhibit refolding of chemically denatured proteins in vitro and induce protein aggregation in live cells via the recruitment of Hsp104 to cytoplasmic foci (Sharma, et al. 2008, Jacobson et al. 2012, 2017). The metal ions are most likely forming polydentate rather than monodentate complexes with unfolded polypeptides inducing their aggregation either through metal complexation or hydrophobic interactions with neighboring unfolded peptides (Tamás et al., 2014). Whether HS-induced protein aggregation can be considered a thiol-stress is uncertain and requires ongoing investigation. Yeast lacking antioxidant enzymes such as superoxide dismutase are sensitive to HS while overexpression can provide protection (Davidson et al.,
Furthermore, prolonged heat stress increases the production of ROS generating primarily protein carbonyls at lysine, arginine, proline, and threonine residues (Berlett and Stadtman, 1997; Dalle-Donne et al., 2003; Verghese et al., 2012). These carbonyl derivatives have the potential to react with cysteine sulphydryls, thus, generating thiol-reactive stress.

**Impacts on Human Health**

Impairment of proteostasis, including the PQC machinery, stress response, and the accumulation of protein aggregates, is linked to diverse human diseases including diabetes, cancers, and, most notably, neurodegenerative disorders such as Alzheimer’s, Parkinson’s and amyotrophic lateral sclerosis (ALS) diseases (Hipp et al., 2014; Valastyan and Lindquist, 2014; Duennwald, 2015). Notably, the majority of these proteopathies are linked to age. In addition to PQC machinery malfunction and aggregation of known proteins, oxidative stress (OS) and metal dyshomeostasis have been implicated, suggesting that disruption of redox balance and, therefore, redox regulation, as a contributing factor to these diseases. Increased production of ROS in cancer cells due to “hypermetabolism” has been well established as a main pathological characteristic (Wang et al., 2019). The increasing generation of ROS is utilized as a sensor to alter metabolism via redox signaling and promote cell proliferation and survival. Additionally, oxidative damage via ROS-induced protein carbonylation has been observed in patients with the neurodegenerative disorder ALS (Matschke et al., 2019). This finding extends to other known neuro-proteopathies (discussed below) perhaps due to neurons high susceptibility to OS and its damage as result of high energy production and decreased levels of glutathione.

Metal toxicity and metal dyshomeostasis contribute to onset and pathology of the age-related neurodegenerative diseases Parkinson’s and Alzheimer’s (AD) by inducing oxidative stress and disrupting neurotransmission (Caudle et al., 2012; Greenough et al., 2013). In AD, the formation of extraneuronal aggregate plaques of the amyloid-beta (Aβ) metalloprotein is a hallmark of disease progression (Greenough et al. 2013). Additionally, these Aβ plaques contain a high level of metal ions. A proposed mechanism of Aβ oligomer neurotoxicity is the binding of
metal ions induces aggregation and ROS production. This supports that maintaining the redox balance is important for normal cell function and disease states associated with impaired protein homeostasis.

Outstanding Questions

To better understand proteotoxicity caused by thiol-reactive stress disruption of redox balance, I sought to identify endogenous yeast proteins whose folding and/or stability is negatively impacted by different modes of thiol stress resulting in protein aggregation. The majority of studies in the field utilize chaperones as proxies for endogenous protein aggregation. Though useful in monitoring proteostasis, this doesn’t provide insight into the number or stress-specificity of endogenous protein targets. Proteomic studies using mass spectrometry-based identification of aggregated proteins in response to HS has provided targets to characterize; however, the connection between redox-sensitive proteins and stress-induced protein aggregation has yet to be made. Following identification of endogenous redox-active and folding-sensitive model, I aim to establish a redox-influenced mechanism of protein aggregation through modification of cysteine thiols. This will drive home the relationship between thiol-reactivity and protein misfolding and aggregation. Investigation into the dynamics of molecular chaperones and redox systems with redox-sensitive protein aggregates will give insights into their role in formation and potential clearance. Finally, I aimed to determine whether thiol-stress proteotoxicity established in yeast was conserved in human cells. Additionally, I aimed to provide insight into how folding proteopathies may arise by examining how thiol stress impacts disease-associated alleles of the model protein established in yeast.
Chapter II: Materials and Methods

Note: This chapter was derived from work published by Amy Eileen Ford.

Strains and Plasmids

Yeast — All yeast genomic GFP-protein fusion strains used in this study were obtained from the genomic GFP-protein fusion library (Table 2.1, Invitrogen, Howson et al., 2005). To construct cysteine substitution mutants of Tpi1 (C41A, C126A, and C41A C126A), the pTPI1-TPI-GFP::HISMX6 sequence was amplified using PCR from genomic DNA of the Tpi1-GFP strain and cloned using homologous recombination into the yeast expression vector pRS416. Cysteine substitution to alanine mutations were introduced into the p416TPI-tpi1-GFP vector using the QuickChange Lightning Site-directed Mutagenesis kit and protocol (Agilent). Mutants were verified by DNA sequencing. To construct mutant alleles of Tpi1 associated with TPI Deficiency, tpi1 gene fragments coding for the amino acid substitutions C41Y, G122R, or I170V were synthesized (Genewiz) and amplified by plasmids given with homology to tpi1 and GFP::HISMX6. Using PCR overlap techniques, mutant tpi1-GFP::HISMX6 products were generated. All mutant tpi1-GFP::HISMX6 alleles were amplified from plasmids by PCR and inserted into the BY4741 (MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0) parent strain using homologous recombination. Integration of mutants was confirmed by DNA sequencing. Yeast expression vectors used in this study are the following: p416TEF-GFP, pSSA3-lacZ (Wang et al., 2012a), p416TEF-mTagBFP2, and p416TEF-tpi1-mTagBFP2 (Table 2.2). The GFP and mTagBFP2 fluorescent protein tags were amplified from the pFA6a-link-yomTagBFP2 (Addgene, Lee et al., 2013) and p416CUP1-GFP (Abrams et al., 2014) plasmids by PCR and cloned into the p416TEF vector. All plasmids were constructed using standard restriction enzyme-based cloning methods. The following mammalian expression vectors were used in this study: pCMV-EGFP-C1 and pCMV-EYFP-N1 (Clontech), pCMV-Hsp70-EYFP (Kim et al., 2002), pCMV-EGFP-hTPI, pHSE-Luc (Clontech), and pCMV-RL (Clontech) (Table 2.2). The pCMV-EGFP-hTPI plasmid was constructed by amplifying the human TPI coding sequence (hTPI) from prepared cDNA (HeLa) and cloning into the pCMV-EGFP-C1 vector. All plasmid constructs were verified by enzyme digestion and DNA sequencing.
Human cells — The human cell lines HEK293 (transformed human embryonic kidney cells, ATCC) and HCT116 (human colorectal carcinoma cells, ATCC) were used in this study (Table 2.1). Cells were seeded at 150,000-250,000 cells/well in 6-well tissue culture-treated plates and incubated and incubated at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) high glucose (Hyclone) supplemented with 5% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin. At 50% confluence, cells were transfected with the desired expression vector using JetPrime transfection reagent according to the manufacturer's optimized protocol for each cell line (Polyplus Transfection).
<table>
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Cell Growth Conditions and Treatments

Yeast — All strains used were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) liquid growth medium at 30°C with aeration to logarithmic phase (OD<sub>600</sub> 0.4-0.8). For thiol-stress treatment, strains were incubated with 2.5 mM diamide (dia), 2 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), or 500 μM diethyl maleate (DEM) for 1 h. For cadmium specific treatments, strains were incubated with CdCl<sub>2</sub> (25-1,000 μM) for 0-3 h. For HS, strains were incubated in a 42°C water bath for 15 min. For cycloheximide chase experiment, strains were incubated with 0.1 mg/mL cycloheximide for 15 min prior to addition of 100 μM CdCl<sub>2</sub> or 2 mM dia for 1 h or 42°C HS for 15 min. For glucose starvation/chronological aging, cells were incubated in a 30°C shaking incubator for 4 days and supplemented with 2% dextrose on day 4 for 1 hr. For pH manipulation experiment, stains were grown to logarithmic phase in YPD rich medium, washed, and resuspended in pH 5.0 phosphate buffer. After resuspension, 2 mM 2,4-dinitrophenol (DNP) was added and cells were incubated at 30°C for 1 h. After DNP exposure, cells were washed and resuspended in YPD medium to recover for 1 h at 30°C. For yeast spot assays, serial dilutions of each strain were made (10<sup>-1</sup>-10<sup>-5</sup>) and plated on YPD medium using a 96-well plate and metal pronged grid. Plates were incubated at 30°C and then imaged after 3 days. For liquid growth assays, strains used were grown in YPD-rich medium to logarithmic phase (OD<sub>600</sub> ~0.6) and normalized to an OD<sub>600</sub> of 0.5. Absorbance at 600 nm was measured over 16 h in 15 min intervals using a 96-well format in a plate reader (BioTek Synergy Mx). The OD<sub>600</sub> at each time point was calculated by subtracting the absorbance of the medium alone from each sample.

Human cells — The HEK293 and HCT116 cell lines were seeded at 150,000-250,000 cells/well in 6-well tissue culture-treated plates and incubated in standard growth medium (DMEM high glucose, 5% FBS, and penicillin-streptomycin) at 37°C 5% CO<sub>2</sub>. For heat shock treatment, 60%-80% confluent plates were incubated on a metal plated shelf at 43°C 5% CO<sub>2</sub> for 1 h. For cadmium treatment, 60%-80% confluent plates were incubated at 37°C 5% CO<sub>2</sub> with 50 μM CdCl<sub>2</sub> for 6 h.
**Protein Expression**

Yeast — Cell lysates were obtained by lysing 6 OD units of cells using the glass bead cell lysis procedure (Abrams et al., 2014). The protein concentration of protein was determined by the Bradford assay (Bio-Rad) in a microplate reader (Synergy Mx, Biotek). Protein samples were boiled in SDS-PAGE sample loading buffer and stored at -20°C. Equal protein concentrations of each sample were loaded onto 12% bis-acrylamide/SDS gel and separated by gel electrophoresis. Separated protein samples were transferred to a PVDF membrane and blotted with monoclonal α-GFP (Roche) to detect the GFP fusion protein. α-PGK (Invitrogen) was used as a loading control.

Human cell lines — HEK293 and HCT116 cell lines expressing the desired vectors were detached from dishes using trypsin, washed with PBS, and lysed in RIPA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, and PIC) for 15 min on ice. Lysate were cleared by centrifugation at 15,000 rpm at 4°C for 10 min. The concentration of protein was calculated with the BCA assay (Bio-Rad). Equal concentrations of each sample were loaded onto 12% bis-acrylamide/SDS gel and separated by gel electrophoresis. Separated protein samples were transferred to a PVDF membrane and blotted with monoclonal α-GFP (Roche) to detect the FP fusion protein. α-GAPDH (Santa Cruz Biotechnology) was used as a loading control.

**Fluorescence Microscopy**

Yeast Live Cell Imaging — For all experiments, live cells were wet mounted onto glass slides and imaged immediately using an Olympus IX81 microscope at 100X magnification with FITC filters to detect GFP and a standard UV/DAPI filter to detect BFP2.

Fixed Human Cell Imaging — HEK293 and HCT116 cell lines were seeded onto glass coverslips in tissue culture-treated 6-well plates. The following day cells were transfected with desired expression vectors. On day 3, cells were exposed to the conditions described above, fixed onto coverslips using 4% paraformaldehyde in PBS, incubated with Hoescht/DAPI stain for
nuclear DNA detection, and mounted onto glass slides using VectaShield mounting medium. For each cell type and experimental growth condition, slides were imaged at 63X magnification in multiple Z-planes using a Zeiss Axioskop40 microscope. Appropriate filter sets were used as above.

**Cryo-lysis and Differential Centrifugation**

This method of cell lysis and fractionation was adapted from Wallace et al., 2015. Approximately 18 OD units of cells were incubated in YPD medium, with or without 750 µM CdCl₂, for 1 h at 30°C. Treated cells were collected, washed, re-suspended in 100 µL of lysis buffer (20 mM Tris-HCl [pH 7.9], 0.5 mM EDTA, 10% glycerol, 50 mM NaCl, and protease inhibitor cocktail (PIC), and flash frozen in liquid nitrogen. Glass beads were added to frozen cell pellets and switched from vortex to dry ice for 1 min intervals six times. Following lysis, 200 µL of lysis buffer was added to each sample, the samples were then thawed on ice, and cellular debris was separated from the cell lysate by slow spinning at 3,000 x g at 4°C for 30 s.

Extracted total protein lysate was separated into soluble and insoluble fractions by ultracentrifugation at 100,000 x g at 4°C for 20 min. The supernatant (soluble) fraction was transferred to a new microcentrifuge tube. The insoluble fraction pellets were washed with lysis buffer, centrifuged again at 100,000 x g 4°C for 20 min, and decanted. Washed pellets were sonicated in 50 µL of insoluble protein buffer (8 M urea, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 2% SDS, and PIC) for 10 min in an ambient-temperature water bath sonicator and centrifuged at 20,000 x g at ambient temperature for 5 min. Both soluble and insoluble fractions were boiled in SDS-PAGE sample loading buffer and stored at -20°C.

**β-glo Assay**

BY4741 strain expressing the pSSA3-lacZ plasmid were grown to exponential phase (OD₆₀₀ 0.4-0.8) in liquid YPD medium and incubated in the following conditions for 1 h at 30°C: no treatment, 100 µM CdCl₂, 50 µM dithiothreitol (DTT), 50 µM DDT + 100 µM CdCl₂, or 2.5 mM
dia. After treatment, 50 µL of each are added to 50 µL of β-glo reagent (Promega) set up in a solid white 96-well plate and β-galactosidase activity is measured by luminescence in a microplate reader (Biotek Synergy Mx) using suggested protocol.

qRT-PCR

HEK293 and HCT116 cells were seeded at 150,000-250,000 cells/well in 6-well tissue culture-treated plates containing standard growth medium. At 50%-70% confluence, cells were incubated in the growth conditions described above. All treatments were recovered for 6 hr at 37˚C 5% CO₂. Following treatment, cells were detached from the dish using trypsin, washed with PBS, and frozen on dry ice. Frozen cell pellets were stored at -80˚C until processing.

RNA was extracted from treated cell pellets using the TRIZOL reagent (Thermo Fisher). The RNA concentration was calculated using absorbance at 260 nm (peak), 280 nm (ratio), and 320 nm (reference) determined using a nanodrop plate reader (Synergy Mx, Biotek), and 10-20 µg of each sample was treated with Turbo DNase (Invitrogen). Following DNase treatment, RNA was purified by phenol/chloroform extraction, the concentration was calculated as before, and 1 µg of RNA was converted to cDNA using the iScript cDNA synthesis kit and protocol (Bio-Rad). mRNA expression of the Hsf1-induced genes HSPA1A (HSP70) and DNAJ1A (HSP40) were measured by quantitative, real-time PCR using iTaq Universal SYBR Green Supermix (Bio-Rad) and calculated using standard methods (Nolan et al., 2006). mRNA expression of GAPDH was used as a normalization control.

Dual-luciferase Reporter Assay

On day 1, HEK293 and HCT116 cells were seeded at 70,000/40,000 cells/well in 24-well tissue culture-treated plates containing standard growth medium. On day 2, cells were transfected with 0.025/0.05 µg of pCMV-RL and 0.25/0.5 µg of pHSE-Luc (FFL) using JetPrime transfection reagent according to the manufacturer’s protocol (Polyplus Transfection). On day 3, cells were incubated in the growth conditions described above. All treatments were recovered
for 6 h at 37°C 5% CO₂. Following treatment, cells were lysed by rocking at ambient temperature for 15 min using lysis buffer from Dual-Luciferase Reporter kit (Promega). Cell lysate was clarified using a short spin and stored at -80°C.

To measure luciferase activity, 20 µL of lysate from each cell type and condition was added to a 96-well plate and luminescence was read following addition of assay reagents (Promega) using a plate reader and dispenser apparatus (BioRad Synergy Mx). FFL activity was quantitated by normalizing to RL activity.

**Statistical Analysis**

Statistical significance was determined using an unpaired Student’s t-test with Welch’s correction performed using QuickCalcs and Prism (version 7.0c, GraphPad Software, Inc.).
Chapter III: Identification of Redox Sensitive, Aggregation Prone Proteins

Note: This chapter was derived from work published by Amy Eileen Ford.
Introduction

Proteomic studies investigating the redox status of proteins found that the oxidation state of Cys-containing proteins increased when exposed to an oxidative environment through environmental or genetic means (Brandes et al., 2011; Brandes et al., 2013; Le Moan et al., 2006). Each of these studies found that cytoplasmic protein thiols are most sensitive to oxidative stress and function in a variety of essential cellular processes—redox systems, energy metabolism, protein folding, and translation. However, it remains unclear whether redox status directly impacts endogenous protein folding and stability. Yanyu Wang, a previous graduate student in the Morano Laboratory, began investigating the connection between OS and protein folding in the cytosol using *S. cerevisiae* as a model (Wang et al. 2012). Using an HSE-lacZ reporter fusion as a proxy for the activation of the HSR by Hsf1 (Morano and Thiele 1999), thiol-reactive compounds, including cadmium, hydrogen peroxide, and diethyl maleate, were found to induce the HSR suggesting potential endogenous protein misfolding in response to the unfavorable folding environment. Each of the thiol-modifiers activated the HSR to varying degrees. In particular, the heavy metal cadmium (Cd) resulted in the strongest response with a ten-fold Hsf1 induction compared to hydrogen peroxide at only four-fold (Wang et al., 2012). Furthermore, the authors found the two solvent exposed cysteines of Ssa1 played a role in this thiol stress-mediated Hsf1 activation, albeit through an unknown mechanism. Therefore, we hypothesized changes in cytosolic redox balance via thiol-specific stress are proteotoxic resulting in endogenous protein misfolding and aggregation.

Visualization of protein misfolding and aggregation in live cells by fluorescence microscopy through the use of fluorescent protein tags is a widely used and established method in the field (Jacobson et al., 2012; Miller et al., 2015). In this chapter, I utilized the commercially available yeast genomic ORF-GFP collection (Howson et al., 2005) to screen a selection of redox sensitive proteins for aggregation in response to thiol-reactive stresses that induce the HSR. Additionally, I developed an alternative method to visualize aggregation via differential centrifugation and Western blot analysis.
Results

Identification of thiol stress-sensitive, aggregation-prone proteins

Given the recognized propensity of thiol compounds to interact with reactive thiol groups, we took advantage of previously published studies that identified *S. cerevisiae* proteins with reactive cysteine residues (Le Moan *et al.*, 2006; Brandes *et al.*, 2011, 2013). To focus our study, I limited my selections to abundant, nuclear/cytoplasmic proteins to facilitate surveillance via fluorescence microscopy. In addition to ten candidates, I included the peroxiredoxin Tsa1, previously shown to localize to cytoplasmic foci upon thiol oxidative stress (e.g., H$_2$O$_2$) and a strain bearing GFP alone (Jang *et al.*, 2004; Weids and Grant, 2014). Strains were visualized by live cell fluorescence microscopy following treatment with the following thiol-reactive compounds for 1 h: 100 µM CdCl$_2$, 2 mM H$_2$O$_2$, and 500 µM DEM (Figure 3.1, 3.2A,B). As expected, Tsa1-GFP formed multiple small foci following cadmium and hydrogen peroxide treatment, confirming that the concentration of each is perceived as a cellular thiol stress (Weids and Grant, 2014). In contrast, the translation factor Eft1-GFP and eight other candidates exhibited a diffuse distribution with no significant change of localization pattern upon thiol stress treatment. However, the glycolytic enzyme triose phosphate isomerase (Tpi1-GFP) exhibited approximately 80% foci formation following cadmium treatment in the cells observed. Though it appears Tdh3 and Pdc1 form foci, the two structures—one small cytoplasmic focus and one larger, potentially nuclear focus—are present in non-stress conditions and this pattern is unchanged following cadmium exposure. Neither the control Tsa1-GFP nor any of the nine candidates were observed to respond to DEM treatment. Importantly, GFP alone did not form foci, indicating that the fluorescent protein tag itself does not aggregate in the presence of thiol-stress and is unlikely to confer aggregation to Tpi1 (Figure 3.2A,B). In short, I have identified Tpi1 as a candidate model endogenous protein that is aggregation sensitive to the thiol stress cadmium.
To confirm conditions used in this study activate the HSR as shown previously, we expressed β-galactosidase under the control of a HSE-containing promoter (pSSA3-lacZ) in untagged BY4741 cells and measured enzymatic activity as a proxy for induction of the HSR following exposure to the thiol-stress cadmium (Figure 3.2C). In agreement with foci formation of Tpi1-GFP and the control Tsa1-GFP, the concentrations of cadmium used in the screen activated the HSR.
Figure 3.1 Identification of redox active, folding sensitive proteins

(A,B) Exponentially grown cells bearing genomic GFP-protein fusions of candidates were visualized by live cell fluorescence microscopy under non-stress conditions (NS) and following 1 h treatment with: 100 μM CdCl$_2$ (Cd), 2 mM H$_2$O$_2$, and 500 μM diethyl maleate (DEM). Scale bar; 10 μm.
Figure 3.2 Tpi1-GFP aggregates in response to cadmium. (A) Exponentially grown cells expressing GFP alone on a plasmid were visualized by live cell fluorescence microscopy in non-stress (NS) and following treatment with 100 µM CdCl₂ for 1 h. Scale; 10 µm. (B) Quantitation of GFP-fusions of Tpi1, Tsa1, and Eft1 and GFP alone for NS and Cd showing the mean percentage of total cells exhibiting foci (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD). ns, not significant, *, p<0.05, **, p<0.001. (C) BY4741 expressing pSSA4-lacZ was incubated at 30°C for 1 h with (+) or without (-) 100 µM CdCl₂ (Cd). Average β-galactosidase activity was measured and normalized to NS (n=3). Error bars indicate standard deviation (SD).
Isolating Cd-induced aggregates

To confirm that the observed foci represented insoluble protein aggregates, I developed and adapted lysis and fractionation protocols from previously published methods using a thermal labile proxy protein firefly luciferase (FFL) fused to GFP and HS to generate FFL aggregates (Abrams and Morano, 2013; Ibstedt et al., 2014; Wallace et al., 2015). FFL is extremely labile and will aggregate after exposure to a mild heat shock of 37°C whereas the GFP tag retains its structure allowing for visualization of FFL aggregates. Typically, these methods utilize a standard glass bead cell lysis protocol followed by sequential high speed centrifugations at 15,000 x g to separate soluble and insoluble protein fractions, which are resolved with the denaturant sodium dodecyl sulfate. Following separation of fractions, I determined the presence or absence of FFL-GFP in the soluble and insoluble fractions using Western blot analysis. Initial testing of the protocol yielded high levels of FFL-GFP in the insoluble fraction even following incubation in optimal conditions at 30°C (Figure 3.3A). This suggested that solubility of proteins may be impacted by the lysis and/or fractionation procedure. Recently, a study focused on identifying heat-induced endogenous aggregation found cryogenic lysis followed by ultracentrifugation at 100,000 x g with a final solubilization of aggregate protein in the stronger denaturant urea, yielded a better in vivo representation of aggregation with little procedural artifacts (Wallace et al., 2015). After modifying the protocol to account for these considerations, the level of FFL-GFP in the insoluble fraction isolated from cells grown in optimal conditions was reduced (Figure 3.3B). Therefore, the cryogenic cell lysis likely prevented aggregation due to the heat generated by the glass bead cell lysis procedure and allowed for better resolution between the soluble and insoluble fractions.
Next, I applied this new method to the thiol-stress folding sensitive protein Tpi1 identified in the above screen. Cells expressing Tpi1-GFP or GFP alone were lysed following a 1 h treatment with 750 µM CdCl₂ and supernatant and pellet fractions were obtained as described in Materials and Methods. Protein fractionation was determined using Western blot analysis (Figure 3.3C). Similar to what I observed by fluorescence microscopy, GFP alone was found nearly exclusively in the soluble fraction in both non-stress and cadmium treatment conditions. In contrast, I observed approximately 40% fractionation of Tpi1-GFP into an insoluble state in the absence of stress, and a further 20% shift following cadmium treatment as determined by densitometry (Figure 3.3D). Despite still producing likely heat-induced aggregation via the cell lysis procedure, these data suggest a fifth of the total available Tpi1 is aggregation sensitive to cadmium exposure.
Figure 3.3 Small fraction of Tpi1-GFP shifts from soluble to insoluble following Cd exposure. (A,B) Western blot analysis of soluble (S) and insoluble (P) protein fractions from cells expressing FFL-GFP exposed to no stress (-), 42°C (A) or 50°C (B) HS (+) for 15 min. S and P fractions were obtained by method described in text. α-GFP monoclonal antibody was used to detect FFL-GFP and a monoclonal α-PGK antibody was used to identify the soluble protein phosphoglycerate kinase as both a fractionation and load control. (C) Western blot analysis of soluble (S) and insoluble (P) protein fractions from cells expressing Tpi1-GFP or GFP alone exposed to no (-) or 750 µM CdCl₂ (+) for 1 h. S and P fractions were obtained using cryo-lysis and differential centrifugation as described in Materials and Methods. α-GFP was used to detect Tpi1-GFP and GFP and α-PGK was used to identify the soluble protein as both a fractionation and load control. (D) The mean percentage of P fraction of total (S+P) in non-stress (NS, -) and Cd (+) stress conditions from (C) was quantitated using densitometry (n=3). Error bars indicate standard deviation (SD). ns, not significant, *, p<0.05.
Discussion

Unlike previous studies that focused solely on chaperone localization, I have found that an endogenous redox sensitive protein, Tpi1, aggregates in response to the heavy metal cadmium. These data connect thiol-redox status identified by proteomic studies with stress-induced protein aggregation established in response to HS. My initial screen only revealed one of the 10 candidates as aggregation prone suggesting that misfolding and aggregation of proteins with reactive cysteines is, perhaps, a rare consequence of thiol stress, despite the previously documented recruitment of Tsa1 and Hsp104 to aggregates in response to cadmium or H₂O₂ treatment (Hanzén et al., 2016; Jacobson et al., 2017). However, based on a proteomic study, only 20% of 290 cysteine-containing proteins detected have an oxidation state greater than 25% (Brandes et al., 2011). Furthermore, only 41 of the 290 are sensitive to changes in redox environment by exogenous peroxide exposure. These data support the conclusion that cytosolic thiol-dependent misfolding and aggregation might be a rare phenomenon. This is in contrast to the parallel scenario in the endoplasmic reticulum, where the majority of resident and secreted proteins contain one or more disulfide bonds that when disrupted genetically or pharmacologically result in severe misfolding and overall proteotoxicity (Walter and Ron, 2011). Perhaps, oxidation by hydrogen peroxide is too transient and quickly reversed by the abundant presence of thioredoxin and Tsa1, thus, Tpi1 aggregation could not occur in the time frame of an hour. Prolonged exposure might overload the thioredoxin system and allow visible detection of aggregation. In the case of DEM exposure, protein alkylation via its monofunctional electrophilic center might not induce a high level of toxicity, despite activation of the HSR. West and coworkers have shown bifunctional electrophiles such as diethyl acetylenedicarboxylate are more potent due to their ability to cross-link proteins and have enhanced toxicity in comparison to monofunctional electrophiles like DEM (West et al., 2011).
Additionally, I successfully developed a method that not only provides another way to show aggregate formation following stress further confirming the Tpi1 foci observed are aggregates, but provides a means to identify redox sensitive proteins via a future proteomic approach. Only a small percentage of fractionated into the insoluble pellet following cadmium exposure, indicating not all of Tpi1 is sensitive to thiol stress. Based on a recent study using Hsp104 to track cadmium-induced aggregation, I suspect this fraction accounts for the newly synthesized Tpi1. This makes sense as Tpi1 possesses two cysteines that are buried within the protein tertiary structure when in the folded state and are only solvent accessible when unfolded such as during synthesis.
Chapter IV: Characterization of Tpi1 Folding Sensitivity

Note: This chapter was derived from work published by Amy Eileen Ford.

Introduction

The heavy metal cadmium reacts with proteins by chelating reactive cysteine sulfhydryl groups forming a strong covalent interaction (Tamás et al., 2014). This thiol interaction induces protein aggregation through either promotion of aberrant interactions of exposed hydrophobic peptide regions or forming multidentate complexes with neighboring cadmium-bound peptides. A previous study using the chaperone Hsp104 to track aggregation found that cadmium induces aggregation of nascent polypeptides which are subsequently degraded via the proteasome (Jacobson et al., 2017). However, an endogenous target was not identified. Initial screening of GFP-protein fusions identified Tpi1 as folding-sensitive to the thiol-stress cadmium (refer to Chapter III).

In addition to metal chelation, there are multiple classes of thiol-reactive stress including oxidation by ROS, peroxides, or the thiol-specific oxidant diamide, and thiol adducts by organic electrophiles such as diethyl maleate. In contrast to chelation, oxidation is generally more transient and resolved readily by the thioredoxin system (Poole, 2015). Each of these different modes of thiol stress have been shown to induce the HSR, suggesting protein misfolding perhaps due to thiol modification (Wang et al., 2012). Furthermore, previous work found co-incubation of cells with the thiol-reactive compound celastrol and the reducing agent dithiothreitol (DTT) inhibited induction of the HSR suggesting thiol-reactivity based protein misfolding (Trott et al., 2008). Unlike these known thiol stressors, the primary mechanism of HS misfolding is due to thermal denaturing of protein; however, some consequences of HS may be further attributed to thiol stress as shown by induction of the OSR and ROS production (Davidson et al., 1996; Chen et al., 2009). Therefore, I hypothesized thiol-reactive stress is proteotoxic and induces thiol-dependent aggregation.

Oxidative protein damage is a known physiological consequence of aging, however, whether the accumulation of ROS is the cause of this damage was uncertain (Muller et al., 2007). Brandes and colleagues investigated this theory by monitory the redox status of thiol-containing proteome in chronological aging yeast (Brandes et al., 2013). The authors found that not ROS
accumulation but a loss of reducing equivalents leads to redox collapse and oxidation of protein thiols. Reducing equivalents such as NADH⁺ are produced by the consumption of glucose and are utilized by the thioredoxin system to maintain a reducing cytosolic environment (López-Mirabal and Winther, 2008). Thus, I hypothesized chronological age with glucose depletion over time is a thiol-reactive stress.

In this chapter, I further characterized Tpi1 sensitivity to cadmium and establish a mechanism of aggregation. Additionally, I determine folding sensitivity to other modes of thiol stress or redox imbalance including OS and glucose starvation in chronologically aged cells.

**Results**

*Cadmium-Induced Aggregation*

To further characterize the effects of cadmium-based thiol stress on localization of Tpi1, I incubated the strain bearing Tpi1-GFP for 1 h in increasing concentrations of CdCl₂ (0-1,000 µM) and visualized aggregate formation by fluorescence microscopy (*Figure 4.1*). Aggregate formation was observed at 25 µM CdCl₂ and both the total fraction of cells with foci and the number of foci per cell increased in a dose-dependent manner. The frequency of foci formation plateaued at 750 µM CdCl₂, but even at this concentration the majority of Tpi1-GFP signal remained diffuse.
Figure 4.1 Cd folding sensitivity is dose-dependent. (A) Exponentially grown Tpi1-GFP was incubated in increasing concentrations of Cd as indicated for 1 h and visualized by live cell fluorescence microscopy. Representative images of cells exposed to 0, 25, 100, and 750 µM Cd are shown. Scale; 10 µm. (B) Quantitation of the mean percentage of total cells from (A) exhibiting foci (n=3). Inset shows gradual increase in foci number at lower Cd concentrations. (C) Quantitation of the relative percentage of cells from (A) with 1, 2, 3, or 4+ foci per cell following exposure to 25, 100, and 750 µM Cd. Error bars indicate standard deviation (SD).
Interestingly, only a fraction of Tpi1-GFP appeared to aggregate and form foci, while the remainder was diffuse throughout the cytosol (Figure 3.1A). This is in contrast to Tsa1-GFP, which appeared to wholly transition into discrete foci upon stress. It was observed previously that cadmium-dependent recruitment of Hsp104-GFP to aggregates/foci was blocked by pre-treatment of cells with cycloheximide to inhibit protein synthesis, demonstrating that cadmium selectively affects nascent proteins (Jacobson et al., 2017). I therefore treated Tpi1-GFP, Tsa1-GFP, and Hsp104-GFP cells with 100 µM CdCl₂ for 1 h after a 15 min incubation with or without 0.1 mg/mL cycloheximide (Figure 4.2). Treatment with cycloheximide alone had no effect on Tpi1-GFP, but completely inhibited Tpi1-GFP aggregation by cadmium. This data is in agreement with the inhibition of cadmium-induced Hsp104-GFP foci shown here and in a previous study (Jacobson et al., 2017). Interestingly, Tsa1-GFP forms foci despite cycloheximide pre-treatment indicating the response of the thiol-specific chaperone is unaffected. These findings demonstrate that overall cadmium proteotoxicity, as evidenced by Hsp104 behavior, as well as folding inhibition of Tpi1 specifically, occur solely with nascent polypeptides. This model also explains our observations that a significant fraction of presumably folded and stable Tpi1-GFP remains soluble in the presence of cadmium (Figure 3.3C,D).
Figure 4.2 Cd targets nascent polypeptides of Tpi1-GFP. (A) Exponentially grown cells bearing Tpi1-GFP, Tsa1-GFP, or Hsp104-GFP were visualized by live cell fluorescence microscopy after no stress (NS), 15 min cycloheximide (CHX), 1 h 100 µM CdCl₂ (Cd), and 15 min pre-treatment with CHX followed by 1 h 100 µM CdCl₂ (CHX → Cd). Representative images for each are shown. Scale bar, 10 µm. (B) Quantitation of the mean percentage of total cells with foci (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD). ns, not significant, **, p<0.001.
I next determined the kinetics of Tpi1-GFP aggregate formation and persistence. Formation of Tpi1-GFP aggregates after the addition of 100 µM CdCl₂ was monitored over the course of 3 h at 30 min intervals. Discernable foci were detected within 30 min and nearly all cells observed contained aggregates at 1 h (Figure 4.3A-C). Subsequently, the fraction of total cells with aggregates and the number of aggregates per cell diminished and were mostly cleared by 3 hr. These data indicate a rapid response to cadmium exposure, followed by slower clearance of the aggregates, suggesting adaption to the presence of the metal. In addition, I monitored the fate of cadmium-induced Tpi1-GFP aggregates after cadmium washout by visualizing cells exposed to 100 µM CdCl₂ for 1 h and then recovered in rich medium lacking cadmium (Figure 4.4A,B). In contrast to aggregate dynamics in the presence of cadmium, Tpi1-GFP was completely diffuse throughout the cytosol within the hour, suggesting a rapid full recovery likely due to aggregate clearance.
Figure 4.3 Aggregate formation and adaption during Cd exposure. (A) Exponentially grown cells bearing Tpi1-GFP were incubated with 100 µM CdCl₂ and culture samples visualized by live cell fluorescence microscopy at 30 min intervals for 3 h. Representative images for the indicated time points are shown. Scale bar is 10 µm. (B) Quantitation of the mean percentage of total cells with foci from (A) at each time point (n=3). (C) Quantitation of the relative percentage of cells from (A) with 1, 2, 3, or 4+ foci per cell following Cd exposure at the indicated timepoints (n=3). Error bars indicate standard deviation (SD).
Figure 4.4 Tpi1-GFP aggregates are rapidly cleared upon removal of stress. (A) Exponentially grown cells bearing Tpi1-GFP were incubated in the presence of 100 µM CdCl$_2$ for 1 h to form aggregates, then transferred to medium lacking Cd for an additional 1 h. Representative images at the indicate 0, 30, and 60 min recovery timepoints are shown. Scale bar; 10 µm. (B) Quantitation of the mean percentage of total cells with foci following recovery (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD).
A key feature of cadmium is its propensity to chelate exposed sulfhydryl groups. I therefore postulated that the aggregation dynamics I observed for Tpi1 were due to interactions with at least one of the cysteines previously found to be subject to oxidation (Le Moan et al., 2006). The yeast Tpi1 enzyme is an active homodimer and contains two cysteine residues at positions 41 and 126 that are likely protected in the folded, native conformation (Figure 4.5A, Lolis et al., 1990). C126 was shown to undergo significant oxidation following exposure to hydrogen peroxide or in a thioredoxin system deletion background (Le Moan et al., 2006). To determine whether the observed cadmium-induced aggregation is dependent on the presence of these cysteines, I made single and double alanine substitutions in the genomic GFP-protein fusion (C41A, C126A, and C41A C126A) (Figure 4.5B). Loss of a functional Tpi1 (tpi1Δ) causes a growth defect in media containing glucose as the sole carbon source indicating Tpi1 is essential for growth on glucose alone (Compagno et al., 2001). Expression and stability of all three cysteine mutants was comparable to that of wild type and had no detectable effects on growth on glucose (Figure 4.5C-E).

Under non-stress conditions, all three mutants were localized diffusely throughout the cytosol, similar to wild type Tpi1-GFP, in agreement with our protein expression and cell viability data (Figure 4.6A, B). Following 1 h of treatment with 100 µM CdCl₂, Tpi1-GFP bearing either the single cysteine substitution aggregated at a level indistinguishable from wild type. Strikingly, Tpi1-GFP C41A C126A failed to aggregate under the same conditions suggesting __.

As another method to determine cysteine-dependent protein misfolding, I incubated BY4741 expressing the pSSA3-lacZ reporter with 50 µM dithiothreitol (DTT) or 100 µM CdCl₂ alone or together for 1 h and measured β-galactosidase activity as a proxy for HSR activation (Figure 4.6C). As expected, DTT alone did not induce the HSR whereas cadmium did; however, simultaneous treatment with both cadmium and DTT resulted in reduced activity. These observations support my hypothesis that cadmium proteotoxicity is a thiol-dependent process and indicate that both cysteines must be present to confer folding sensitivity to Tpi1.
Figure 4.5 Cysteine mutagenesis of Tpi1-GFP does not adversely alter protein expression or growth on glucose. (A) Ribbon structure of the yeast Tpi1 homodimer with cysteines 41 and 126 indicated (orange) in each monomer (PDB: 1YPI). (B) Location of engineered alanine substitutions in chromosomal TPI1-GFP allele. (C) Western blot analysis of protein expression of Tpi1-GFP WT, C41A, C126A, and C41A C126A. α-GFP was used to identify the protein-GFP fusion and α-PGK was used as a load control. (D) Cell viability of Tpi1-GFP WT, C41A, C126A, and C41A C126A as determined by spot dilution assay. Wedge indicates serial 10-fold culture dilutions. (E) Cell viability of Tpi1-GFP WT, C41A, C126A, and C41A C126A as determined by liquid growth assay (n=2).
Figure 4.6 Cd-induced Tpi1-GFP aggregation is thiol-dependent. (A) Exponentially grown cells bearing Tpi1-GFP WT, C41A, C126A, or C41A C126A were incubated in non-stress conditions (NS) or in the presence of 100 µM CdCl₂ for 1 hr and visualized by live cell fluorescence microscopy. Scale bar; 10 µm. (B) Quantitation of the mean percentage of total cells with foci (n=3). Quantitation of the mean percentage of total cells with foci following recovery (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD). ns, not significant, ***, p<0.0001. (C) BY4741 expressing the pSSA3-lacZ reporter was incubated in the following conditions for 1 h at 30°C: no treatment (-), 100 µM CdCl₂, 50 µM dithiothreitol (DTT), or 50 µM DDT + 100 µM CdCl₂. Average β-galactosidase activity was measured and normalized to no treatment (n=3). Error bars indicate standard deviation (SD).
Oxidative Stress-Induced Aggregation

Although peroxide did not induce aggregation of Tpi1 or the other candidates tested (Figure 3.1), the thiol-specific oxidant diamide was shown to activate the HSR 6-fold more than peroxide suggesting it is potentially a stronger thiol modifier (Wang et al., 2012). I therefore investigated whether diamide would induce Tpi1 aggregation when peroxide could not by incubating Tpi1-GFP and Tsa1-GFP with 2.5 mM diamide for 1 h at 30°C (Figure 4.7A). Like cadmium, diamide induced aggregation of a fraction of Tpi1, as well as the total pool of the chaperone Tsa1 to foci. To determine if the fraction that aggregates is composed of nascent polypeptides versus folded native protein, I incubated cells with 2.5 mM diamide for 1 h after a 15 min incubation with or without 0.1 mg/mL cycloheximide (Figure 4.7A). Pre-treatment with cycloheximide negated diamide-induced aggregation of Tpi1-GFP, indicating folding sensitivity of newly synthesized polypeptides of Tpi1. Similar to cadmium exposure, Tsa1-GFP foci formation was uninhibited indicating cycloheximide does not affect chaperone recruitment. Furthermore, this concentration of diamide did indeed induce the HSR as determined by β-galactosidase activity of the pSSA3-lacZ reporter (Figure 4.7B). In conclusion, diamide is a thiol-reactive proteotoxic stress as it activates recruitment of the thiol-dependent chaperone Tsa1 and targets nascent Tpi1 similarly to the thiol-chelator cadmium.
Figure 4.7 Diamide induces aggregation of nascent Tpi1-GFP. (A) Exponentially grown cells bearing Tpi1-GFP or Tsa1-GFP were visualized by live cell fluorescence microscopy after no stress (NS), 15 min cycloheximide (CHX), 1 h 2.5 mM diamide (dia), and 15 min pre-treatment with CHX followed by 1 h 2.5 mM dia (CHX → dia). Representative images for each shown. Scale is 10 µm. (B) BY4741 expressing pSSA4-lacZ was incubated at 30°C for 1 h with (+) or without (-) 2.5 mM diamide (dia). Average β-galactosidase activity was measured and normalized to NS (n=3). Error bars indicate standard deviation (SD).
There is some speculation and evidence that HS induces oxidative or thiol stress. To determine whether Tpi1 was folding labile to HS and this stress targeted nascent polypeptides as shown with the thiol-specific stressors cadmium and diamide, I incubated Tpi1-GFP, Hsp104-GFP, and Tsa1-GFP at 42°C for 15 min with or without 15 min pre-treatment of 0.1 mg/mL cycloheximide (Figure 4.8A). Following HS, Tpi1-GFP and the chaperones Hsp104-GFP and Tsa1-GFP formed foci indicating protein aggregation. Pre-treatment with cycloheximide prevented HS-induced aggregation of Tpi1-GFP and localization of Hsp104-GFP to foci. Tsa1-GFP still formed foci indicating recruitment of this chaperone is uninhibited. These data agree with previous studies on HS and cadmium-induced protein aggregation that showed temperature and thiol stress impact nascent polypeptides (Medicherla and Goldberg, 2008; Jacobson et al., 2017). To determine whether indeed HS induces thiol stress, I incubated Tpi1-GFP WT, C41A, C126A, and C41A C126A at 42°C for 15 min and compared cysteine mutant localization to WT using fluorescence microscopy (Figure 4.8B). Both C41A and C126A single cysteine mutants formed foci following HS similarly to WT. Interestingly, C41A C126A did not form foci indicating one cysteine must be present to enable HS-induced Tpi1 aggregation. These data support the hypothesis that HS produces thiol stress at least sensed by Tpi1 and induces aggregation of Tpi1 in a cysteine-dependent manner.
Figure 4.8 HS induces aggregation of Tpi1-GFP in a thiol-dependent manner. 
(A) Exponentially grown cells bearing Tpi1-GFP or Hsp104-GFP were visualized by live cell fluorescence microscopy after no stress (NS), 15 min cycloheximide (CHX), 15 min at 42°C (HS), and 15 min pre-treatment with CHX followed by 15 min at 42°C (CHX → HS). Representative images for each shown. Scale bar; 10 µm. (B) Exponentially grown cells bearing Tpi1-GFP WT, C41A, C126A, or C41A C126A were incubated in non-stress (NS) or 42°C for 15 min and visualized by live cell fluorescence microscopy. Representative images for each shown. Scale bar; 10 µm.
Glucose Starvation-Induced Aggregation

I explored another mode of redox imbalance/thiol stress by monitoring foci formation of Tpi1-GFP in chronologically aging yeast. In yeast, chronological age is determined by measuring the growth of a cell culture over time until the culture reaches stationary phase. Brandes and colleagues found that in chronologically aging yeast cultures the proteome undergoes thiol oxidation due to loss of reducing equivalents (NADPH) brought about by glucose depletion (Figure 4.9A, Brandes et al., 2013). I hypothesized that this oxidation would induce aggregation of Tpi1. I monitored Tpi1-GFP aggregate formation in chronologically aging yeast under continuous culture in rich medium for 4 d (Figure 4.9B-C). On day 1, as expected, I observed little to no aggregation of Tpi1-GFP, but as the days progressed, the percentage of cells with detectable aggregates steadily increased to a level comparable to that observed with cadmium treatment. This data is consistent with Tpi1 becoming more oxidized over time. Interestingly, glucose supplementation of the day 4-aged culture resulted in clearance of Tpi1-GFP aggregates, indicating that no additional aggregates were formed after glucose addition and that previously existing aggregates were rapidly turned over.

Because at least one Tpi1 thiol (C126) had been previously identified to be oxidized in a thioredoxin mutant background (Le Moan et al., 2006), I predicted that the redox imbalance in aging/starving cells may mimic thiol chelation by impacting Tpi1 cysteines. To verify that the observed aggregation was indeed thiol specific, I examined the cysteine mutants C41A, C126A, and C41A C126A (Figure 4.9B,C). As observed with cadmium treatment, the behavior of Tpi1 with each single mutation was nearly identical to wild type, while the double mutant displayed little to no foci formation over the course of the experiment. These findings are consistent with the model that glucose starvation due to chronological aging induces a thiol-specific stress, and that at least in the case of Tpi1, cysteines in the primary sequence of the protein render it folding labile under such conditions.
Figure 4.9 Chronological aging induces cysteine-dependent Tpi1-GFP aggregation. (A) Illustration of aging/starvation experiment. (B) Tpi1-GFP WT, C41A, C126A, and C41A C126A expressing cells were inoculated into rich medium and grown without additional supplementation for 4 d. On day 4, cells were supplemented with 2% glucose. Representative images from live cell fluorescence microscopy are shown for each day (d1-4) and after 1 h supplementation (d4+glu). Scale bar; 10 µm. (C) Quantitation of the mean percentage of total cells with foci for each strain (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD). ns, not significant, *, p<0.05, **, p<0.001.
**pH-driven Aggregation**

Although cadmium is thought to induce terminally damaged protein (Jacobson et al., 2017), it would be interesting to determine whether Tpi1 could form adaptive assemblies that are reversed upon removal of stress. It is known that the prion forming protein Sup35 can undergo pH-driven reversible aggregation (Franzmann et al., 2018). Following generation of an acidic pH lower than 5.0, Sup35 formed gel-like aggregates that disassembled when re-introduced into an optimal folding environmental pH. To determine whether changes in pH can induce Tpi1 aggregation, I incubated Sup35-GFP or Tpi1-GFP expressing cells in a pH 5.0 phosphate buffer with or without 2 mM of the proton carrier 2,4-dinitrophenol (DNP) for 1 h to generate an acidic pH (Figure 4.10). Without the addition of DNP, Sup35-GFP and Tpi1-GFP remained diffuse similar to incubation in YPD. After DNP exposure, Sup35-GFP formed foci in agreement with the previous study by Franzmann and coworkers (Franzmann et al., 2018). Interestingly, Tpi1-GFP formed foci as well indicating it is pH-folding sensitive. To determine whether these aggregate assemblies are reversible, I incubated each strain in pH 5 phosphate buffer with DNP for 1 h and then recovered in YPD for 1 h. Both Subp35-GFP and Tpi1-GFP foci were no longer observed suggesting potential re-solubilization of aggregates.

Sup35 assemblies are formed through the self-interaction of negatively charged amino acid clusters and amyloid-like domains within the primary sequence (Franzmann et al., 2018). To rule out the possibility that the pH folding sensitivity of Tpi1 is cysteine-dependent, I examined Tpi1-GFP C41A, C126A, and C41A C126A in the same conditions as above (Figure 4.10). Tpi1-GFP cysteine mutants formed foci in response to acidification by DNP and disassembled similarly to wildtype. These data indicate Tpi1 is sensitive to acidic pH through a thiol-independent mechanism.
Figure 4.10 pH-driven reversible aggregation of Tpi1-GFP. Exponentially grown cells bearing Sup35-GFP or Tpi1-GFP WT, C41A, C126A, or C41A C126A were visualized by live cell fluorescence microscopy in the following conditions: YPD medium, pH 5 phosphate buffer with (+) or without (-) 2 mM of the proton carrier 2,4-dinitrophenol (DNP) for 1 h, and 1 h incubation in pH 5 buffer + DNP followed by 1 h recovery in YPD. Representative images are shown. Scale bar; 10 µm.
Discussion

Further characterization of Tpi1 folding sensitivity to cadmium has shown that nascent polypeptides of Tpi1 are the primary target and cadmium induces aggregation in a thiol-dependent manner establishing a mechanism for thiol-stress disruption of proteostasis. These aggregates are processed through potential chaperone-directed proteosomal degradation or re-folded to allow entry into the soluble protein pool as shown by chaperone recruitment and clearance over time. These findings extend to other types of thiol-stress including oxidation by diamide or HS and glucose starvation-mediated redox imbalance in aging cells. Moreover, the Tpi1 cysteines appear to present a folding liability under challenge from multiple thiol stressors despite differences in thiol-reactivity. Finally, Tpi1 is folding labile to other thiol-independent or thiol-nonspecific stresses including HS and acidic pH.

All thiol-reactive stresses examined in this chapter showed thiol stress targets nascent Tpi1 as determined by translation inhibition by cycloheximide. Alternatively, inhibition of Tpi1 aggregation could be explained by loss of expression of an interacting partner or chaperone that is required for folding and/or stability. Although cycloheximide prevents translation of induced chaperone mRNA, the abundant levels of chaperone proteins remain largely unchanged. Furthermore, Tpi1 folding isn’t likely dependent on a specialized chaperone such as Hsp90 (Johnson, 2012). These facts exclude loss of a chaperone as a potential explanation for inhibition of cadmium-dependent aggregation. With regards to an interacting partner protein, Tpi1 is not known to function within a complex, excluding this model as well.

Interestingly, I observed differences in the response to thiol stress between the chaperones Hsp104 and Tsa1. Loss of recruitment of Hsp104 indicates this chaperone only responds to misfolding and aggregation of newly synthesized protein. In contrast, Tsa1 undergoes thiol-dependent oxidation in response to thiol stress such as peroxide activating its chaperone activity and recruitment to protein aggregates. This known redox regulation of activity might explain why Tsa1 recruitment is uninhibited by CHX suggesting endogenous protein aggregation besides Tpi1 or nascent protein. I have not ruled out that thiol stress impacts folded, native protein as
another target with exposed cysteines has yet to be identified. Furthermore, there is no known redox regulation of Hsp104 indicating it could respond differently to thiol-reactive stress versus non-thiol-specific stress.
Chapter V: PQC and Redox System Dynamics

Note: This chapter was derived from work published by Amy Eileen Ford.

Introduction

Molecular chaperones such as Hsp104 or Tsa1 (in the case of oxidatively damaged proteins) have been utilized to track formation of protein aggregates (Jacobson et al., 2017; Weids and Grant, 2014). As controls for protein aggregation, I have shown the chaperones Hsp104-GFP and Tsa1-GFP form foci in response to different thiol-stresses suggesting sites of protein aggregation (Chapter 4). Several non-membrane bound compartments induced after cytotoxic stresses have been identified and their components characterized via fluorescence reporter tagging and microscopy (Escusa-Toret et al., 2013; Miller et al., 2015). Chaperones and other stress-related proteins have been shown to localize into distinct stress foci following different stimuli including Hsp104 and Hsp42 to indicate general quality control compartments (Q-body), the peroxiredoxin Tsa1 to recognize thiol-specific aggregates, Pub1 to represent formation of stress granules (SG), and Edc3 to indicate processing bodies (P-body). The latter two composed of both protein and RNA components (Buchan et al., 2008; Kshirsagar and Parker, 2004). Constituents of each compartment are either recruited to sites of aggregation or involved in their assembly with potential PQC roles in protein degradation, soluble and aggregate protein sequestration, and controlled protein trafficking.

In addition to protein folding surveillance and stress adaption via stress bodies, the cell employs redox systems (i.e. the thioredoxin system) to monitor protein thiol status, an integral component of protein folding. Loss of a functional cytosolic thioredoxin system (trx1/2Δ or trr1Δ) increases thiol reactivity and oxidation of cysteine-containing proteins and has been shown to activate the HSR indicating disruption of proteostasis (Le Moan et al., 2006, Sara Peffer, unpublished). Therefore, I propose a role for the thioredoxin system in maintaining thiols in a reduced state to prevent protein misfolding and aggregation.

In this chapter, I determine colocalization of cadmium-induced aggregates with specific stress bodies. Additionally, I provide further support that the thioredoxin system is a component of the cytosolic PQC.
Results

Stress Compartments

I employed GFP-protein fusions to each of the stress markers mentioned above and visualized their localization relative to cadmium-induced Tpi1-BFP2 aggregates to allow simultaneous two-color surveillance. In non-stress conditions, Tpi1-BFP2 was diffuse throughout the cytosol consistent with what we have shown with Tpi1-GFP and indistinguishable from BFP2 expressed alone (Figure 5.1). I noted that the Tpi1-BFP2 fusion required higher concentrations of CdCl₂ to consistently induce aggregation, due possibly to the presence of the wild type copy of Tpi1 expressed in the genome, forming mixed heterodimers. The stress markers Hsp104-GFP, Tsa1-GFP, Hsp42-GFP and Pub1-GFP were likewise primarily diffuse, exhibiting few to no foci (Figure 5.1A-C,E). Edc3-GFP formed several foci per cell, consistent with previous studies demonstrating that PB form constitutively in optimal growth conditions (Decker and Parker, 2012) (Figure 5.1D). Following a 1 h exposure to 750 µM CdCl₂, Tpi1-BFP2 and the stress markers Hsp104-GFP, Tsa1-GFP, Hsp42-GFP formed detectable aggregates in response to the thiol stress (Figure 5.2A). Interestingly, Pub1-GFP remained diffuse throughout the cytosol suggesting cadmium does not induce the formation of SGs. As evidenced by Edc3-GFP foci, PB were maintained, if not slightly increased in the presence of cadmium. As we found with GFP, cadmium did not induce aggregation of BFP2 alone (Figure 5.1).

To determine the nature of Tpi1 aggregates, we quantified the percentage co-localization with the indicated stress markers (Figure 5.2B). Approximately 90% of Tpi1-BFP2 foci co-localized with Hsp104-GFP, Tsa1-GFP, and Hsp42-GFP foci, demonstrating recruitment of these chaperones. Essentially no co-localization was observed with Edc3-GFP foci, and as stated above little to no Pub1-GFP foci were induced during cadmium stress. Conversely, only about 30% of Hsp104-GFP and Tsa1-GFP foci were observed to colocalize with Tpi1-BFP2 suggesting chaperone localization to native Tpi1 aggregation or non-Tpi1-associated protein aggregates (Figure 5.2C).
Figure 5.1 Cd does not induce foci formation of SG or PB and BFP2 does not confer aggregation to Tpi1. (A-E) Exponentially grown cells bearing genomic GFP-protein fusions of Tsa1, Hsp104, Hsp42, Edc3, and Pub1 (chaperone, green) expressing Tpi1-BFP2 or BFP2 alone (BFP2, red) on a plasmid were visualized by live cell fluorescence microscopy in the following conditions: non-stress (NS) and 750 µM CdCl₂ (Cd) for 1 h. Shown are representative images of the NS control for chaperone-GFP strains expressing Tpi1-BFP2 or BFP2 alone and the BFP2 only control following Cd exposure. Scale bar; 10 µm.
Figure 5.2 Tpi1 aggregates recruit protein chaperones to foci distinct from other stress bodies. (A) Exponentially grown cells bearing GFP-protein fusions of Tsa1, Hsp104, Hsp42, Edc3, and Pub1 (stress marker, green) and also expressing Tpi1-BFP2 (Tpi1, false-colored red) on a plasmid were exposed to 750 µM CdCl₂ for 1 h and visualized by live cell fluorescence microscopy. Representative images are shown. Scale bar: 10 µm. (B) Quantitation of the mean percent co-localization (merge) of Tpi1-BFP2 foci containing stress marker foci from (A) (n=3). (C) Quantitation of the mean percent co-localization of Tsa1-GFP and Hsp104-GFP with Tpi1-BFP2 from (A) (n=3). Error bars indicate standard deviation (SD).
We further examined the temporal association of Tpi1-BFP2 with Tsa1-GFP. Cells treated with 750 µM CdCl$_2$ were harvested from batch culture at 15 min intervals for a total of 1 h and co-localization was determined (Figure 5.3). We first observed foci formation of Tpi1-BFP2 at 30 min, consistent with what we observed with Tpi1-GFP, while 0-1 Tsa1-GFP foci were present before cadmium treatment. Between 30-60 min, all Tpi1-BFP2 foci colocalized with Tsa1-GFP and increases in both percent of cells with foci and number of foci per cell were coincident. These results are consistent with chaperones being recruited early to, if not seeding sites of, cadmium-induced protein aggregation.
Figure 5.3 Simultaneous temporal co-localization of Tsa1-GFP and Tpi1-BFP2 aggregates. (A) Exponentially grown cells co-expressing Tsa1-GFP (green) and Tpi1-BFP2 (false-colored red) on a plasmid were exposed to 750 μM CdCl₂ for 1 h and visualized at 15 min intervals by live cell fluorescence microscopy. (B) Quantitation of the mean percentage of total cells with foci from (A) at each time point (n=3). (C) Quantitation of the relative percentage of cells from (A) with 1, 2, 3, or 4+ co-localized foci per cell following Cd exposure at the indicated timepoints (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD).
Role of the Thioredoxin System

To further examine whether absence of a functional thioredoxin system induced endogenous protein aggregation, I made a deletion of *TRR1 (trr1Δ)* in the Tpi1-GFP, Tsa1-GFP, and Hsp104-GFP strains and visualized localization in optimal growth conditions by fluorescence microscopy (Figure 5.4). Similar to wildtype, Tpi1-GFP remained diffuse throughout the cytosol forming no foci indicating no aggregation. The chaperones Tsa1-GFP and Hsp104-GFP appeared to form foci in the *trr1Δ* background suggesting possible disruption in proteostasis, though this was not quantitated.
Figure 5.4 Loss of TRR1 results in foci formation of Tsa1-GFP and Hsp104-GFP. Exponentially grown WT and trr1Δ cells bearing Tpi1-GFP, Tsa1-GFP, or Hsp104-GFP were incubated in optimal growth conditions and visualized by live cell fluorescence microscopy. Representative images are shown. Scale bar, 10 µm.
Discussion

Altogether, these data strongly support a model that Tpi1 forms QB-type aggregates with the chaperones Hsp104, Tsa1, and Hsp42 that are independent of other known stress-inducible protein structures. Moreover, both Hsp104 and Tsa1 are recruited into additional foci independent of Tpi1, consistent with cadmium causing general proteotoxic stress. In contrast to thiol-reactive stress, acidic pH may induce SG or PB as it was shown to drive aggregation of Tpi1 (Chapter IV). It would be interesting to determine if SG or PB markers, Edc3 or Pub1, would be recruited following formation of Tpi1 aggregates rather than immediately as seen with cadmium and QB.

Preliminary results reveal little new evidence for the role of the thioredoxin system in maintaining proteostasis; alas, the connection between the redox system and PQC is inconclusive. Perhaps with loss of the thioredoxin reductase, Tpi1 is more folding-sensitive to additional stress such as peroxide, HS, or cadmium. On the other hand, thiol reduction and, therefore, folding may depend more on the peroxiredoxin Tsa1 rather than thioredoxin. Deletion of thioredoxin or its reductase activates stress responses including the HSR and OSR priming cells with high levels of Tsa1. Furthermore, deletion of the enzyme/chaperone was shown to activate the HSR as well (Sara Peffer, unpublished).
Chapter VI: Conservation of Redox-associated Proteostasis

**Note:** This chapter was derived from work published by Amy Eileen Ford.

Introduction

Tpi1 is an important glycolytic enzyme responsible for converting dihydroxyacetone-1-phosphate to glyceraldehyde-3-phosphate and is conserved from single cell yeast to metazoans. In yeast, proper function is determined by growth on glucose medium or sensitivity to lithium (Shi et al., 2005). Although the structure and primary function of Tpi1 is conserved (53% identity, 68% similarity), the number of cysteines is not. Human TPI (hTPI) has an additional three cysteines that have the potential to be modified by Cd. Despite these differences in TPI, I hypothesize cadmium proteotoxicity is conserved.

In humans, compound heterozygous mutations in this enzyme can cause a rare disease called TPI Deficiency as a result of loss of function (Oláh et al., 2002). This disease is characterized by hemolytic anemia and progressive neurodegeneration leading to death in early childhood. TPI deficiency is rare most likely due to a high occurrence of embryonic lethality. A study in 2004 investigated the mechanism of loss of function by characterizing mutations found in patients with the disease in the budding yeast model (Ralser et al., 2006). Although these mutations could complement tpi1Δ null phenotypes, the authors observed some changes in catalytic activity and dimer formation. This led the authors to conclude the mutations conferred altered folding conformations that prevent proper dimer formation leading to loss of function. Based on these studies, I hypothesized these mutations might render TPI folding sensitive and aggregation prone.

In this chapter, I determine conservation of redox-associated proteostasis by applying the model I have established in yeast to human cells. I also investigate the mechanism of disease of TPI Deficiency by integrating mutations found in patients into haploid S. cerevisiae.
Results

Thiol-stress Proteotoxicity Conservation

Together with previous work, our findings suggest that cadmium is a potent proteotoxic agent in budding yeast, with particular affinity for nascent polypeptides. To extend these findings to humans, where cadmium is considered a deadly poison, we sought to monitor chaperone dynamics in human cells, utilizing HEK293 and HCT116 cell lines. We obtained a previously characterized carboxyl-terminal YFP fusion to the human stress-inducible Hsp70 (HSPA1A) shown to localize to protein aggregates (Kim et al., 2002). Plasmids expressing Hsp70-YFP or YFP alone were transfected into cells and expression assessed by Western blot and fluorescence microscopy. Western blot analysis validated similar expression levels in both cell lines (Figure 6.1A). In non-stress conditions, Hsp70-YFP was localized diffusely throughout the cytosol and mostly excluded from the nucleus as judged by DAPI staining (Figure 6.1B, E). I noted a consistent low level of both YFP and Hsp70-YFP foci (~1-5) in the absence of stress, likely attributable to the transient overexpression system utilized, and therefore chose to quantify aggregation only in cells exhibiting >5 foci. Following exposure to 50 μM CdCl₂ for 6 h, distribution of YFP alone remained unchanged, while Hsp70-YFP formed multiple foci per cell in both cell lines, consistent with Hsp70 localizing to thiol-stress induced protein aggregates. These findings establish that cadmium is a proteotoxic agent in human cells as it is in yeast.

Next, I was interested in determining whether the Tpi1 folding sensitivity we observed in budding yeast was conserved between yeast and humans. I constructed an amino-terminal GFP fusion to the human homolog (hTPI) produced from a mammalian constitutive expression vector. GFP-hTPI and GFP alone were transfected into HEK293 and HCT116 cell lines and expression confirmed by Western blot and fluorescence microscopy (Figure 6.1C-E). While GFP alone remained diffuse in both non-stress and cadmium stress conditions, GFP-hTPI formed a large number of aggregates per cell indicating that human TPI, like the yeast homolog, is highly susceptible to cadmium-induced aggregation.
Figure 6.1 Cd proteotoxicity is conserved between yeast and human cells. (A,D) Western blot analysis of HEK293 and HCT116 cells expressing either Hsp70-YFP/GFP-hTPI or YFP/GFP alone. α-GFP was used to detect Hsp70-YFP and YFP and α-GAPDH was used as a loading control. (B,E) HEK293 and HCT116 cells expressing Hsp70-YFP/GFP-hTPI or YFP/GFP alone were incubated at 37˚C with (Cd) or without (NS) 50 µM CdCl₂ for 6 h. Following incubation, cells were fixed, stained with DAPI, and visualized by fluorescence microscopy. Representative images of each cell type for each condition are shown as indicated. Scale bar; 50 µm. (C,F) Quantitation of the mean percentage of cells with >5 foci per cell (n=3). A minimum of 100 cells were counted per biological replicate. Error bars indicate standard deviation (SD). *, p<0.05, **, p<0.001, ***, p<0.0001.
To ensure the conditions used in our study induce the HSR as we have previously shown in yeast, we measured gene expression by qRT-PCR of two stress induced genes DNAJ1B (Hsp40) and HSPA1A (Hsp70) in both HEK293 and HCT116 cell lines following exposure to HS and Cd (Figure 6.2A). Following a 6 h exposure and 6 h recovery from 50 µM CdCl₂, expression of the stress-related genes in both cell lines was induced similarly to a standard HS treatment at 43°C for 1 h. In addition to measuring mRNA expression, I observed cadmium induction of the HSR in HCT116 cells using a dual-luciferase reporter system for measuring Hsf1 activation (Figure 6.2B). All together, these experiments indicate thiol-reactive stress disrupts proteostasis in human cells comparatively to what I have shown in the model eukaryote yeast.
Figure 6.2 Cd induces the HSR in human cells. (A) Mean mRNA expression quantitated by qRT-PCR of HSPA1A (Hsp70) and DNAJB1 (Hsp40) from HEK293 and HCT116 cells incubated in the following conditions: 37°C (NS), 43°C for 1 h with 6 h recovery at 37°C (HS), and 50 µM CdCl\(_2\) at 37°C for 6 h followed by recovery in medium lacking CdCl\(_2\) for 6 h at 37°C (Cd). Error bars indicate standard deviation (SD). ns, not significant, *, p<0.05, **, p<0.001. (B) HCT116 cells expressing pHSE-FFL and pCMV-RL were incubated in the same conditions as (A) and mean FFL activity was quantitated by normalizing to RL activity determined by a dual-luciferase assay. Error bars indicate standard deviation (SD). *, p<0.05.
Understanding the Mechanism Behind Human TPI Deficiency

Here, I applied the mutations found in patients with TPI Deficiency to the yeast model and focused directly on protein folding versus function as this was to be determined previously unaffected (Ralser et al., 2006). To determine whether these mutations render Tpi1 aggregation prone or hypersensitive to stress, I made single amino acid substitutions in the genomic Tpi1-GFP fusion background previously generated (C41Y, G122R, and I170V) (Figure 6.3A) and visualized their localization in non-stress and stress conditions compared to wildtype by fluorescence microscopy (Figure 6.3B). In non-stress conditions, all mutants are observed diffuse throughout the cytosol indicating no inherent folding defect. When exposed to a low concentration of 50 µM CdCl₂, Tpi1-GFP mutants formed foci to a level comparable to wildtype. Additionally, exposure to a mild HS of 33°C for 15 min resulted in no foci formation suggesting no increased sensitivity to HS. Together, these data indicate each mutation alone is not folding sensitive or at least not more so than wildtype.
Figure 6.3 TPI Deficiency-associated mutations do not result in aggregation. (A) Ribbon structure of the yeast Tpi1 homodimer with each amino acid mutation indicated in at least one monomer (PDB: 1YPI). (B) Location of engineered amino acid substitutions in chromosomal TPI1-GFP allele. Red star (\*) indicates mutation. (C) Exponentially grown cells bearing Tpi1-GFP WT, C41Y, G122R, or I170V were incubated in non-stress conditions (NS), in the presence of 50 \( \mu \text{M} \) CdCl\(_2\) for 1 h (Cd), or 15 min at 33°C (HS) and visualized by live cell fluorescence microscopy. Representative images shown. Scale bar; 10 \( \mu \text{m} \).
Discussion

Here, I have found that thiol stress proteotoxicity, specifically cadmium, is conserved between yeast and human cells through the observation that human Hsp70 localizes to foci following stress and this extends to my model protein Tpi1 first identified in yeast. Although thiol-stress folding sensitivity is conserved, I did not find further evidence for TPI Deficiency mutant folding defects; thus, the mechanism of disease remains elusive. There might be differences in folding sensitivity between the yeast and human homologs or at least in cases when only one allele is present. It would be interesting to determine if stresses that did not induce aggregation of the yeast Tpi1 homolog, such H₂O₂ and DEM, would impact aggregation of human TPI based on this folding sensitivity difference. This is supported by studies showing differences in minimum cadmium cell toxicity between human cells and yeast. In cardiomyocytes 0.1 µM CdCl₂ is cytotoxic (Limaye and Shaikh, 1999) whereas in yeast, 50 µM of CdCl₂ was shown as cytotoxic (Wu et al., 2016). Nevertheless, my data and others support that disruption of TPI structure through thiol modification or mutation has the potential to negatively impact protein folding.
Chapter VII: Discussion and Future Directions
Summary

The eukaryotic cytosolic proteome is vulnerable to changes in proteostatic and redox balance caused by temperature, pH, oxidants and xenobiotics. Cysteine-containing proteins are especially at risk because the thiol side chain is subject to oxidation, adduction and chelation by thiol-reactive compounds. All of these thiol-modifiers have been demonstrated to induce the heat shock response and recruit protein chaperones to sites of presumed protein aggregation in the budding yeast *Saccharomyces cerevisiae*. However, endogenous targets of thiol stress toxicity responsible for these outcomes are largely unknown. My work has determined that changes in cytosolic redox balance via thiol-specific stresses, including cadmium, diamide, and glucose starvation are proteotoxic (*Figure 7.1*). Emerging from the ribosome, newly synthesized cysteine-containing peptides are in a reduced state. Exposure to thiol-specific stress, including xenobiotics like cadmium and the loss of the reducing power of redox systems, results in the modification of protein thiols by covalent interactions and oxidation. These non-native modifications to proteins result in an inability to properly fold, leading to the formation of protein aggregates. The thiol-reactive stress and subsequent aggregation is sensed by the cytoplasmic protein quality control network that activates stress responses and recruits chaperones such as Hsp104 and Tsa1. These chaperones assist in clearing existing aggregates and preventing further damage. Above all, protein aggregation as a result of exposure to thiol-specific stress extends to human cells; thus, establishing a conserved mechanism and model for redox-associated proteostasis.
Figure 7.1 Model for redox-associated protein homeostasis
A cysteine-containing polypeptide (dash line) is synthesized from ribosome (gray) with reduced (SH) and redox active (S') thiols. Thiol-reactive stress modifies (X) the thiols inducing aggregation. To adapt and prevent further protein aggregation, stress responses (OSR/maroon and HSR/pink) are activated increasing chaperone and detoxifying enzyme levels. Q-body associated (Hsp104/blue and Hsp42/orange) and thiol-dependent (Tsa1/red) chaperones are recruited to sites of aggregation to sequester and disassemble aggregate for degradation by the proteasome (purple). The thioredoxin system (green) reduces modified protein thiols to prevent aggregation. C, cytoplasm and N, nucleus.
Identification of Redox-sensitive, Aggregation Prone Proteins

My study’s generalizability to other cysteine-containing proteins and the overall proteome is limited because of the identification of only one target using a small fluorescence-based screen of 10 candidates. Following an initial screen, we identified the glycolytic enzyme Tpi1 as folding-sensitive to thiol-reactive stress. Although the other potential candidates contained cysteines and were identified as redox active, they did not exhibit any visible folding defects. For example, the translation elongation factor Eft1 did not aggregate in response to 100 µM CdCl₂ or other thiol-stresses tested despite being identified as redox active and containing a total of seven cysteines located both buried and exposed within the folded, native structure. This was surprising. Therefore, I hypothesize cysteine containing proteins are differentially affected by thiol-specific stress.

Potential characteristics that may govern sensitivity are location of cysteine residues in the N-terminal region, functional domains, and solvent accessible or disordered regions as well as the number of cysteines in these regions. In fact, failure to detect aggregation of other candidates might be due to general folding sensitivity or stability. Aggregation may be dose dependent and require a more severe level of stress to misfold and form aggregates. It would be interesting to examine candidates during HS or chronological age as their mechanism of action is proposed as less direct. These functional and structural properties will give insight into why some cysteine-containing proteins are susceptible to misfolding by thiol-specific stress and others are not.

When considering the role of thiol sensitivity, it is important to distinguish between cysteine modification as a programmed regulatory signal versus accidental consequence. As per mentioned in the Introduction, redox-dependent cysteine modification is utilized to regulate various components of the PQC machinery such as the Hsp70 chaperone function in regulating the UPR and HSR stress responses. Unlike the Hsp70 regulatable cysteines, Tpi1’s two cysteines are buried within the protein 3D structure rather than solvent exposed. Although decreased metabolism and growth is advantageous during stress, this striking difference in
cysteine accessibility suggests Tpi1 thiol sensitivity is most likely an accidental consequence to thiol stress or redox imbalance.

By using a more proteomic approach in future studies, my screen’s sensitivity and target pool can be enhanced. This method will allow comparisons between proteomic studies that are focused exclusively on either stress-induced protein aggregation or thiol reactivity. Utilizing this an advantage, future studies can better identify candidates based on known folding sensitivity to non-redox associated stress. A proteome wide screen will not only complement the proteomic studies identifying redox active proteins, but also identify which of those redox active proteins are physiologically relevant. To conduct this screen, one could isolate thiol stress induced protein aggregates via the method I have developed (Chapter III) and identify protein content by simple unlabeled or iTRAQ-labeled mass spectrometry of the insoluble fractions of both non-stress and thiol-stress conditions. Not only would this enable one to discern any significant fold change differences but compare the functional and structural properties discussed above.

**Thiol-dependent Aggregation**

We determined via cysteine-mutagenesis that cadmium-induced aggregation of Tpi1 is thiol-dependent. The presence of a single cysteine enables cadmium to cause protein misfolding and aggregation, though, only when in the unfolded state like during synthesis. This suggests that cadmium directly interacts with cysteines from multiple peptide chains, forming a bidentate complex. From here, complexation of cadmium molecules that are bound to nascent chains or that interact with hydrophobic patches in neighboring peptides will promote aggregation that is visible by fluorescence. Although diamide was not tested on Tpi1 cysteine mutants, I predict the mechanism would be similar and require the presence of at least one cysteine to cause aggregation through direct modification. Diamide has the potential to generate higher ordered oxidative forms such as disulfides due to its potency as a thiol-specific oxidant (Kosower and Kosower, 1995). Disulfide bonds are more stable than sulfenic acid mono-oxidation increasing the propensity for the protein to misfold and aggregate.
When comparing cadmium or diamide to another form of thiol-reactive stress addressed here—glucose starvation—the mechanism of action is hypothesized to be more indirect. Depletion of glucose over time results in a loss of reducing equivalents (e.g. NADPH) that are used to power the redox systems (Brandes et al., 2013). An inability to maintain a reducing cytosol leads to oxidation of protein thiols. These modifications are able to persist allowing higher ordered oxidation such as disulfide bonds, sulfinic or sulfonic acids, which promotes misfolding and aggregation. It will be interesting to determine whether other known thiol modifiers, such as peroxide or organic electrophiles, can induce protein aggregation; it will also be useful to distinguish the differences in the mechanisms of action using thiol modification capture methods in combination with the aggregate detection methods used in this study.

Unlike the thiol-specific modifiers addressed above, HS induced thiol-dependent aggregation is surprising and the mechanism unclear. Typically, HS induces protein carbonyls through the generation of ROS at other amino acids such as arginine and threonine (Berlett and Stadtman, 1997; Dalle-Donne et al., 2003; Verghese et al., 2012); however, I found HS-induced aggregation of Tpi1 was cysteine-dependent. It is possible carbonyl derivatives react with the sulfydryl group of cysteines to induce Tpi1 aggregation. To test this, HS-induced Tpi1-GFP aggregates can be isolated using the cryo-lysis following by differential centrifugation method I developed and probed for carbonylation using a carbonyl moiety specific antibody (Levine, 2002). It would be interesting if isolation of aggregates from Tpi1-GFP C41A C126A showed a loss of carbonyl detection. Furthermore, HS-generated ROS may overload the thioredoxin system blocking maintenance of redox balance. A prolonged oxidative environment would allow stable oxidation and, therefore, protein aggregation. On the other hand, it is possible HS simply causes thermal denaturing of folded Tpi1 allowing modification of its cysteines via the homeostatic level of oxidative potential in the cell. However, I showed HS-induced aggregation of TPI1 is abolished with cycloheximide pre-treatment excluding this as the likely scenario. Thiol-dependent aggregation of Tpi1 in response to HS is most likely less direct.
Role of PQC and Redox Systems

It is possible that cadmium indirectly negatively impacts folding by interacting with molecular chaperones, as has been shown with oxidation of the bacterial and human Hsp70 (Winter et al., 2005). However, if the case, I would have observed aggregation of Tpi1 regardless of the presence or absence of its cysteines. It has been shown by measuring the activity of the protein relative to cadmium concentration that cadmium interferes with spontaneous refolding of chemically denatured proteins in vitro (Sharma et al. 2008). Importantly, inhibition could only be attenuated with the addition of ATP-dependent chaperones such as the E.coli Hsp70 system DnaK/DnaJ/GrpE and not glutathione. Interestingly, I found DTT, another reducing agent like glutathione, to only slightly reduce cadmium activation of the HSR. Together, these data suggest chaperone availability is more important than reducing power. Perhaps overexpression of chaperones can prevent cadmium-induced aggregation of Tpi1 through either sequestering cadmium or “holdase” protection. Conversely, inhibition of chaperone function through mutagenesis or chemical inhibitors may produce hypersensitivity. For instance, Hsp70 cysteine mutagenesis within the nucleotide binding domain lead to constitutive activation of the HSR suggesting thiol-stress might inactivate Hsp70 functions (Wang et al., 2012a; Zheng et al., 2016). Both experiments would enhance our understanding of the molecular chaperone role in maintaining proteostasis during redox imbalance.

In addition to sequestering or inactivating chaperones, cadmium may interfere with the cytoplasmic thioredoxin system preventing the maintenance of the reducing environment required to maintain cysteines in their reduced state. I have shown deletion of thioredoxin reductase had no impact on Tpi1 folding, but did induce foci formation of the chaperones Hsp104 and Tsa1. It would be interesting to determine if loss of TRR1 or the thioredoxin-dependent chaperone TSA1 increases Tpi1 folding sensitivity to cadmium or other thiol-stress exposure such as hydrogen peroxide, which was not visible by fluorescence microscopy in a wildtype background.
While proteotoxic stress clearly induces the misfolding of nascent proteins and their subsequent terminal aggregation via hydrophobic interactions, recently a subset of these structures has been found to include mature, folded polypeptides that reversibly localize together as a stress adaptation (Wallace et al., 2015a; Saad et al., 2017). These assemblies are often pH driven and associated with stress granules (Munder et al., 2016; Franzmann et al., 2018). These reversible aggregates have gel-like properties consisting of stretches of negatively charged amino acids and amyloid-like domains such as in the prion forming protein Sup35. I have shown Tpi1 forms foci similarly to Sup35 following acidification by sorbic acid or DNP. However, foci formation of Tpi1 was not impacted by the absence of either or both its cysteines indicating it is not a thiol-related process. This data is further supported by the observation that cadmium does not induce stress granule formation and no co-localization was observed with Tpi1 aggregates. Although an interesting finding, the property of Tpi1 that enables this pH-dependent foci formation is unknown as Tpi1 contains no obvious amyloid- or prion-like region. Rather, thiol-stress induced Tpi1 aggregates recruit chaperones involved in general protein quality control (Escusa-Toret et al., 2013; Miller et al., 2015). Typically, this aspect of PQC is thought to deal with terminally misfolded protein. Tpi1 is assembled into Q-bodies by Hsp104, Hsp42, and Tsa1 sequestering the thiol-dependent misfolded protein into multiple, dispersed cytoplasmic aggregates. Here, they are most likely trafficked between the cytosol and nuclear JUNQ/INQ compartment with inevitable degradation.

After examining the formation dynamics, we found that cadmium-induced Tpi1 aggregates had cleared, albeit through an undetermined mechanism. In general, cytoplasmic protein aggregates can either be re-solubilized with the assistance of chaperones or degraded via the eukaryotic proteasome (Chen et al., 2011). In addition, clearance may be observed through the elimination of aggregates from daughter cells and retention in mother cells following cell division (Zhou et al., 2011, 2014). Newly synthesized proteins are particularly susceptible to heat-induced misfolding and aggregation and have been shown to be degraded rapidly upon heat stress (Medicherla and Goldberg, 2008). There is evidence that, unlike nascent
polypeptides, heat induced aggregation of fully folded proteins results in complete disaggregation and refolding (Wallace et al., 2015).

A study focused on the metalloid arsenite and the chaperone Hsp104 found that clearance of aggregates is carried out by chaperone-assisted proteosomal degradation (Jacobson et al., 2012). Again, the authors found this to be true for cadmium in a later study (Jacobson et al., 2017). Co-localization analysis indicated chaperones involved in Q-body and JUNQ/INQ formation are immediately recruited to the site of Tpi1 aggregate formation. It is reasonable to surmise on the basis of previous studies and co-localization data presented here that cadmium-induced Tpi1 aggregates are degraded via the proteasome and this is a chaperone-mediated process. Using chemical inhibitors and single-cell monitoring over time, chaperone function involvement in formation and the precise mode of clearance could be specifically determined. Whether or not clearance can further be attributed to cellular division and retention in mother cells may depend on Cd uptake and reactivity kinetics. To start, aggregate formation can be monitored in mother and daughter cells by separating the two populations.

**Human Conservation and Protein Folding Disease**

Many of these proteins identified as redox-active and/or aggregation prone have human protein homologs that could be implicated in protein misfolding diseases. Indeed, compound heterozygous mutations in Tpi1 are associated with the rare human disease TPI Deficiency (Oláh et al., 2002). Previous structural and functional studies have found these mutations most likely alter the folding structure causing an inability to properly form the active homodimer conformation leading to loss of function and lethality (Ralser et al., 2006). Although my work did not provide additional evidence for mutation-based folding defects, I have found Tpi1 to be folding-sensitive to stress that impacts structural integrity and prevents folding: a parallel scenario to mutation-caused changes. That is to say the mutation of cysteine 41 to tyrosine mimics thiol modification. Furthermore, stress, including redox-dependent modification of cysteines, might exacerbate
folding instability caused by mutations. However, it is possible mutation of cysteines might be an evolutionary adaption to prevent accidental modification during stress.

Additionally, there might be differences in folding sensitivity between the yeast and human homologs or at least in cases when only one allele is present. Human TPI is potentially more sensitive to changes that might affect the overall protein structure and folding. Perhaps, the model in yeast is oversimplified necessitating the need to lean towards replicating compound mutations as seen in humans. To do so, one could create diploid yeast with homo and hetero alleles of each mutation identified in human patients including C41Y, G122R, I170V, and E104D. The presence of two mutated alleles may increase severity of the observed phenotype found by Ralser et al. Furthermore, it will be interesting to examine the other mutations in concert with E104D or homozygous E104D as this mutation causes the most severe form of the disease. This will provide a better understanding of folding sensitivity and mechanism of disease.

Though I was not nearly as comprehensive in my analysis in human cells as in yeast, I have established cadmium is proteotoxic in human cells and human TPI is folding sensitive to the heavy metal. As stated in Chapter 6, the human TPI homolog has an additional three cysteines that have the potential to be modified by thiol-reactive stress. If the model holds true, I predict all cysteines must be absent in order to abrogate cadmium-induced aggregation. Furthermore, I showed in yeast that the chaperones Hsp104 and Tsa1 co-localize with Tpi1 aggregates. The hHsp70-YFP has been shown previously to localize to human disease related aggregates (Kim et al., 2002). Based on this evidence, I expect Hsp70 would co-localize with hTPI aggregates. This could be determined by co-immunofluorescence of GFP-hTPI aggregates and a specific antibody to the inducible Hsp70. Following these analyses, I can definitively declare my model for redox-associated proteostasis is conserved.

Conclusions

Together, my work establishes a model for endogenous thiol-dependent protein misfolding and aggregation that can apply to future studies. Future studies addressed above will
further develop the model and provide insights into potential protein folding diseases such as TPI Deficiency. Overall, my research solidifies the link between redox buffering and proteostasis as a conserved eukaryotic cooperative protein quality control system.
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Vita

Amy Eileen Ford was awarded a bachelor’s in science in Biology at the University of North Texas (UNT) in Denton. Ms. Ford completed a Howard Hughes Medical Institute Undergraduate Research Internship at UNT and a Molecular Basis of Infectious Diseases research training program at University of Texas Health Science Center McGovern Medical School. Ms. Ford is pursuing a doctoral degree in Microbiology and Molecular Genetics at the MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. Ms. Ford has received multiple travel awards to present at professional conferences, including a Gordon Research Conference. Ms. Ford has authored a chapter in Heat Shock Proteins in Signaling Pathways textbook and has a research publication in Molecular Biology of the Cell. She is a member of the American Society of Microbiology. Married to Darrell Ford, Ms. Ford is a dog-lover, adopting playful and mischievous Moose, a chocolate Labrador. Ms. Ford enjoys game nights with friends, jigsaw puzzles and cooking.