Pharmacologic Approaches for Adapting Proteostasis in the Secretory Pathway to Ameliorate Protein Conformational Diseases

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Maintenance of the proteome, ensuring the proper locations, proper conformations, appropriate concentrations, etc., is essential to preserve the health of an organism in the face of environmental insults, infectious diseases, and the challenges associated with aging. Maintaining the proteome is even more difficult in the background of inherited mutations that render a given protein and others handled by the same proteostasis machinery misfolding prone and/or aggregation prone. Maintenance of the proteome or maintaining proteostasis requires the orchestration of protein synthesis, folding, trafficking, and degradation by way of highly conserved, interacting, and competitive proteostasis pathways. Each subcellular compartment has a unique proteostasis network comprising common and specialized proteostasis maintenance pathways. Stress-responsive signaling pathways detect the misfolding and/or aggregation of proteins in specific subcellular compartments using stress sensors and respond by generating an active transcription factor. Subsequent transcriptional programs up-regulate proteostasis network capacity (i.e., ability to fold and degrade proteins in that compartment). Stress-responsive signaling pathways can also be linked by way of signaling cascades to nontranscriptional means to reestablish proteostasis (e.g., by translational attenuation). Proteostasis is also strongly influenced by the inherent kinetics and thermodynamics of the folding, misfolding, and aggregation of individual proteins, and these sequence-based attributes in combination with proteostasis network capacity together influence proteostasis. In this review, we will focus on the growing body of evidence that proteostasis deficits leading to human pathology can be reversed by pharmacologic adaptation of proteostasis network capacity through stress-responsive signaling pathway activation. The power of this approach will be exemplified by focusing on the ATF6 arm of the unfolded protein response stress responsive-signaling pathway that regulates proteostasis network capacity of the secretory pathway.

Eukaryotic protein homeostasis, or proteostasis, is maintained by a diverse number of integrated pathways that sometimes complement each other and in other scenarios compete to

regulate the function of the proteome (Morimoto 1998; Balch et al. 2008; Morimoto and Cuervo 2009; Powers et al. 2009; Jarosz et al. 2010; Taipale et al. 2010; Hartl et al. 2011). Com-

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partment-specific stress-responsive signaling pathways regulate the capacity of the proteostasis network in that compartment, utilizing sensors that detect higher than normal levels of protein misfolding and/or aggregation (DiDomenico et al. 1982; Morimoto 1998; Mori 2000; Schröder and Kaufman 2005; Marciniak and Ron 2006; Ron and Walter 2007; Westerheide et al. 2009; Walter and Ron 2011). Activation of a stress-responsive signaling pathway associated with a particular cellular compartment results in generation or activation of a transcription factor that orchestrates a transcriptional program to enhance the proteostasis capacity of that compartment. Folding capacity almost always increases in concert with degradation capacity, highlighting the delicate balance between protein production, folding, and degradation (Balch et al. 2008; Morimoto and Cuervo 2009; Lee et al. 2010). Another key feature of the restoration of proteostasis associated with stress-responsive signaling is reduced transcription of normal cellular messenger RNAs (mRNAs), reduced splicing of normal transcripts, and reduced translation of preexisting mRNAs (Yost et al. 1990; Shang et al. 2007; Ghosh et al. 2010). Importantly, these mechanisms sharply decrease the proteome load on the proteostasis network, maximizing the possibility that proteostasis will be reestablished (Yost et al. 1990; Ron and Walter 2007; Shang et al. 2007; Ghosh et al. 2010; Walter and Ron 2011). When proteostasis is restored through these highly orchestrated responses, stress-responsive signaling subsides owing to the production of negative regulators such as chaperones that bind to the stress sensor.

Ribosome-associated chaperones (Maier et al. 2005; Merz et al. 2008; Preissler and Deuerling 2012; Chartron et al. 2016) hand off proteins to multiple folding pathways, including the HSP70-Hsp40-nucleotide exchange factor folding pathway, the Hsp90-cochaperone-folding pathway, or the TRiC chaperonin-folding pathway in the cytosol (Ellis and Hartl 1999; Young et al. 2004; Tang et al. 2007; Yam et al. 2008; Voisine et al. 2010). How these proteostasis network pathways work together and create emergent capacity as a system is only poorly understood. The ubiquitin proteasome system is

intimately linked to each of these chaperone systems by kinetic partitioning; futile attempts at folding eventually redirect terminally misfolded substrates to ubiquitin conjugation-mediated degradation (Leggett et al. 2002; Lecker et al. 2006; Finley 2009). Proteasome degradation rids the cell of dangerous aggregation-prone species and reduces the load on the proteostasis network. Similar partitioning, sometimes utilizing ubiquitin conjugation, links unsuccessful folding by chaperone pathways to the lysosomal degradation of these nonnative conformations via autophagy (Cuervo and Dice 1996; Kruse et al. 2006; Tsvetkov et al. 2010; Wong and Cuervo 2010; Arias and Cuervo 2011). There appear to be compensatory mechanisms that up-regulate the proteasome when autophagy is impaired and vice versa (Lamark and Johansen 2010; Zhu et al. 2010; Chen and Yin 2011).

While we are far from a systems-level understanding of the functions and regulation of the proteostasis network (Vidal et al. 2011), we do know enough about stress-responsive signaling pathways to begin to manipulate them to down- and up-regulate proteostasis network components in the appropriate stoichiometry to reestablish proteostasis (Mori 2000; Boyce et al. 2005; Schröder and Kaufman 2005; Shang et al. 2007; Walter and Ron 2011; Calamini et al. 2012; Gallagher and Walter 2016; Gallagher et al. 2016). This evolved regulatory strategy appears to be able to alleviate the deficiencies of proteostasis that lead to specific diseases when we are young (Westerheide et al. 2004, 2009; Westerheide and Morimoto 2005; Dai et al. 2007; Balch et al. 2008; Mu et al. 2008a,b; Cowen et al. 2009; Powers et al. 2009; Whitesell and Lindquist 2009; Tsytler et al. 2011). In model organisms, stress-responsive signaling pathways are attenuated upon aging via epigenetic and likely other mechanisms (Morley et al. 2002; Cohen et al. 2006, 2009, 2010; Ben-Zvi et al. 2009; Labbadia and Morimoto 2015; Solis et al. 2018). The ultimate goal is to be able to activate stress-responsive signaling pathways in the elderly exhibiting degenerative diseases, including neurodegenerative diseases.

Notably, the misfolding and/or aggregation of individual vulnerable proteins not only com-

promise their own folding, but that of additional proteins sharing common proteostasis pathways (Powers et al. 2009). Even one mutated aggregation-prone protein can consume considerable proteostasis network capacity, putting numerous members of the proteome that require this capacity at risk of misfolding and aggregation (Gidalevitz et al. 2006). In turn, this leads to wider proteome misfolding or aggregation if stress-responsive signaling cannot efficiently reestablish proteostasis (Balch et al. 2008). Thus, small-molecule, stress-responsive signaling pathway activators are one way to counter the influence of a misfolding- or aggregation-prone protein in an organism and holds high promise for increasing health span, the number of years free of significant pathology (Westerheide et al. 2004, 2009; Mu et al. 2008b).

In contrast to adapting proteostasis network activity (i.e., the biology of protein homeostasis), another approach is to make the energetics of a specific misfolding-prone protein less problematic, utilizing small-molecule-binding-associated stabilization (Miroy et al. 1996; Sawkar et al. 2002, 2006; Cohen and Kelly 2003; Hammarstrom et al. 2003; Razavi et al. 2003; Johnson et al. 2005; Tojo et al. 2006; Yu et al. 2007; Choi et al. 2010; Johnson et al. 2010; Bulawa et al. 2012; Makley et al. 2015). While the sequence of a protein specifies its folding, unfolding, misfolding energetics, as well as its concentration-dependent aggregation kinetics, the energetics of an individual mutant protein prone to misfolding can be selectively tuned if the folded state of that protein has a hydrophobic depression or cavity where a small molecule can bind (Miroy et al. 1996; Sawkar et al. 2002, 2006; Cohen and Kelly 2003; Hammarstrom et al. 2003; Razavi et al. 2003; Johnson et al. 2005; Tojo et al. 2006; Yu et al. 2007; Choi et al. 2010; Bulawa et al. 2012; Makley et al. 2015). Small molecules that bind to the native state of mutant proteins lower their folding free energy and thus increase the folded population relative to the unfolded, misfolded, and aggregated states. After folding is complete, pharmacologic chaperone binding to the native state can slow unfolding events that can lead to proteolysis and/or aggregation, hence the reason some of

these pharmacologic chaperones are called kinetic stabilizers (Johnson et al. 2005; Sekijima et al. 2006; Tojo et al. 2006; Bulawa et al. 2012). Indeed, targeting a particular misfolding-prone protein with a small molecule that binds to its native state not only can restore proteostasis of that energetically compromised protein, but this strategy also has the promise of restoring proteostasis to other energetically compromised proteins that require the same proteostasis network components for folding (Gidalevitz et al. 2006). Now that there are quite a few pharmacologic chaperones/kinetic stabilizers available to stabilize individual proteins, some of which are regulatory agency approved, it is important to try these at restoring proteostasis in diseases of interest where the misfolding-prone protein itself lacks available kinetic stabilizers (Miroy et al. 1996; Fan et al. 1999; Fan 2001, 2003; Sawkar et al. 2002; Hammarstrom et al. 2003; Razavi et al. 2003; Sekijima et al. 2006; Tojo et al. 2006; Bulawa et al. 2012). For example, if a given secreted misfolding-prone protein linked to a disease for which there is no disease-modifying drug therapy utilizes the endoplasmic reticulum (ER) Hsp70-40-nucleotide exchange factor pathway for folding, it is possible that transthyretin and α 1 antitrypsin pharmacologic chaperones/kinetic stabilizers could ameliorate a misfolding disease caused by a protein that they do not target. The hypothesis is that by making wild-type transthyretin or α 1 antitrypsin folding more efficient pharmacologically, this would take a load off the ER Hsp70-40-nucleotide exchange factor pathway. This strategy might afford the Hsp70-40-nucleotide exchange factor pathway sufficient capacity to restore proteostasis to a disease-associated protein that also utilizes the same proteostasis pathway.

In this review, we focus on pharmacological adaptation of the ATF6 arm of the unfolded protein response stress-responsive signaling pathway for rebalancing proteostasis in the ER and downstream secretory pathway (Yoshida et al. 1998; Nadeau et al. 2006, 2007; Wiseman et al. 2010; Plate et al. 2016a; Paxman et al. 2018). This approach is appealing because enhancement of the biology of proteostasis might

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be capable of ameliorating complex multifaceted problems and holds particular promise in combating aging-associated degenerative maladies linked to secreted proteins (Shoulders et al. 2013; Cooley et al. 2014). However, proteostasis networks are finely tuned and highly integrated, hence manipulating them does raise the specter of unintended consequences, especially if alterations are carried out directly (e.g., by targeting Hsp90) as opposed to activating a given stress-responsive signaling pathway that transcriptionally controls Hsp90. Hence, we focus on altering proteostasis network capacity through stress-responsive signaling pathways, namely, the ATF6 pathway. That said, I see a future where pharmacologic chaperones/kinetic stabilizers will be used in combination with stress-responsive signaling pathway modulators (Mu et al. 2008b). Stress-responsive signaling pathway activators resculpt the folding free energy diagram up to, but not including, the native state, lowering aggregation and in some cases catalyzing folding (i.e., pushing the protein toward the native state) (Fig. 1). In contrast, a pharmacologic chaperone binds to the native state of a protein, lowering its free energy and increasing the population of the folded state by pulling the polypeptide from less well-folded states (Fig. 1; Schröder and Kaufman 2005; Ron and Walter 2007; Mu et al. 2008b; Wiseman et al. 2010; Walter and Ron 2011).

THE PROTEOSTASIS CHALLENGES OF THE ENDOPLASMIC RETICULUM SECRETORY COMPARTMENT

Approximately one-third of the proteome traffics through the ER to the Golgi and onto the lysosome and the extracellular space, by way of highly dynamic networks of membrane-delimited vesicles traveling along microtubules (Fellwell et al. 2001; Schröder and Kaufman 2005; Ron and Walter 2007). The vesicles bud from one secretory pathway subcompartment and fuse with the next in a highly regulated manner. The secreted proteome faces a number of challenges, including extreme crowding, the requirement to make stabilizing disulfide bonds by ox-

idation, the high kinetic energy of protein collisions that can facilitate aggregation and lead to gain-of-toxic-function diseases (e.g., liver cancer linked to misfolded and misassembled $\alpha 1$ antitrypsin accumulating in the hepatocyte ER), and the low folded free energy state of the secreted proteome (Eriksson et al. 1986). Consequently, secreted proteins in the ER are susceptible to misfolding and degradation, potentially leading to loss-of-function diseases such as the lysosomal storage diseases wherein an energetically compromised mutant protein is largely degraded by the proteasome or the lysosome (Cohen and Kelly 2003; Mu et al. 2008b). Numerous secreted proteins are cotranslationally modified by branched glycans attached to the side chain of Asn. This so-called *N*-glycosylation process is key to both folding efficiency and function for both intrinsic reasons (the conserved *N*-glycan makes native state stabilizing interactions with the protein) (Culyba et al. 2011) and extrinsic reasons (*N*-glycosylated glycoproteins use the glycan and protein-binding chaperones calnexin and calreticulin pathways for folding and degradation, pathways that are only found in the ER) (Yoshida 2003; Williams 2006; Hebert et al. 2014).

The proteostasis pathways of the ER include the Hsp70-Hsp40-nucleotide exchange factor and Hsp90-cochaperone pathways, which play important roles in folding and degradation of the secreted proteome (Hartl et al. 2011). However, the proteostasis pathways unique to the ER are equally critical: the *N*-glycan- or lectin-binding calnexin- and calreticulin-based proteostasis pathways that help *N*-linked glycoproteins to fold or direct them to degradation through the glucosidases that target chronically misfolded proteins to ER-associated degradation (ERAD) (Ellgaard et al. 1999; Williams 2006; Hebert et al. 2014). There are also numerous protein-processing proteases and protein-folding enzymes in the secretory pathway, including disulfide oxidases and disulfide isomerases, to name a few (Schröder and Kaufman 2005; Ron and Walter 2007; Hartl et al. 2011).

All proteins residing in and passing through the secretory pathway are encoded by nuclear genes. These proteins are cotranslationally in-

Figure 1. Pharmacologic stress-responsive signaling pathway activation coordinates transcription and translation of chaperone pathways and folding enzymes that resculpt the folding free energy diagram of the mutant protein-of-interest in the endoplasmic reticulum (ER), enhancing its folding and minimizing misfolding, increasing the population of the mutant protein in the folded state. Pharmacologic chaperones bind to the folded state of the mutant protein, lowering its free energy, further increasing its population. Collectively, these mechanisms of action create more folded mutant protein that can engage the trafficking machinery, leading to increased function. (Figure from Mu et al. 2008b; reprinted, with permission, from Elsevier © 2008.)

serted into the ER through the translocon, a complicated machine that enables membrane proteins to be inserted into the membrane with the proper topology and fold while simultaneously allowing soluble ER proteins to pass through the translocon in a largely unfolded

conformation that is read out by the oligosaccharyl transferase enzyme complex that sometimes attaches an *N*-glycan when it detects an Asn-xxx-Ser/Thr “sequon” (Shan and Walter 2005; Johnson 2009; Larkin and Imperiali 2011). There is no mechanism for degrading

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nonnative proteins within the confines of the ER that we know of (Nakatsukasa and Brodsky 2008). Instead, these conformationally abnormal proteins are delivered by vesicle trafficking to the lysosome by one of several possible mechanisms (Kruse et al. 2006; Kon and Cuervo 2010) or they are exported into the cytoplasm for degradation by the proteasome, in the process called ERAD (Werner et al. 1996; Brodsky and McCracken 1997; McCracken et al. 1998; McCracken and Brodsky 2003; Brodsky 2012). Both pathways are complex, highly regulated, and incompletely understood.

STRESS-RESPONSIVE SIGNALING PATHWAYS TO MAINTAIN PROTEOSTASIS IN THE ENDOPLASMIC RETICULUM, DOWNSTREAM ORGANELLES, AND IN THE EXTRACELLULAR SPACE

Historically, it was mysterious how proteostasis problems within the membrane-delimited ER could be perceived, and responded to, by regulatory mechanisms involving transcription in the nucleus and translation in the cytoplasm. The solution is now well understood: there are

three key stress-responsive signaling pathways associated with the stress sensors IRE1, ATF6, and PERK (Fig. 2), which have one domain in the ER to sense excessive proteome misfolding and/or aggregation and another in the cytoplasm to enable the transcriptional and translational responses (Mori 2000; Schröder and Kaufman 2005; Ron and Walter 2007; Walter and Ron 2011). As nonnative proteins accumulate within the ER lumen, they recruit away chaperones that negatively regulate each of the three stress sensors. Alternatively, aggregates may directly interact with the stress sensors to activate them. Activation is associated with changes in the conformation and/or quaternary structure of the stress sensor that transduces a signal across the ER membrane to each of the stress sensor's cytoplasmic effector domains (Fig. 2; Mori 2000; Schröder and Kaufman 2005; Ron and Walter 2007; Li et al. 2010; Walter and Ron 2011). This ultimately results in the generation or activation of a transcription factor, leading to an increase or reduction of hundreds of proteostasis network components that create emergent functions, restoring proteostasis. Chaperone levels rise as a result of the

Figure 2. Simplified depiction of the three arms of the unfolded stress response stress-responsive signaling pathway courtesy of Lars Plate. ER, Endoplasmic reticulum; ERAD, ER-associated degradation. (Figure from Wiseman et al. 2010; adapted, with permission, from Elsevier © 2010.)

transcriptional program, and they bind to the stress sensors turning them off.

DISCOVERING AND VALIDATING SMALL-MOLECULE ACTIVATORS OF THE ATF6 ARM OF THE UNFOLDED PROTEIN RESPONSE

Historically, activation of all three arms of the unfolded protein response (UPR) has been achieved by thapsigargin treatment (inhibitor of the Ca^{2+} ATPase SERCA that transfers Ca^{2+} from the cytosol to the ER) or tunicamycin administration (inhibitor of N-linked glycosylation) (Mori 2000; Schröder and Kaufman 2005; Ron and Walter 2007; Li et al. 2010; Walter and Ron 2011). Either treatment leads to misfolding and aggregation of the secreted proteome in the ER, which activates stress-responsive signaling by causing proteotoxic stress. While these pharmacologic agents have served investigators well in terms of delineating the signaling pathways for each arm of the UPR, clearly pharmacologically enhancing protein misfolding and aggregation is undesirable for treating proteinopathies.

The small-molecule BIX achieves expression of the ATF6-target gene BiP through an ATF6-dependent mechanism. However, this pharmacologic agent does not induce other genes associated with the ATF6 transcriptional program (Kudo et al. 2008), making it unlikely that BIX will be useful for ameliorating proteinopathies. Regulation of all target genes in the transcriptional response is likely required to get maximal benefit from stress-responsive signaling.

Thus, the effort of the Wiseman and Kelly Laboratories to use a transcriptional profiling approach early in the drug discovery process, before functional assessment, ensured that the small molecules we were pursuing preferentially activated the ATF6 transcriptional program, as opposed to global UPR activation or activation of other stress-responsive signaling pathways (Shoulders et al. 2013). The activation of ATF6 involves trafficking of the full-length ATF6 protein from the ER to the Golgi, where ATF6 is processed by site-1 and site-2 proteases (Ye et al. 2000a,b). This processing releases the active cy-

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tosolic bZIP transcription factor, enabling its nuclear localization at which location the transcription factor can bind so-called ERSE sites in DNA as a dimer, leading to transcriptional reprogramming (Fig. 2; Yoshida et al. 1998; Nandanaka et al. 2006, 2007).

We employed a cell-based reporter high-throughput screen, together with counter-screens to eliminate compounds that activated undesirable stress-responsive signaling pathways, followed by transcriptional profiling to identify nontoxic small molecules that preferentially activated the ATF6 transcriptional program (Calamini et al. 2012; Plate et al. 2016a). The goal was to identify ATF6 activators that transcriptionally phenocopy the ability of the ATF6 transcription factor generated at a physiological concentration by chemical genetic means to preferentially activate the ATF6 transcriptional program (Shoulders et al. 2013). The screen employed a three-tiered strategy to identify small molecules that preferentially activated the ATF6 arm of the UPR (Plate et al. 2016a). A cell-based transcriptional reporter assay, wherein a fragment of the BiP (ER Hsp70) promoter driving expression of firefly luciferase (ERSE-FLuc) was employed (Yoshida et al. 1998; Nandanaka et al. 2006, 2007). The dependence of ERSE-FLuc activation on XBP1s and ATF6 in HEK293DAX cells that stably express tet-inducible XBP1s and a trimethoprim-regulated dihydrofolate reductase (DHFR)-ATF6 revealed that BiP is preferentially induced by ATF6 (Lee et al. 2003; Shoulders et al. 2013). The ERSE-FLuc reporter was stably transfected into a HEK293T-Rex cell clone that was employed to screen the 644,951 small-molecule Scripps Drug Discovery Library to identify molecules that activate the ERSE-FLuc reporter with robust statistics (Plate et al. 2016a). A subset of these molecules was previously shown to activate the cytosolic heat shock response utilizing an analogous transcriptional reporter assay and were therefore eliminated from further consideration (Calamini et al. 2012). The top 281 compounds remaining were counterscreened using a luciferase reporter assay reporting on the activation of the IRE1/XBP1s arm of the UPR. Two-thirds of the screening hits activate the ERSE-FLuc

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reporter >2-fold better than the XBP1s-RLuc reporter, indicating ATF6 arm-selective UPR activation and demonstrating the power of the screening strategy. Multiplex gene expression (MGE) profiling was then performed on all 281 hits individually in HEK293T-Rex cells to identify compounds that preferentially activate the ATF6 transcriptional program relative to other possibilities. The Wiseman Laboratory recently developed a targeted RNA-seq approach that scrutinizes the selectivity of stress-responsive pathway activation utilizing multiple reporter genes from each transcriptional program. As the RNA-seq methodology decreases in price, it will soon be practical to carry out an RNA-seq experiment to evaluate the extent that an ATF6-activating compound recapitulates the transcriptional reprogramming selectivity afforded by the transcription factor itself.

The small molecule *N*-(2-hydroxy-5-methylphenyl)-3-phenylpropanamide **147** (Fig. 3) up-regulates the majority of transcripts up-regulated by the ATF6 transcription factor itself, based on an RNA-seq experiment, although the amplitude of changes in transcript levels is about 50% that afforded by the ATF6 transcription factor (Plate et al. 2016a). Of all the molecules we analyzed in detail thus far, compound **147** appears to be the most selective ATF6 activator. The 2-amino-*p*-cresol substructure comprises the majority of the top 79 ATF6 activators exhibiting good selectivity for activation of the ATF6 transcriptional program over the other UPR transcriptional programs, as well as other stress-responsive signaling pathways. The activation of the ATF6 arm of the UPR by **147** requires that ATF6 is present. No **147** ATF6 acti-

vation is observed in ATF6^{-/-} mouse embryonic fibroblasts (Plate et al. 2016a). Compound **147** also requires site-1 protease activity for ATF6 activation, as pharmacological inhibition of site-1 protease antagonizes the function of **147** (Plate et al. 2016a).

MEDICINAL CHEMICAL AND CHEMICAL BIOLOGICAL EFFORTS SUGGEST THAT **147** REACTS WITH ER DISULFIDE ISOMERASES ACTIVATING THE ATF6 ARM OF THE UPR

The ATF6 arm of the UPR appears to be activated in response to ER stress through a process involving reduction of inter- and intramolecular disulfides in ATF6 that results in formation of a reduced ATF6 monomer that is trafficking competent (Nadanaka et al. 2006, 2007). The hypothesis is that this reduced ATF6 monomer traffics to the Golgi where it is processed by the site-1 and site-2 proteases to generate the *N*-terminal fragment of ATF6, the active transcription factor responsible for the transcriptional program (Ye et al. 2000a,b).

The acetylated 2-amino-*p*-cresol substructure in **147** and related ATF6 selective activators originating from the high-throughput screen suggested to us that **147**-dependent ATF6 activation may involve metabolic oxidation of **147** by P450s to form a quinone methide that preferentially reacts with a protein(s) leading to ATF6 activation (Paxman et al. 2018). This idea is consistent with the result that **147** becomes inactive when cotreated with the P450 inhibitor resveratrol (Paxman et al. 2018). Analogs of **147** wherein the methyl or the hydroxy group are substituted are inactive, also indicating that functional groups required for forming the quinone methide are critical for ATF6 activation (Paxman et al. 2018). Fortunately, we were able to prepare a version of **147** bearing an alkyne (i.e., **147-alkyne**) (Fig. 4), that activates the ATF6 arm of the UPR analogously to **147**. The quinone methide was envisioned to form an irreversible covalent conjugate with cellular proteins, including the target(s) responsible for activation of the ATF6 arm of the UPR (Fig. 4). A copper-catalyzed azide-alkyne cycloaddition

Figure 3. Line drawing depiction of the chemical structure of the small-molecule ATF6 selective activator **147**.

Figure 4. Line drawing depiction of the chemical structure of the small-molecule ATF6 selective activator **147-alkyne** employed for target identification using affinity chromatography mass spectrometry–mass spectrometry. **147-alkyne** undergoes oxidation to form a QM or a QI, which gets labeled covalently by protein conjugates. (Figure adapted from Paxman et al. 2018 and reprinted under the terms of the Creative Commons Attribution License.)

or (CuAAC) “click” reaction (Besanceney-Webler et al. 2011) between an azide-functionalized biotin (Yang et al. 2010) and the alkyne now covalently attached to its target enables affinity chromatography–mass spectrometry–mass spectrometry bottom-up mass spectrometry to identify the target or targets of **147-alkyne** (Paxman et al. 2018).

We showed that **147** activates the ATF6 arm of the UPR in an ALMC-2 plasma cell line derived from a light chain amyloidosis patient. We prepared lysates from ALMC-2 cells treated with **147-alkyne** for 18 h, and the biotin affinity tag was clicked on to facilitate affinity chromatography using streptavidin beads. The **147-alkyne**-based protein conjugates isolated by this approach were subjected to SDS-PAGE. In-gel digestion of the **147-alkyne** conjugates followed by mass spectrometry–mass spectrometry characterization identified four ER protein disulfide isomerases (PDIA1, PDIA4, PDIA6, and TXNDC5), as prevalent bands on the SDS-PAGE that were efficiently eliminated by adding a 5-fold excess of **147** together with **147-alkyne** during the 18-h treatment period (Paxman et al. 2018). An affinity chromatography mass spectrometry–mass spectrometry experiment employing **147-alkyne**, with and without compet-

itor **147** (no affinity tag), employing tandem mass tags for multiplexing identified another two protein disulfide isomerases (PDIA3 and TMX1) in addition to the four disulfide isomerases (PDIA1, PDIA4, PDIA6, and TXNDC5), already identified by SDS-PAGE, that form conjugates with **147** (Paxman et al. 2018). Disulfide isomerases caught our attention because it is logical to see how impeding their activity through **147** conjugation could lead to net reduction of ATF6, permitting its trafficking and enabling ATF6 activation (Nadanaka et al. 2006, 2007). The observed modification of ER-localized proteins by **147** that results in activation of the ATF6 arm of the UPR is not a general feature of other small-molecule quinone methides, which modify most thiol-containing proteins in multiple compartments of the cell (Backus et al. 2016). Collectively, these data support the notion that **147** is a prodrug that gets activated by P450 in or proximal to the ER, activating the ATF6 arm of the UPR by leading to net reduction of ATF6 (Paxman et al. 2018). A relevant example of a more typical quinone methide is celastrol, which is triterpenoid natural product that harbors a quinone methide that activates numerous stress-responsive signaling pathways (targets in numerous compartments), including

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the antioxidant response and the heat shock response (Westerheide et al. 2004; Trott et al. 2008).

To further scrutinize the proposed mechanism of action of **147**, a genetic approach was applied, wherein PDIA1, PDIA3, PDIA4, PDIA5, or PDIA6 were short hairpin RNA (shRNA)-depleted by 25%–50% based on PCR assessment. These results demonstrate that modest depletion of a subset of PDIs selectively inhibits **147**-dependent activation of ATF6, suggesting that covalent modification of PDIA3, PDIA4, and PDIA5 contribute to ATF6 activation by **147** (Paxman et al. 2018).

Additional studies on the mechanism of action of **147** examined a set of small molecules called Ceapins discovered by the Walter group (Gallagher and Walter 2016; Gallagher et al. 2016). Ceapins inhibit ER ATF6 activation by stress through stabilizing ATF6 oligomers in the ER and preventing ATF6 monomer trafficking to the Golgi. Stabilization of ATF6 oligomers by Ceapins should inhibit **147**-dependent ATF6 activation by preventing disulfide-mediated disruption of the ATF6 oligomers into the reduced monomers required for trafficking. Indeed, **147**-dependent activation of ATF6 was inhibited by coadministration of increasing concentrations of the Ceapin, CP7 (Paxman et al. 2018). These data are consistent with **147** playing an important role in promoting ATF6 disulfide reduction through protein disulfide isomerase conjugation and ATF6 dissociation prior to ATF6 trafficking to the Golgi for proteolytic activation. Thus, the small molecule **147** appears to be a prodrug converted into a quinone methide electrophile in or in proximity to the ER, which preferentially activates the ATF6 signaling pathway without activating other stress-responsive signaling pathways (Paxman et al. 2018). Other stress-responsive signaling pathways can be activated by similar electrophiles, including the IRE1 and PERK UPR signaling pathways, the heat shock response, and the NRF2 oxidative stress response. Thus, it is remarkable that transcriptional profiling by RNA-seq and other approaches demonstrates how selective ATF6 activation by **147** is (Plate et al. 2016a; Paxman et al. 2018).

TOWARD AMELIORATION OF DISEASES THROUGH ATF6 ACTIVATION

The capacity of the UPR stress-responsive signaling pathways to distinctly influence ER proteostasis by transcriptional reprogramming and translational attenuation has significant potential to alleviate proteinopathies associated with secreted proteins (Hetz 2012). Here I will focus on the potential of activating the ATF6 arm of the UPR for ameliorating diverse proteinopathies.

In collaboration with Chris Glembotski, an expert in myocardial ischemia/reperfusion injury, the Glembotski, Wiseman, and Kelly Laboratories demonstrated that selective pharmacologic activation of the ATF6 arm of the UPR by IV administration of **147** (2 mg/Kg) during reperfusion preserves heart function in a murine model of myocardial ischemia/reperfusion injury (Blackwood et al. 2019). The ATF6 target genes were up-regulated in the heart after IV injection of **147**, with transcript levels reaching a maximum level at 24 h and falling to baseline over the course of a week. To examine the influence of ATF6 activation by **147** in various murine tissues, an inactive control compound that closely resembles **147** or **147** were administered by IV, and the effects of these compounds on tissue damage were examined in the heart via the acute ischemia/reperfusion murine model, in the murine kidney via transient unilateral renal portal system occlusion, and in the brain via transient unilateral middle cerebral artery occlusion (Blackwood et al. 2019). Notably, even when administered only at the time of reperfusion, **147** significantly decreased infarct sizes in all three tissues when measured 24 h after reperfusion (Blackwood et al. 2019). Thus, pharmacologic activation of the ATF6 stress-responsive signaling pathway is a proteostasis network-reprogramming-based therapeutic strategy for ameliorating ischemia/reperfusion damage, highlighting the potential of arm-selective UPR activation for treating a wide range of diseases (Hetz 2012; Plate et al. 2016b; Blackwood et al. 2019).

The systemic amyloid diseases result from the secretion of destabilized, properly folded amyloidogenic proteins into the blood that un-

dergo conformational excursions during circulation in the extracellular space, facilitating aggregation into a spectrum of proteotoxic aggregates including amyloid fibrils (Eisele et al. 2015). The pharmacologic and genetic evidence that protein aggregation causes the destruction of postmitotic tissue leading to neurodegenerative diseases and degenerative diseases in general is compelling (Coelho et al. 2012, 2013; Maurer et al. 2018). We showed that chemical genetic up-regulation of the ATF6 transcription factor increases ER quality control stringency for the amyloidogenic protein transthyretin, preferentially reducing secretion of disease-associated transthyretin variants proportional to the variants' tetramer destabilization (Shoulders et al. 2013; Chen et al. 2014). Decreased variant transthyretin secretion slows extracellular, concentration-dependent aggregation of transthyretin. Thus, the selective small-molecule activator of ATF6 **147** was expected to reduce the secretion of destabilized mutated transthyretin from hepatocytes, wherein **147** activates the ATF6 arm of the UPR. Hepatocytes pretreated with **147** reduced A25T transthyretin secretion by 45%, without altering wild-type transthyretin secretion or the secretion of the endogenous secreted proteome, according to ³⁵S metabolic labeling (Plate et al. 2016a). Thus, it seems clear the ATF6 activation has the potential to ameliorate human amyloid diseases by enhancing ER quality control (i.e., degradation of mutant secreted amyloidogenic proteins).

CONCLUSION

Considerable effort goes into validating a pharmacologic agent, such as the ATF6 activator **147** outlined above. However, once **147** pharmacodynamics are validated, the small molecule can be used in a spectrum of disease models to discern utility. Having a validated ATF6 activator with established murine tissue pharmacodynamics, even allows negative data in murine models of disease to be interpreted with some confidence. Pharmacologic activators of additional stress-responsive signaling pathways either have been or are in the process of being validated. Having this collection of stress-

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responsive signaling pathway activators will be very useful for establishing clinical uses for a diverse collection of stress-responsive signaling pathway modulators.

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