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Chemical Biology Framework to Illuminate Proteostasis

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Abstract

Protein folding in the cell is mediated by an extensive network of >1,000 chaperones, quality control factors, and trafficking mechanisms collectively termed the proteostasis network. While the components and organization of this network are generally well established, our understanding of how protein-folding problems are identified, how the network components integrate to successfully address challenges, and what types of biophysical issues each proteostasis network component is capable of addressing remains immature. We describe a chemical biology–informed framework for studying cellular proteostasis that relies on selection of interesting protein-folding problems and precise researcher control of proteostasis network composition and activities. By combining these methods with multifaceted strategies to monitor protein folding, degradation, trafficking, and aggregation in cells, researchers continue to rapidly generate new insights into cellular proteostasis.

1. INTRODUCTION

Protein homeostasis, or proteostasis, refers to the state in which the localization, concentration, and conformation of cellular proteins are optimal. Proteostasis is managed by the proteostasis

network, a complex collection of factors involved in preventing, identifying, and resolving protein-folding challenges. In metazoan cells, this network is individually optimized within specific subcellular compartments. The expression and activity of diverse network components that mediate protein translation, folding, transport, and degradation are up- and downregulated as required to effectively respond to intrinsic proteomic demands and extrinsic environmental stressors. Dynamic regulation of the proteostasis network is mediated by compartment-specific stress responses, including the heat shock response (HSR) in the cytosol and nucleus (1), the unfolded protein response (UPR) in the secretory pathway (2), and the mitoUPR in mitochondria (3). These stress responses sense emerging protein-folding problems and then signal to the cellular protein-production machinery to alter the nascent protein load and to the nucleus to resculpt proteostasis networks and resolve the proteostatic challenge.

Failed proteostasis, whether due to dysregulated expression of proteostasis network components, excessive external stress, or destabilizing mutations in client proteins, often leads to disease. Pathology may arise due to loss-of-function, in which a protein within a critical pathway fails to fold and the consequent absence of function disrupts a critical biological activity. Examples include cystic fibrosis (4) and the lysosomal storage diseases (5). Alternatively, disease can arise by gain-of-function pathways in which misfolded proteins and/or aggregated client proteins either are directly toxic or otherwise deleteriously affect critical cellular mechanisms. Examples include neurodegenerative disorders like Alzheimer's and Parkinson's diseases. Importantly, the distinction between loss-of-function and gain-of-function pathologies is fuzzy, with many diseases displaying features of both. In the collagenopathies, mutations that cause collagen misfolding are widely assumed to cause a loss-of-function phenotype, but emerging evidence emphasizes the likely importance of the gain-of-function effects of collagen misfolding that disrupt the endoplasmic reticulum (ER) and overall cell health (6, 7). Similarly, in the neurodegenerative disorders it remains unclear whether there may be an unappreciated key function of the aggregating, disease-associated proteins such as amyloid-β and α-synuclein (8). Regardless of the detailed origins of pathology, pharmacologic resculpting of the cellular proteostasis network to improve the cell's capacity to address protein misfolding and aggregation is one appealing strategy to address these diverse disorders.

Maladaptive functioning of the proteostasis network can also promote disease. For example, cancer cells often face chronic proteostatic challenge owing to their rapid growth, high mutation

rates, and exposure to problematic environments (e.g., hypoxia and nutrient starvation). Many cancers display chronic overexpression of proteostasis machinery components (9, 10), likely to address these challenges. As such, cancer cells can be particularly susceptible to chemotherapies that disrupt proteostasis network pathways (11, 12). Pathogens also often hijack their host's proteostasis network to support folding of their own proteins. In particular, many viruses rely on the proteostasis network of both the cytosol and the ER to fold proteins necessary for their infection and propagation (13). This phenomenon was recently shown to have important implications for the ability of viruses to rapidly adapt to environmental pressures (14–17). Targeted disruption of host proteostasis networks may, therefore, prove to be an effective antiviral therapeutic strategy (18, 19).

Fundamental interest in the mechanisms of cellular protein folding motivates much work in the proteostasis field. From the perspective of biomedical research, it is also critical that we develop a thorough understanding of the strategies and molecular mechanisms by which cells facilitate productive protein folding and respond to protein misfolding. Until such understanding is achieved, both the identification of correct proteostasis network components as targets for therapeutic development and any potential deleterious effects that may result from modulating these pathways will remain unclear.

2. EXPERIMENTAL FRAMEWORK: SELECTING CONTEXT

In this review, we present a comprehensive framework the chemical biologist or biochemist can deploy to illuminate metazoan proteostasis (**Figure 1*a***). The first step is to select an appropriate experimental substrate or context for cellular protein-folding studies. After an appropriate client is chosen, the next stage is to selectively perturb pathways within the proteostasis network. Following such perturbation, the impact on client folding, quality control, and trafficking can be assessed using any of a wide variety of assays. By quantifying which aspects of protein folding, degradation, or localization are altered by proteostasis network modulation, the relative roles of diverse components of the proteostasis network can be distinguished. The information obtained can then guide further in vitro studies to define molecular mechanisms. Alternatively, the results can inform the choice of targets for the development of drug-like small molecules for testing in the preclinical setting.

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Figure 1 A chemical biology–based experimental framework to illuminate proteostasis. (*a*) Following selection of compelling experimental contexts displaying interesting protein-folding challenges, small-molecule perturbation of the proteostasis environment is combined with assays to monitor protein folding, trafficking, and quality control to provide insight into proteostasis. (*b*) Examples of designer proteins used to study proteostasis are shown: destabilized variants of firefly luciferase (FLucDM) and HaloTag [HaloTag(K73T)], two proteins whose wild-type forms are able to fold under basal proteostasis conditions. These destabilized variants rapidly misfold and/or aggregate when exposed to proteotoxic stress. Misfolded FLucDM can be detected by loss of luciferase activity, while aggregation of HaloTag can be detected by using a solvatochromic ligand (AgHalo 550) whose fluorescence is induced within the hydrophobic environment of protein aggregates. (*c*) The location of the proteostasis boundary is determined by the composition and activities of the cellular proteostasis network (149). Protein variants falling within this boundary have sufficiently high stability and folding rate, as well as a low misfolding rate, allowing them to function. Perturbing the proteostasis network can shift the proteostasis boundary, thereby modifying the accessible mutational landscape and providing insight into the functions of the perturbed proteostasis network components. Panel *c* adapted with permission from Reference 149.

There are many factors to consider when selecting an experimental context in which to investigate proteostasis mechanisms. Localization of the protein client to be considered is key. For example, secreted and lysosomal proteins can be used to gain insights into ER proteostasis, as the ER is the site for their folding and quality control. In contrast, membrane proteins access both the cytosolic and ER proteostasis networks, providing an opportunity to study the interplay between these pathways. A second consideration is the ease of assessing protein folding, localization, and degradation, which is largely determined by the availability of activity or folding assays and high-quality antibodies. Further, while the choice of context is often guided by the opportunity to gain important insights into proteostasis mechanisms, substrates with disease relevance can yield a value-added benefit owing to direct pathologic relevance.

2.1. Endogenous Clients as Paradigms for the Study of Cellular Proteostasis

Endogenous proteins can provide compelling experimental contexts for studies of cellular proteostasis. One advantage is that these clients have co-evolved with their cognate proteostasis networks, ensuring the biological relevance of insights obtained. On the other hand, serious technical limitations can be encountered with respect to the availability of assays to detect activity, folding, degradation, and trafficking. This experimental trade-off must be considered when choosing to use an endogenous client versus a designer probe of cellular proteostasis (see Section 2.2). One widely employed solution to this problem is fusion of the endogenous protein

to a fluorescent or luminescent protein or antibody epitope tag to facilitate detection, although such fusion can adversely affect the properties of the endogenous client.

Wild-type proteins with unusual properties, such as large size, atypical conformation, or complex folding pathways, can provide particularly useful experimental contexts to obtain novel insights into proteostasis. Examples include large, fibrillar proteins and complex membrane proteins. Collagen, for example, displays the unusual attributes of a folding process that begins at the extreme C terminus, a fibrillar structure that precludes trafficking in normal secretory vesicles, and a complex assembly pathway that renders quality control challenging. Studies of wild-type collagen proteostasis continue to yield fresh perspectives on mechanisms of secretory pathway proteostasis (20), including the recent discovery of a mechanism for ER-to-lysosome-associated degradation (21).

Another valuable approach is to leverage the genetics of protein misfolding disease to inform the choice of substrates that seem likely to rely on unique or understudied aspects of the proteostasis network. Numerous genetic disorders feature mutations that cause rapid protein degradation owing to inefficient protein folding. The proteins involved provide uniquely suitable paradigms to study mechanisms of quality control. For example, regulation of the proteostasis of membrane proteins has been extensively studied by using destabilized, disease-causing variants of the cystic fibrosis transmembrane conductance regulator (CFTR) (22). Mutant β-glucocerebrosidase and other enzymes involved in the lysosomal storage diseases (5), as well as the null–Hong Kong variant of α-1-antitrypsin (23), have been used to understand how the ER attempts to solve proteostatic challenges associated with misfolding soluble proteins within the secretory pathway.

Gain-of-toxic-function diseases associated with the accumulation of protein aggregates, either within or outside cells, can also provide valuable experimental contexts to study proteostasis mechanisms. Proteostasis failures associated with the accumulation of protein aggregates in the cytosol and nucleus have been studied by using proteins involved in neurodegenerative disorders, such as huntingtin, amyloid, and tau (24, 25). The availability of protein variants with diverse aggregation propensities has proved invaluable for the discovery of aggregation-related proteostasis mechanisms. In the secretory pathway, the rapidly aggregating Z variant of α-1-antitrypsin provides a similar option to understand how the ER addresses protein aggregation (26, 27). Notably, many protein aggregation disorders are associated with

extracellular aggregation of a destabilized protein. Such model systems can be valuable for elucidating how the ER partitions between quality control and secretion of misfolded proteins. Options include destabilized, disease-causing variants of transthyretin and immunoglobulin light chain, which form amyloid deposits if they escape ER quality control and instead are secreted into the extracellular milieu (28, 29).

2.2. Designer Proteins to Investigate Cellular Proteostasis

While endogenous proteins have many advantages, the challenges associated with developing effective assays to quantify their folding and partitioning between fates of aggregation, degradation, and/or secretion can be substantial. To address this issue, numerous groups have focused on the development of model substrates specifically designed to rely on proteostasis mechanisms but with properties that make their folding state and localization much more straightforward to assay in a cellular setting (**Figure 1*b***).

The most widely employed examples of these designer proteins are enzymes with easy optical readouts based on luminescence or fluorescence that also display a chaperone dependence when expressed in cells. The introduction of mutations that further impair either the kinetic or thermodynamic properties of these nonnative model proteins can enhance dependence on cellular proteostasis mechanisms (30–33). Folding and expression of these sensors following various perturbations of the proteostasis network can then be followed by assaying the relative enzymatic activity, as in the case of destabilized firefly luciferase or retroaldolase (31, 32). Fusion of such constructs to fluorescent proteins has also been used to monitor aggregation via fluorescent resonance energy transfer, as in the case of destabilized barnase variants (30). As just one example of the potential power of these methods, firefly luciferase–based studies recently assisted the discovery of new mechanisms for quality control in the nucleolus (34).

Designer aggregating proteins have also recently been developed. Most significantly, a destabilized variant of HaloTag covalently labeled with an environmentally sensitive fluorescent ligand can be used to effectively report on intracellular protein aggregation (35, 36).

2.3. The Evolutionary Biology Paradigm

Recent work has revealed that the mutational landscape accessible to a protein evolving in the cellular context depends critically on the composition and activities of the proteostasis network (14–17, 37) (**Figure 1*c***). This concept was first pioneered in studies of heat shock protein 90

(Hsp90) function by Rutherford & Lindquist (38). It follows that a third compelling experimental context to study mechanisms of proteostasis is to examine the mutational landscape accessible to a client protein in different proteostasis environments. For example, if inhibiting the activity of a specific chaperone precludes access to a subset of mutations, that information provides a foundation to explore the types of biophysical problems that chaperone is capable of addressing. Such a mechanism can be particularly relevant in certain cancers, where chronic upregulation of chaperones appears to accelerate mutation-mediated drug resistance onset (39).

A key challenge for leveraging evolution to study proteostasis is the low mutation rate and slow growth of metazoan cells. This issue has been addressed in a number of different ways. Most recently, rapidly proliferating and mutating RNA viruses that hijack metazoan proteostasis networks have been leveraged to elucidate how mammalian chaperones solve protein-folding problems (14, 15). A similarly effective strategy can be the application of deep mutational scanning libraries of protein variants in the context of perturbed proteostasis environments (16, 17). Excitingly, the development of two analogous mammalian phage-assisted continuous evolution (mPACE) methods for continuous directed evolution of proteins of interest in mammalian cells expands the possible scope of evolutionary biology studies beyond just pathogen proteins to the actual endogenous clients of cellular chaperones and quality control mechanisms (40, 40a). Looking forward, the continued development of new tools for targeted mutagenesis within specific regions of genomes is likely to further increase opportunities to apply in vivo evolution to the proteostasis field (41, 42, 42a).

3. STRATEGIES TO PERTURB THE PROTEOSTASIS NETWORK

With an experimental substrate or context for studying the cellular proteostasis network selected, the next step (**Figure 1*a***) is to perturb potentially relevant pathways within the proteostasis network and assess the consequences for the folding of the client protein of interest. The proteostasis network is organized around multiple stress-responsive regulatory nodes that regulate defined subsets of proteostasis network components (**Figure 2*a***). These nodes are typically differentiated by subcellular compartment, with each organelle containing unique nodes that can be activated by protein misfolding within that compartment. Global modulation of the proteostasis environment within a particular subcellular environment can, therefore, be achieved by targeting the appropriate stress-responsive regulatory nodes. Alternatively, individual

proteostasis network components can be targeted, either alone or in combination with regulation of proteostasis nodes, to gain detailed insight into the roles of a single chaperone or quality control factor.

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Figure 2 General strategies for pharmacologic perturbation of the proteostasis network. (*a*) Organization of the proteostasis network by (often) compartmentalized regulatory nodes. The accumulation of misfolded proteins activates regulatory nodes, which then remodel proteostasis network composition in the associated subcellular compartment to resolve proteotoxic stress. (*b*) Chemical genetic strategies can marry the advantages of chemical and genetic approaches for perturbation of the proteostasis network, while minimizing the disadvantages associated with each approach. (*c*) Genetic modulation of endogenous gene expression through the use of CRISPR-dCas9. In combination with TADs (in CRISPR activation) or transcriptional repressors (e.g., KRAB, in CRISPR interference), dCas9 is used to regulate gene expression. (*d*) Dosable and temporal control of protein levels (and therefore activity) can be achieved by modulating proteasomal degradation using destabilized domains fused to the protein of interest. Orthogonal control of multiple proteostasis components can also be achieved in a single cell by using unique destabilized domain/ligand pairs, such as FKBP/Shield-1 or DHFR/trimethoprim. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, catalytically inactive CRISPR-associated protein 9; sgRNA, single guide RNA; TAD, transcriptional activation domain.

Historically, proteostasis network perturbation was achieved through treatment with stressors that induced upregulation of chaperones and quality control mechanisms by causing extensive protein misfolding. Examples include treatment with dithiothreitol to reduce disulfide bonds and activate the UPR, high temperature to induce the HSR, or arsenite to cause oxidative damage. While these strategies are powerful for defining the composition and regulatory flow of the proteostasis network, they have limited value for mechanistic studies because they are accompanied by severe and widespread protein misfolding, protein aggregation, and cellular toxicity. Stress-independent methods for perturbing the proteostasis network are much preferred, as they sidestep these issues. The need for stress-independent perturbation strategies has driven the development of a vast suite of methods for precision modulation of proteostasis using pharmacologic and chemical genetic methods. In the sections below, we emphasize both the power and the limitations of these chemical biology approaches to provide fresh insights into proteostasis mechanisms.

3.1. Genetic Versus Chemical Approaches to Perturb the Proteostasis Network

There are three general strategies available for stress-independent perturbation of the proteostasis network: genetic, chemical, and chemical genetic. Each affords advantages and disadvantages (**Figure 2*b***). The genetic approach is perhaps most widely used. Proteostasis network components can be overexpressed, knocked down, or knocked out, either transiently or stably. Genetic regulation of endogenous proteostasis components is also now possible through the use of clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) technology. In CRISPR systems, single guide RNAs are used to target the bacterial endonuclease Cas9 to specific genomic loci (43). By using a catalytically inactive Cas9, known as dCas9, in combination with transcription activation domains (in CRISPR activation) or KRAB transcription repressor domains (in CRISPR interference), targeted modulation of endogenous gene expression levels can be achieved (44, 45) (**Figure 2*c***).

These genetic strategies can be very effective. They are highly specific, as the only direct perturbation is to the manipulated gene, and they are easily adapted to high-throughput screens. Moreover, they can be applied in a straightforward manner to virtually any component of the proteostasis network. On the other hand, genetic approaches lack temporal control, and the kinetics of modulation are slow. The dynamic, highly adaptive nature of the proteostasis network often, therefore, results in compensatory effects in response to such genetic manipulation that can mask important phenotypes. Furthermore, overexpression can lead to chaperones or quality control factors expressed at far beyond physiologically relevant levels. Both overexpression and knockout can also disrupt important aspects of proteostasis network stoichiometry, resulting, for example, in an imbalance of chaperone levels relative to their cognate co-chaperones.

As an alternative to genetic modulation, small molecules can be used to directly inhibit or activate both regulatory nodes and specific components of the proteostasis network. The direct chemical approach affords tight temporal control, rapid reversibility, and high dosability. These advantages can provide more relevant biological insights, avoiding cellular compensation and ensuring the physiologic significance of results obtained. Moreover, studies with drug-like small molecules can feed directly into preclinical drug development. However, there are also significant disadvantages associated with this approach. Most significantly, reliable and potent pharmacologic modulators of the activities of the vast majority of proteostasis network components and nodes are currently unavailable. Further, no small molecule is perfectly

selective, and pleiotropic effects owing to off-target activity can present a serious problem that is often challenging to diagnose.

Chemical genetic strategies merge the advantages of genetic and direct chemical approaches, while sidestepping most of the disadvantages (**Figure 2*b***). For example, small-molecule regulation of CRISPR-Cas9 transcriptional modulators can be achieved by controlling recruitment of transcriptional regulation domains to dCas9 by inducible dimerization using the FKBP/FRB system, by regulating the stability of dCas9 transcriptional regulators using destabilized domains (**Figure 2*d***), or by directly inhibiting the DNA-binding activity of dCas9 (46–48). With these and other related systems, the transcription of proteostasis genes can be selectively and orthogonally tuned to precisely engineer unique proteostasis environments to study protein folding. Similarly sophisticated chemical genetic techniques are now available to control most aspects of stress-responsive signaling pathways, including the UPR and HSR, with small molecules (reviewed in Section 3.2). These chemical genetic strategies combine the high specificity and flexibility of traditional genetic manipulation with the dynamic and temporal control made possible by small molecules.

3.2. Global Remodeling of Cellular Proteostasis Environments by Chemically Targeting Stress-Responsive Signaling Pathways

Modulating stress-responsive signaling pathways is a powerful approach to both significantly and specifically perturb localized cellular proteostasis environments. Numerous methods are now available to chemically modulate the HSR and the UPR. We note that there are also unique aspects of stress-responsive signaling and the proteostasis network within the mitochondria (49), but as small-molecule-based techniques to modulate these pathways are less developed, they are not reviewed here.

3.2.1. Modulating the cytosolic and nuclear proteostasis environment via chemical control of heat shock factor 1.

The composition of the cytosolic and nuclear proteostasis networks is largely controlled by the master regulator of the HSR—heat shock factor 1 (HSF1) (1). In the absence of stress, sequestration of HSF1 by cytosolic chaperones as well as intramolecular interactions between coiled-coiled domains (HR-A/B and HR-C) within HSF1 cooperate to maintain HSF1 largely in a monomeric state within the cytosol (50, 51). Following stress onset, the accumulation of misfolded proteins titrates chaperones away from HSF1, triggering HSF1 trimerization and

transport to the nucleus. There, HSF1 upregulates transcription of heat shock proteins and other components of the proteostasis network (52) (**Figure 3*a***). Further fine-tuning of HSF1 transcriptional activity is mediated by various posttranslational modifications, including phosphorylation, acetylation, and SUMOylation (9, 53–56). Negative feedback loops involving these modifications, as well as sequestration by excess chaperones, act to reduce HSF1 transcriptional activity back to basal levels after protein-misfolding stress is addressed (54).

<COMP: PLEASE INSERT FIGURE 3 HERE>

Figure 3 Modulation of cytosolic and nuclear proteostasis by targeting HSF1. (*a*) The HSF1 transcription factor is the master regulator of the heat shock response. Cytosolic chaperones bind within the RD and maintain HSF1 in a monomeric state. The RD also contains sites for posttranslational modifications that can further fine-tune transcriptional activation. Flanking the RD are two heptad repeat regions (HR-A/B and HR-C) that facilitate a switch between intermolecular and intramolecular interactions following the titration of chaperones off HSF1 by the accumulation of misfolded proteins. (*b*) Methods for genetic regulation of HSF1 are shown. By deleting amino acids 186–202 of the RD, cHSF1 can be created. A potent dominant-negative construct can then be created by additional deletion of the TAD from cHSF1 to create dn-cHSF1. (*c*) Small-molecule modulators can affect HSF1 activity. Celastrol activates HSF1 in a stress-dependent manner, while $I_{HSF}115$ can achieve inhibition. (*d*) Dosable chemical genetic regulation of HSF1 activity through the combination of DD technology with constitutively active (FKBP.cHSF1) or dominant-negative constitutively active (DHFR.dn-cHSF1) HSF1 variants. Abbreviations: cHSF1, constitutively active HSF1; DBD, DNA-binding domain; DD, destabilized domain; dn-cHSF1, dominant-negative constitutively active HSF1; HSE, heat shock element; HSF1, heat shock factor 1; RD, regulatory domain; TAD, transcription activation domain.

Stress-independent induction of HSF1 activity provides a compelling means to globally enhance the cytosolic and nuclear proteostasis environments. Inhibition of basal HSF1 activity could, on the other hand, yield depleted proteostasis environments that render protein folding challenging. Preventing stress-responsive HSF1 activation could also provide a mechanism to sensitize cells to proteotoxic stress while leaving protein folding under basal conditions unaffected. Unfortunately, genetic methods to modulate HSF1 activity have limited applicability. HSF1 knockdown or knockout is an option, but cells can compensate over time (57). HSF1 overexpression is also not particularly effective for inducing transcriptional activity, as even the overexpressed protein is held largely inactive (58). Overexpression of constitutively active HSF1 (cHSF1) variants created by deletion of the regulatory domain (59) (**Figure 3*b***) can partially

overcome these limitations, but the resulting unnaturally high levels of HSF1-mediated transcription are generally toxic (52). Thus, chemical and chemical genetic tools are critical for probing the HSF1-regulated proteostasis network.

Unfortunately, direct chemical tools to control HSF1 activity in a stress-independent manner remain severely limited. In particular, since the activity of transcription factors like HSF1 is dependent on difficult-to-target protein–protein and protein–DNA interactions, designing potent modulators presents a significant challenge. Some success in developing HSF1 inhibitors has been achieved through disrupting binding of HSF1 to regulatory DNA sequences through the use of RNA aptamers, or by impairing formation of intramolecular coiled-coils within the HSF1 trimer through the use of peptides (60, 61), but such molecules are typically not cell permeable. Small molecules that alter protein translation can also inhibit HSF1 (62), but the off-target consequences of this indirect mechanism are problematic. High-throughput screening methods have also recently identified several compounds that prevent recruitment of HSF1 to DNA; however, the mechanism of action for many of these compounds is either poorly understood or mediated by upstream effectors that are also involved in other pathways (63–66). Perhaps most promising is the recent identification of $I_{HSF}115$ (67) (**Figure 3*c***), a compound that showed considerable potency in cell-based experiments.

The development of small-molecule-based, stress-independent activators of HSF1 signaling has proven even more challenging. Extant HSF1 activators function by increasing protein misfolding via chaperone inhibition (e.g., Hsp90 inhibitors) or, like celastrol (**Figure 3*c***), are reactive small molecules that likely induce significant protein misfolding and thereby induce HSF1 by causing stress (68–70).

In summary, both genetic and chemical approaches to tune HSF1 activity remain quite immature and have numerous caveats. In contrast, chemical genetic approaches have proven very effective for mechanistic studies. The current most potent and selective strategy to inhibit HSF1 is based on a cHSF1 variant lacking the transcription activation domain (71) (**Figure 3*b***). Expression of a chimeric protein composed of this dominant-negative constitutively active form of HSF1 (dn-cHSF1) fused to a destabilized domain confers small-molecule-dependent, dosable inhibition of endogenous HSF1 (**Figure 3*d***). In the absence of other effective HSF1 inhibition strategies, this approach has increasingly been used to elucidate consequences of HSF1 inhibition (17, 72). Small-molecule-controlled, dosable, and stress-independent HSF1 activation

has been similarly achieved by fusing cHSF1 to a destabilized domain (52, 73) (**Figure 3*d***).

3.2.2. Modulating the endoplasmic reticulum proteostasis environment via the unfolded protein response.

Several features differentiate regulation of the UPR in the ER from that of the HSR in the cytosol. First, activation of stress-responsive signaling in the ER requires signaling from the ER lumen to the nucleus by way of transmembrane sensors to induce transcriptional upregulation of ER proteostasis components. Second, whereas the HSR has one primary regulatory node, the ER proteostasis network has three, each of which has its own unique transmembrane sensor and transcriptional effector. These three parallel signaling pathways are initiated by the ER transmembrane sensor proteins PERK, IRE1, and ATF6, which in turn induce the ER proteostasis network–regulating transcription factors ATF4, XBP1s, and ATF6f, respectively (**Figure 4*a***).

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Figure 4 Modulation of secretory pathway proteostasis via the endoplasmic reticulum (ER)'s unfolded protein response (UPR). (*a*) The three arms of the UPR, regulated by the ER transmembrane sensors PERK, IRE1, and ATF6, respectively. Each transmembrane sensor initiates a signaling cascade that in turn activates a unique transcriptional effector to upregulate expression of ER quality control factors and chaperones. The PERK arm of the UPR can also feed into the eIF2α-mediated integrated stress response to reduce protein translation. (*b*) Perturbation of the UPR by targeting either the ER transmembrane sensors or their associated transcriptional effectors. Downstream components within the PERK signaling pathway can be targeted as well. Some small-molecule modulators of the transmembrane sensors are now available, but direct chemical control of the transcriptional effectors still relies on chemical genetic methods. (*c*) Approaches for chemical genetic control of UPR transmembrane sensors. Dimerization of PERK can be regulated by fusing the protein to a chemically inducible dimerization domain, Fv2E, regulated by the small-molecule ligand AP20187. Selective IRE1 activation can be achieved using a bump–hole approach, in which a mutant of IRE1 containing a hole within the adenosine triphosphate–binding pocket selectively binds to the bumped ligand 1NM-PP1 to promote IRE1 dimerization/oligomerization and initiate IRE1's ribonuclease activity. (*d*) Approaches for chemical genetic activation and inhibition of the UPR transcription factor ATF6f that employ destabilized domain (DD) technology. Additional abbreviation: NA, not applicable.

The UPR has been reviewed in detail elsewhere (2, 74). We briefly summarize it here (**Figure 4*a***) to provide context for discussion of chemical biological regulation of the pathway. The ER transmembrane sensors are activated following titration of the ER-resident Hsp70

chaperone BiP off the sensors by the accumulation of misfolded proteins in the ER lumen (75), and by other related mechanisms (76, 77). Disassociation of BiP induces dimerization of PERK, thereby activating PERK's cytosolic kinase domain. The activated kinase domain phosphorylates multiple targets, including the eukaryotic initiation factor eIF2α (78), which is also an important player in the integrated stress response induced by oxidative stress, amino acid starvation, and more (79). Phosphorylated eIF2α attenuates global protein translation while selectively upregulating translation of the transcription factor ATF4. ATF4 induces the GADD34 phosphatase, which can then dephosphorylate eIF2α to relieve the translational repression. Activation of the second arm of the UPR is analogous, involving BiP release followed by IRE1 dimerization and oligomerization. This self-assembly activates the cytosolic kinase domain, causing *trans*-autophosphorylation; *trans*-autophosphorylation engenders a conformational change that activates IRE1's cytosolic ribonuclease (RNase) domain, removing an intron from the inactive form of the XBP1 transcription factor XBP1u. Translation of the spliced XBP1 transcript, termed XBP1s, then causes upregulated expression of ER chaperones and quality control factors (80). IRE1's RNase domain can also degrade several other ER-associated messenger RNAs (81). ATF6 is the third arm of the UPR. It is an ER-resident transmembrane protein that traffics to the Golgi apparatus upon BiP disassociation. There, ATF6 undergoes proteolytic processing to release a cytosolic transcription factor termed ATF6f (82).

Because each arm of the UPR activates a unique subset of chaperones and quality control factors, stress-independent, arm-specific UPR modulation results in distinctive ER proteostasis environments (83). Therefore, arm-specific activation can be more valuable for mechanistic studies than global UPR induction. Fortunately, stress-independent direct small-molecule approaches to modulate individual UPR arms are considerably more advanced than those for the HSR (**Figure 4*b***); some have already been successfully tested in animal models of disease (84). Numerous valuable chemical genetic techniques are also available.

Because PERK activity is transmitted by phosphorylation of substrates by PERK's cytosolic domain, kinase inhibitors with reasonable selectivity can be used to inhibit PERK (85). Selective small-molecule PERK activators that function by directly engaging PERK are, in contrast, not currently available. Chemical genetic control of PERK activity has, however, been achieved by fusion of the ligand-inducible dimerization domain Fv2E to PERK (**Figure 4*c***). In this system, PERK activity is induced by the small-molecule dimerizer AP20187 (86). It is also possible to

modulate downstream PERK signaling by targeting the GADD34 phosphatase that controls the phosphorylation status of eIF2α and thus the translational repression mediated by PERK. Molecules such as Sephin1 have been proposed to inhibit phosphatases involved in eIF2α dephosphorylation and thereby extend the time course of stress-induced protein translation inhibition (87, 88), although the mechanism remains controversial (89). Alternatively, because PERK-mediated eIF2α phosphorylation represses protein translation by attenuating the guanine nucleotide exchange factor eIF2B, preventing inactivation of eIF2B by using the small molecule ISRIB can sidestep protein translation repression (90). It is important to note that these modulators of eIF2α phosphorylation status are not specific UPR modulators, as the eIF2α pathway is the key nexus of the integrated stress response, which is also related to many types of non-ER stress (79).

The PERK arm of the UPR largely functions through modulating protein translation, playing a minimal role in transcriptional remodeling of the ER proteostasis network (the PERK-induced transcription factor ATF4 drives apoptosis in response to chronic ER stress). Modulators of the IRE1-XBP1s and ATF6 UPR arms are, therefore, typically more effective for perturbing ER proteostasis network composition. ATF6 activation and inhibition are both now possible with selective small molecules or via chemical genetic approaches (**Figure 4*c***). The small molecule AA147 promotes selective ATF6 activation by inhibiting protein disulfide isomerases that regulate ATF6 activity (91, 92). While this mechanism may involve some induction of ER protein misfolding, any such effect is likely minimal as the other arms of the UPR are not activated by AA147 treatment. Ceapin-1 is a potent inhibitor of ATF6 (93), functioning by recruiting ATF6 to peroxisomes via a neomorphic interorganelle tether (94). A final option is chemical genetic control of the ATF6f transcription factor itself. Deletion of ATF6's ER-lumenal and transmembrane domains generates the active form of the transcription factor. However, as is also true for overexpression of cHSF1, nonphysiologic high levels of ATF6f expression are toxic. To address this issue, fusion to destabilized domains can confer dosable chemical control of ATF6f activity in cells (73, 83). This approach can also be used to confer chemical control of the activity of a dominant-negative form of ATF6f to inhibit both ATF6f- and XBP1s-mediated transcriptional remodeling (73) (**Figure 4*d***).

Direct pharmacologic inhibitors of IRE1 are also available. Inhibition can be achieved by targeting the RNase domain, which results in impairment of XBP1 splicing and downstream

gene induction (95, 96). Since IRE1 autophosphorylation is required to activate the RNase domain, a second class of IRE1 inhibitors has focused on inhibition of the kinase domain (97, 98). Activation of IRE1 can also be achieved by small-molecule binders to the kinase domain that induce a conformational change promoting oligomerization and endonuclease activation (90, 98). However, pleiotropic consequences of treating cells with these kinase inhibitors currently limit their broad use, and improved IRE1 activators are still needed. In the interim, chemical genetic techniques to modulate the IRE1 arm of the UPR are available. Chemical control of IRE1 activation can be endowed using a bump–hole strategy in which an IRE1 variant containing a mutation within the kinase domain is paired with an engineered small-molecule binder that selectively interacts with the modified IRE1 kinase domain (99) (**Figure 4*b***). Alternatively, tetracycline repressor-regulated control of XBP1s levels has proved to be an effective strategy for temporal control of XBP1s activity (83, 100–102). The lack of dosable control of XBP1s levels that is associated with tetracycline repressor regulation does not appear to be very problematic because, unlike the case for ATF6f and cHSF1, XBP1s overexpression is typically nontoxic.

3.3. Targeting Individual Components of the Proteostasis Network by Using Small Molecules

Modulating the activity of the master regulators of proteostasis (e.g., HSF1, IRE1, or ATF6) results in global remodeling of the proteostasis network in subcellular compartments. Such broad-scale remodeling is both powerful and biologically relevant, but it can also convolute mechanistic evaluation of individual component functions. Chemical biology strategies to directly modulate the activities of individual chaperones or quality control factors can, therefore, be invaluable. Unfortunately, potent and selective small molecules for this purpose are available for only a few proteostasis network components, with perhaps the greatest successes to date in the areas of modulating the adenosine triphosphate (ATP)-dependent heat shock protein–type chaperones and protein degradation mechanisms. Both are briefly discussed below. For the many proteostasis network components that lack selective small-molecule modulators, a valuable alternative is application of chemically controlled variants of the CRISPR interference and CRISPR activation systems, discussed above (46–48), to dosably control the levels of any desired proteostasis network component.

3.3.1. Targeting adenosine triphosphate–dependent chaperones and chaperone complexes. The Hsp90 chaperone is one of the most abundant proteins in the cell. This chaperone's abundance and importance promoted the early development of potent Hsp90 ATPase pocket-binding inhibitors, including STA-9090 and geldanamycin (103, 104). The availability of these and related compounds underpinned rapid progress in understanding the functional consequences of Hsp90 in proteostasis and beyond (38), highlighting the value of chemical methods to perturb proteostasis mechanisms. However, a key issue is the existence of several Hsp90 isoforms, including Hsp90α and Hsp90β in the cytosol, Grp94 in the ER, and TRAP1 in the mitochondria. Owing to the considerable structural similarity within the ATP-binding pockets of Hsp90 isoforms, early Hsp90 inhibitors displayed pan-isoform activity, convoluting mechanistic interpretation. Excitingly, recent work has seen substantial success in the development of isoform-specific Hsp90 inhibitors. In one approach, selectivity between the ER-resident isoform Grp94 and the cytosol-resident isoforms Hsp90α and Hsp90β has been achieved by exploiting unique structural features within the Grp94 ATP-binding pocket (105, 106). Alternatively, mitochondrial targeting moieties have been used to preferentially inhibit the mitochondria-resident Hsp90 isoform TRAP1 (107). Hsp90β-specific inhibitors have also recently emerged (108). The availability of these new compound classes is opening new doors in continued efforts to understand roles of Hsp90 chaperones in proteostasis (109).

The related Hsp70 chaperone systems have also been successfully targeted in cells via selective and potent small molecules from several compound classes (110, 111). However, the issue of isoform selectivity remains to be addressed, as there are up to 13 distinctive Hsp70 isoforms in cells.

A key issue associated with inhibition (or genetic knockdown) of the Hsp90 and Hsp70 chaperones is compensatory stress-response activation that can make it difficult to attribute phenotypes directly to inhibition of the chaperone versus stress-response-mediated proteostasis network remodeling. In particular, Hsp90 inhibition rapidly induces the HSR via HSF1. In this context, the availability of destabilized domain-regulated dn-cHSF1 can be particularly valuable to uncouple the direct consequences of Hsp90 inhibition from the compensatory consequences of HSF1 activation (71, 72).

The efficiency and client selectivity of the ATP-driven chaperones depends, in large part, on associated co-chaperones (112, 113). Furthermore, remodeling of chaperone complexes is

observed during disease, suggesting that cells alter both chaperone expression and co-chaperone interactions to effectively address protein-folding demands (11). To further probe the roles of co-chaperone complexes, recent efforts have led to the development of compounds that modulate protein–protein interactions between chaperones and their cognate co-chaperones (114, 115). Although strategies for only a few co-chaperones are currently available, they nonetheless present an intriguing new opportunity to gain insight into the mechanistic roles of chaperone complexes in directing client protein fates.

3.3.2. Small-molecule modulation of protein degradation pathways.

Limited availability of chemical and chemical genetic methods to modulate the activities of the quality control factors that identify misfolded proteins and target them to degradation is a significant challenge in the proteostasis field. On the other hand, the development of pharmacologic modulators of the major protein degradation pathways, including the ubiquitin-proteasomal and autophagy-lysosomal systems, is more advanced.

Modulation of the ubiquitin-proteasome system has primarily focused on pharmacologic inhibition of the proteasome protease activity, for which several selective small molecules with varying selectivity have been developed (116). Perhaps the most commonly used compound is the peptide aldehyde MG-132 (117). There are now, however, many compounds that more specifically target the proteasome, such as bortezomib (12). These compounds constitute preferred options for perturbing proteasome function. Proteasomal degradation of many substrates can also be impaired by preventing upstream ubiquitination by E3 ligases. Of particular interest, small molecules that selectively impair substrate ubiquitination by inhibiting specific E3 ligase–client interactions have begun to emerge (118).

Activation of macroautophagy in cells is a multistep process in which a cellular region is entrapped within a membrane to form an autophagosome, which then fuses with a lysosome to degrade the enclosed materials. Autophagy inhibitors and activators have been developed with varying specificity against several steps of autophagy (119). Inhibitors of PI3K/Vps34, such as 3-methyladenine and wortmannin—both of which are nonspecific—inhibit nucleation and elongation of autophagosome membranes (120, 121). Alternatively, autophagosome–lysosome fusion can be prevented by using bafilomycin A1 (122). The mechanism of action for many widely used autophagy inhibitors has been disputed. Nonetheless, these compounds provide useful handles to perturb disposal of aggregated proteins in proteostasis studies.

ER proteins exist in a subcellular compartment that lacks its own protein degradation factors. Thus, misfolded or aggregated proteins in the ER require specialized machinery for transport to cytosolic degradation mechanisms. These transport machineries provide a unique set of additional targets for the design of small molecules that influence ER protein degradation. Of particular interest, inhibition of ER-associated degradation can be achieved by targeting VCP/p97, an ATPase involved in protein extraction from the ER prior to targeting to the proteasome. Several potent inhibitors of p97 have recently been developed (123, 124), providing a useful strategy to perturb ER proteostasis. However, it should be noted that p97 plays roles in numerous cellular functions beyond just ER-associated protein degradation. Results from p97 inhibition must, therefore, be interpreted with caution. In contrast to ER-associated degradation, we currently lack chemical methods to selectively modulate ER-to-lysosome-associated degradation without globally perturbing autophagy (21).

4. METHODS TO MONITOR EFFECTS OF PROTEOSTASIS PERTURBATION

Proteostasis network perturbations lead to the identification of nodes and components within the network that play an important role in the folding, quality control, or trafficking of the client(s) selected for study (**Figure 1*a***). The next step is to apply appropriate assays to understand the mechanistic or biophysical origins of observed effects. One approach is to directly observe changes in protein activity, half-life, or conformation in the cellular context as a consequence of proteostasis network perturbation. Alternatively, studies revealing the interactions between proteostasis network components and the client of interest can reveal critical mechanisms that regulate folding, trafficking, and quality control. In the sections below, we describe some of the prominent assays that can be applied to read out the consequences of proteostasis network perturbation for client proteins of interest.

4.1. Monitoring Protein Half-Life in the Cell

One frequent consequence of proteostasis modulation is an alteration of protein half-life. Intracellular steady-state analyses of protein levels cannot provide insight into rates of degradation or trafficking. An ideal alternative is to directly determine the half-life of a protein by using pulse-chase techniques. Cycloheximide chase experiments are one option, but the requirement for inhibition of new protein translation can be deleterious. A preferred, if labor-

intensive, approach is to metabolically label newly synthesized proteins via a brief pulse with radioactive (^{35}S) cysteine and methionine (**Figure 5*a***). The life cycle of the radiolabeled proteins produced during the pulse can then be quantitatively determined following immunoprecipitation of the protein of interest. Global analysis of endogenous protein half-lives across the entire proteome can also be achieved via stable isotope labeling with amino acids in cell culture (SILAC), by briefly labeling newly synthesized proteins with stable isotopes and using quantitative proteomics to determine the isotopic ratio of each protein across the proteome (125).

<COMP: PLEASE INSERT FIGURE 5 HERE>

Figure 5 Methods to assay proteostasis in cells. (*a*) Pulse-chase analysis. This approach involves metabolically labeling newly synthesized proteins with heavy or radioactive amino acids and following the decay of fluorescent signal from a tagged substrate after a stimulatory light pulse. (*b*) Approaches for in vivo fluorescence lifetime analysis. To compare protein half-life between proteostasis environments or mutational variants, the relative fluorescence intensity of the fluorescently tagged protein of interest can be compared with an internal fluorescent control expressed from an internal ribosomal entry site. Alternatively, tandem fluorescent reporters in which proteins with different fluorescent maturation rates are fused to the protein of interest can also be used. (*c*) Evaluating protein conformational changes in vivo. The accumulation of misfolded or unfolded proteins in a cell can be assessed by comparing the footprint of chemical labeling of solvent-exposed cysteine residues, or by the partial proteolysis of enzymatically accessible unfolded regions. (*d*) Real-time in vivo evaluation of protein aggregation with AggTag. A protein of interest is fused to either a HaloTag or a SnapTag and covalently labeled with an AggTag probe containing a molecular rotor or solvatochromic fluorophore. Protein aggregation activates ligand fluorescence by exposing the ligand to a rigid and/or hydrophobic microenvironment. (*e*) Cellular thermal shift assays to evaluate proteome stability. Cells with unfavorable proteostasis environments may leave proteins vulnerable to heat-induced aggregation, thereby altering the aggregation temperature. Results can be assessed via immunoblotting or proteome-wide via mass spectrometry.

Since these isotope labeling techniques require bulk analysis of a cell population, information on changes in protein half-life at the single-cell level is lost. Moreover, the methods are labor intensive. To overcome these limitations, recent efforts have focused on developing real-time methods for monitoring protein half-life in single cells by incorporating fluorescence markers on proteins of interest. Analogous to metabolic pulse-chase, a fluorescence marker on a protein of interest can be temporally induced, either chemically, by covalent modification of SnapTag or HaloTag with a fluorescent small molecule, or by using a photoconvertible fluorescent protein (126–128) (**Figure 5*a***). Alternatively, a snapshot of relative protein stability can be obtained by

comparing the fluorescence of the tagged protein of interest to an internal control expressed from the same messenger RNA through the use of an internal ribosomal binding site, or by using tandem fluorescent reporters composed of two fluorescent proteins with different maturation rates (129, 130) (**Figure 5*b***). In both methods, the fluorescence of the reporter can be tracked by using flow cytometry or fluorescent microscopy, allowing for single-cell, real-time analysis of protein turnover. Studies using these assays on a substrate of interest in the context of proteostasis network perturbation can provide deep insight into how a particular stress response or chaperone influences the trafficking and quality control of its client proteins.

4.2. Observing Protein Conformational Changes in Response to Proteostasis Network Perturbation

Pulse-chase methods report on protein half-life but provide little or no direct information regarding how proteostasis network perturbation influences protein folding and conformation. Several complementary cell-based assays can provide relevant information in this regard. Loss of secondary and tertiary structures associated with localized unfolding can be detected through enzymatic or chemical modification of protein backbones or side chains (**Figure 5*c***). One prevalent enzymatic approach is limited proteolysis, in which cell lysates are briefly exposed to low concentrations of proteases to cleave solvent-exposed regions of proteins. Detection of cleaved peptides can be achieved across the proteome by using this strategy in tandem with mass spectrometry analysis in limited proteolysis mass spectrometry (LiP MS) (131). Alternatively, chemical modification of accessible, nucleophilic cysteine thiols within intact cells can selectively label unfolded protein regions, providing both a visual method to quantify cellular protein unfolding and a mass spectrometry–based technique to identify proteins that preferentially unfold in response to stress (132).

Another strategy leverages the fact that unfolded and partially unfolded proteins are susceptible to aggregation. Aggregated proteins are often resistant to sodium dodecyl sulfate detergent-mediated solubilization, and can therefore be physically isolated from soluble proteins by using differential centrifugation, size exclusion chromatography, or filter-trap assays. Alternatively, a variety of fluorescence-based methods have been developed to detect protein aggregates in living cells. Environmentally sensitive fluorescent dyes such as Thioflavin T can be used to detect amyloid-like aggregates (133). Aggregation-prone proteins may also be genetically fused to a fluorescent protein, enabling the use of imaging approaches to determine

aggregate load and distribution across cell populations by identifying fluorescent puncta with either microscopy or flow cytometry (71, 134) (**Figure 5*d***). Recent improvements to fluorescent methods include the development of the AggTag approach, in which an environmentally sensitive small-molecule fluorophore is covalently linked to a protein of interest to assess aggregation in real time (135) (**Figure 5*d***). Fluorescence-based imaging approaches can be used to evaluate characteristics of protein aggregates at high resolution, including quantity, size, and localization. Advanced fluorescence imaging techniques, such as fluorescence recovery after photobleaching and fluorescence lifetime imaging, can also be employed to evaluate the dynamics of protein aggregates (136, 137).

Protein stability in cells can be directly assessed by using the cellular thermal shift assay (CETSA) (138) (**Figure 5*e***). In CETSA, intact cells are briefly heated to promote irreversible protein aggregation, followed by immunoblotting to determine the fraction of a given protein that remains soluble at a particular temperature. While CETSA has traditionally been applied to assess changes in protein stability as a consequence of small-molecule binding, recent work has leveraged the approach to assess how loss of protein *O*-glycosylation destabilizes proteins in cells (139). Furthermore, CETSA can be used in tandem with mass spectrometry to probe proteome-wide changes in protein stability (138). This strategy is likely to prove very valuable for unbiased, simultaneous assessment of how a given proteostasis network perturbation affects the foldedness of thousands of proteins in a single experiment.

4.3. Interactome Analyses to Discern Origins of Proteostasis Alterations

Since the folding and quality control pathway for any given client is unique, different proteostasis network components are required to maintain a particular protein's functionality. Proteostasis network perturbations can change these interactions with a client protein and thereby change the client protein's fate. These changes in the client interactome can provide deep insight into the roles of particular proteostasis network nodes and components in solving protein-folding problems. Numerous developments in the past few years have improved our ability to quantitatively evaluate protein interactomes as a consequence of a perturbation.

Direct interactions between proteostasis components and client proteins can be identified by using coimmunoprecipitation to enrich the binding partners of a protein of interest from cellular lysate, following which quantitative mass spectrometry can be used to identify the interactors under different conditions (**Figure 6*a***). However, since many proteostasis network interactions

are transient, the combination of cell lysis and the need for stringent wash steps during coimmunoprecipitation will result in many false negatives (140). Chemical or enzymatic modification of neighboring proteins in intact cells prior to lysis can be used to immortalize and/or enrich transient or low-affinity interactions (**Figure 6*b***). Chemical cross-linking agents are widely employed to covalently tether interacting proteins, most commonly by using the cell-permeable, lysine-reactive cross-linker dithiobis(succinimidyl propionate) (20, 141). Alternatively, enzymatic labeling of proximal proteins with biotin can be achieved by fusing the protein of interest to the promiscuous biotin ligase BirA, or to a peroxidase such as APEX2 that converts biotin-phenol to biotin-phenoxyl radicals (142–145). The labeled proteins can then be enriched by streptavidin pull down and identified by mass spectrometry. These strategies can be very effective to identify transient interactors but must be carefully optimized to avoid false positives.

<COMP: PLEASE INSERT FIGURE 6 HERE>

Figure 6 Mapping protein interactomes. (*a*) Immunoprecipitation can be used to identify interactions between endogenous proteostasis network components and client proteins. (*b*) Enrichment of low-affinity or transient interactions can be enhanced by using covalent cross-linking reagents or by labeling proximal proteins via fusing the protein of interest to a promiscuous biotin ligase (BirA/BioID) or APEX2. (*c*) To amplify the signal from low-expressing interactors, semi-high-throughput luciferase screens such as the luminescence-based mammalian interactome (LUMIER) assay can be used.

Mass spectrometry–based approaches for interactome analysis preferentially identify interactors that are highly expressed and therefore may miss low-abundance proteostasis network components. To amplify the signal of low-abundance interactors, semi-high-throughput screens for protein–protein interactions can be used. A particularly promising implementation is the luminescence-based mammalian interactome (LUMIER) assay, in which a library of FLAG-tagged bait proteins is transfected into cells expressing a luciferase-tagged protein of interest (146–148) (**Figure 6*c***). Protein–protein interactions can then be confirmed and quantified by detecting luminescence following cell lysis and immunoprecipitation on plates coated with an anti-FLAG antibody.

5. PERSPECTIVE

The strategy of perturbing the proteostasis network in the context of a carefully chosen substrate or experimental substrate followed by assaying the consequences is among the most successful approaches for elucidating mechanisms of proteostasis. Integration with small-molecule control of the proteostasis network can ensure the physiologic relevance of findings, yielding valuable insights with direct relevance to disease treatment.

These studies can be followed up by efforts to understand mechanisms of proteostasis maintenance at the molecular level. For example, individual proteostasis network components with important functions identified via in vivo perturbation experiments can often be further studied by reconstituting the individual components in vitro. Alternatively, biophysical studies on kinetic and thermodynamic aspects of protein folding can be used to contextualize alterations in enzymatic function and protein degradation observed upon cellular proteostasis network perturbation.

Aside from laying a strong foundation for mechanistic studies, the chemical biology framework for studying proteostasis can also be valuable because it provides a direct avenue to translational research. The identification of nodes and components of the proteostasis network that solve protein-folding and quality control challenges in disease-relevant contexts without deleterious effects can direct attention to appropriate therapeutic targets. For example, recent preclinical studies have indicated modulation of the ATF6 pathway in the UPR as a compelling target in both ischemia (84) and light chain amyloidosis (91). Similarly, modulating the HSR and the activities of certain chaperones could prove valuable for antiviral and anticancer therapy (11, 19, 39).

Looking forward, we anticipate the continued development of increasingly effective chemical biology methods with high potency and selectivity for modulation of the proteostasis network, as well as the development of improved assays for assessing mechanisms of proteostasis in cells. As these tools are applied across increasingly diverse model systems, our fundamental understanding of how cells solve protein-folding problems will greatly improve. Further, we expect the identification and validation of many new therapeutic modalities with applicability across a broad range of diseases.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that

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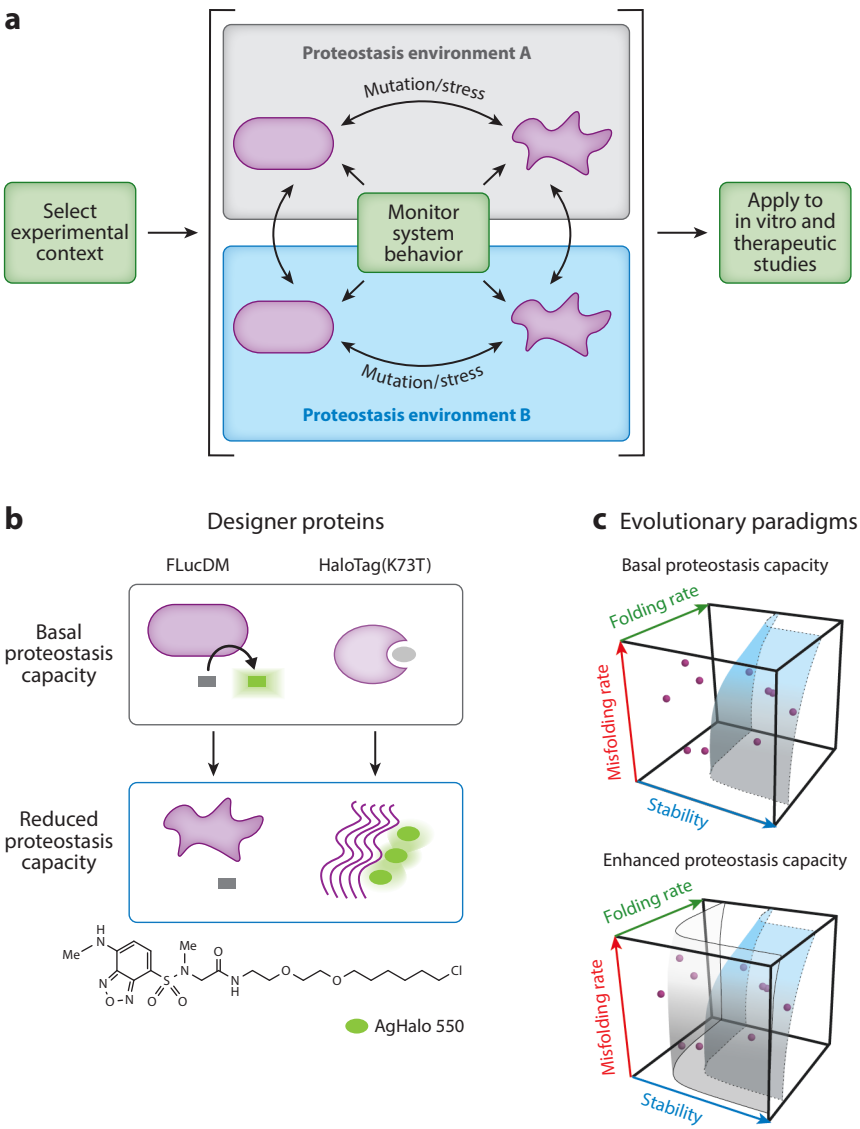
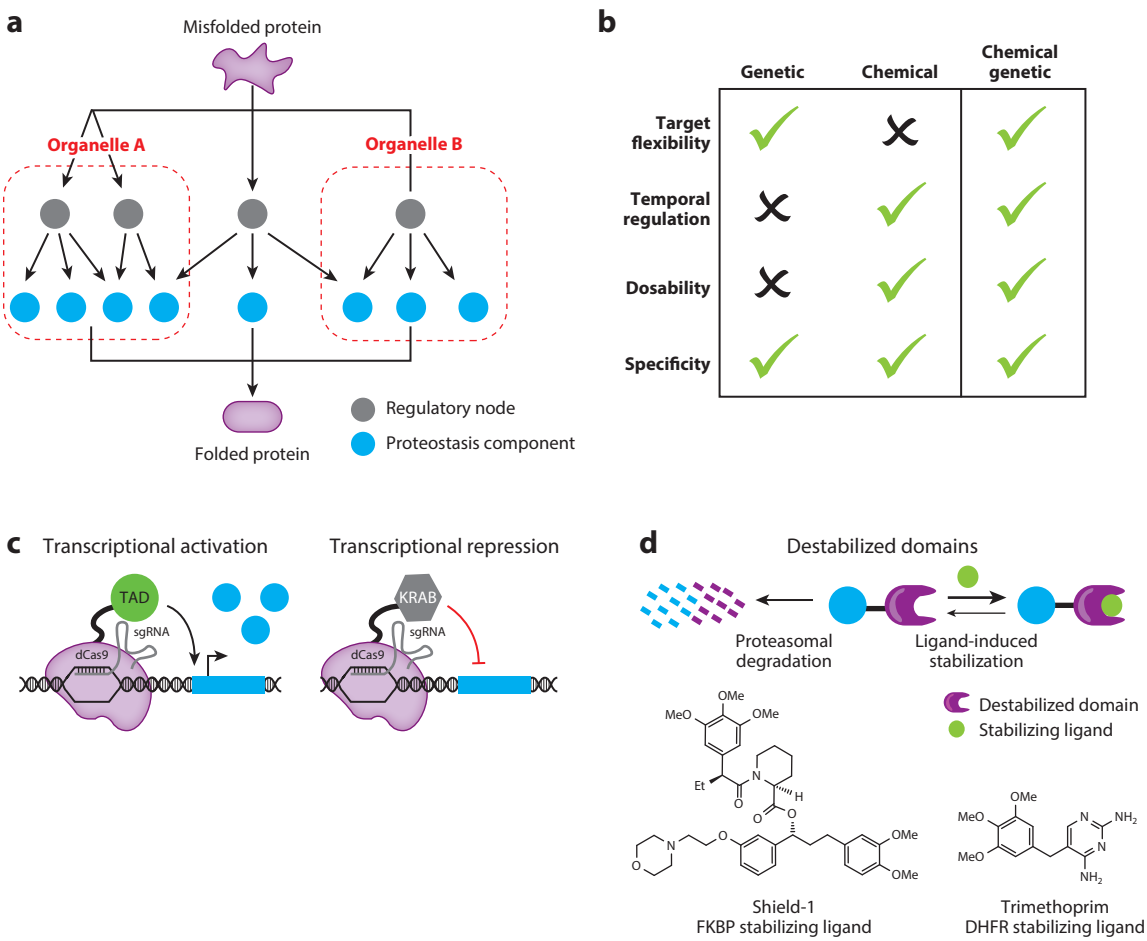
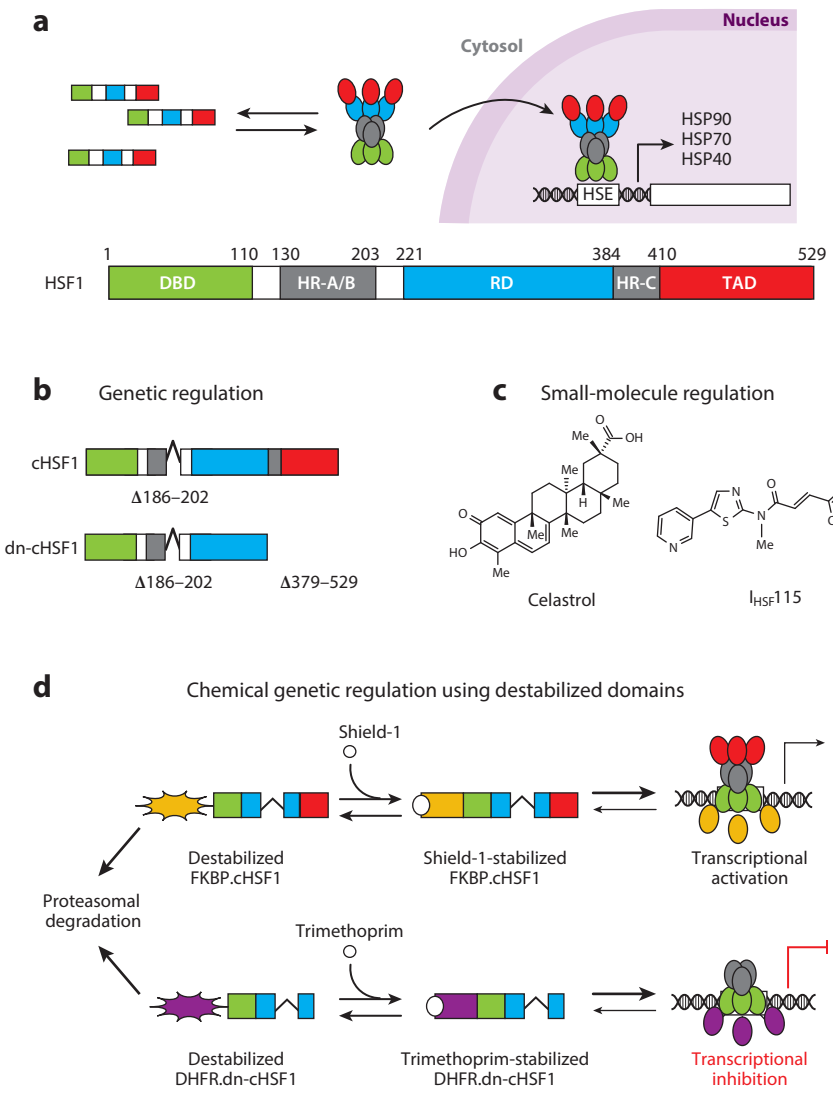
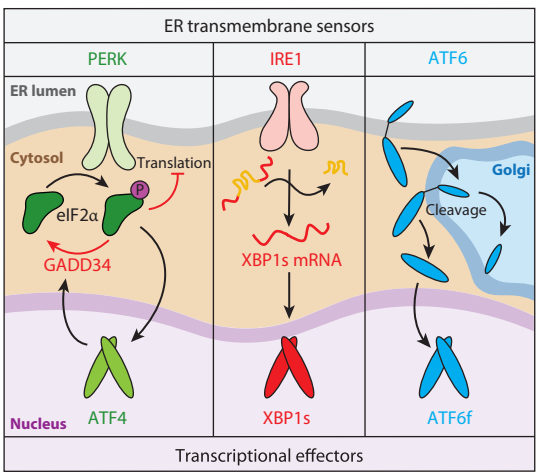






a Signaling pathways

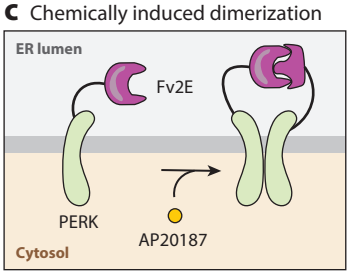


b Methods for regulation

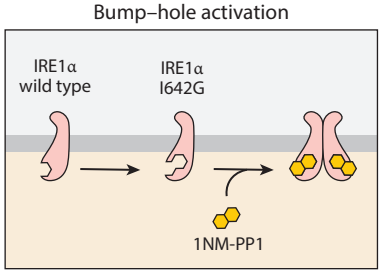
		PERK	IRE1	ATF6
Inhibition	Direct	CCT020312	Kinase inhibitors	AA147
	Chemical genetic	PERK-Fv2E	Bump–hole	NA
Activation	Direct	Kinase inhibitors	4μ8c MK0186893	Ceapins
	Chemical genetic	NA	NA	NA

		eIF2α	XBP1s	ATF6f
Inhibition	Direct	Sephin1 Raphin1	NA	NA
	Chemical genetic	NA	DD-XBP1s	DD-ATF6f(1–373)
Activation	Direct	ISRIB	NA	NA
	Chemical genetic	NA	NA	DD-ATF6f(171–373)

c Chemically induced dimerization



Bump–hole activation



d Destabilized domains