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Current Opinion in Cell Biology

Chemical and/or biological therapeutic strategies to ameliorate protein misfolding diseases

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Inheriting a mutant misfolding-prone protein that cannot be efficiently folded in a given cell type(s) results in a spectrum of human loss-of-function misfolding diseases. The inability of the biological protein maturation pathways to adapt to a specific misfolding-prone protein also contributes to pathology. Chemical and biological therapeutic strategies are presented that restore protein homeostasis, or proteostasis, either by enhancing the biological capacity of the proteostasis network or through small molecule stabilization of a specific misfolding-prone protein. Herein, we review the recent literature on therapeutic strategies to ameliorate protein misfolding diseases that function through either of these mechanisms, or a combination thereof, and provide our perspective on the promise of alleviating protein misfolding diseases by taking advantage of proteostasis adaptation.

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Current Opinion in Cell Biology 2011, **23**:231–238

This review comes from a themed issue on
Cell regulation
Edited by Laurie Glimcher and Claudio Hetz

Available online 9th December 2010

0955-0674/$ – see front matter

DOI 10.1016/j.ceb.2010.11.002

Introduction

Normal physiology hinges on the quality and concentration of proteins making up an organism's proteome. The protein homeostasis, or proteostasis, network is a highly conserved biological system designed to maintain the high quality and physiological concentration of proteins composing the proteome in spite of mutations, a constantly changing cellular and/or extracellular environment, and in the face of viral infections and other extrinsic stresses [1••,2]. The proteostasis network comprises several integrated and competing biological pathways, including ribosomal protein synthesis, chaperone/co-chaperone – and enzyme-mediated folding, proteasome– and lysosome-associated degradation pathways, vesicular trafficking pathways, etc. (Figure 1) [3–5]. The proteostasis networks in each subcellular compartment have unique attributes, and are independently regulated by stress-responsive signaling pathways that attempt to match proteostasis network capacity with demand, principally by coordinated transcriptional and translational upregulation of deficient pathways [6,7]. There are also post-translational mechanisms to match proteostasis network capacity with demand [8•,9,10].

The competition between protein folding and degradation is a key feature of proteostasis network function (Figure 1). Mutant proteins that do not fold efficiently at a given proteostasis network capacity are largely degraded by the proteasome or by lysosomes, leading to the loss-of-function phenotype of numerous protein misfolding diseases like cystic fibrosis [11] and the lysosomal storage diseases [12].

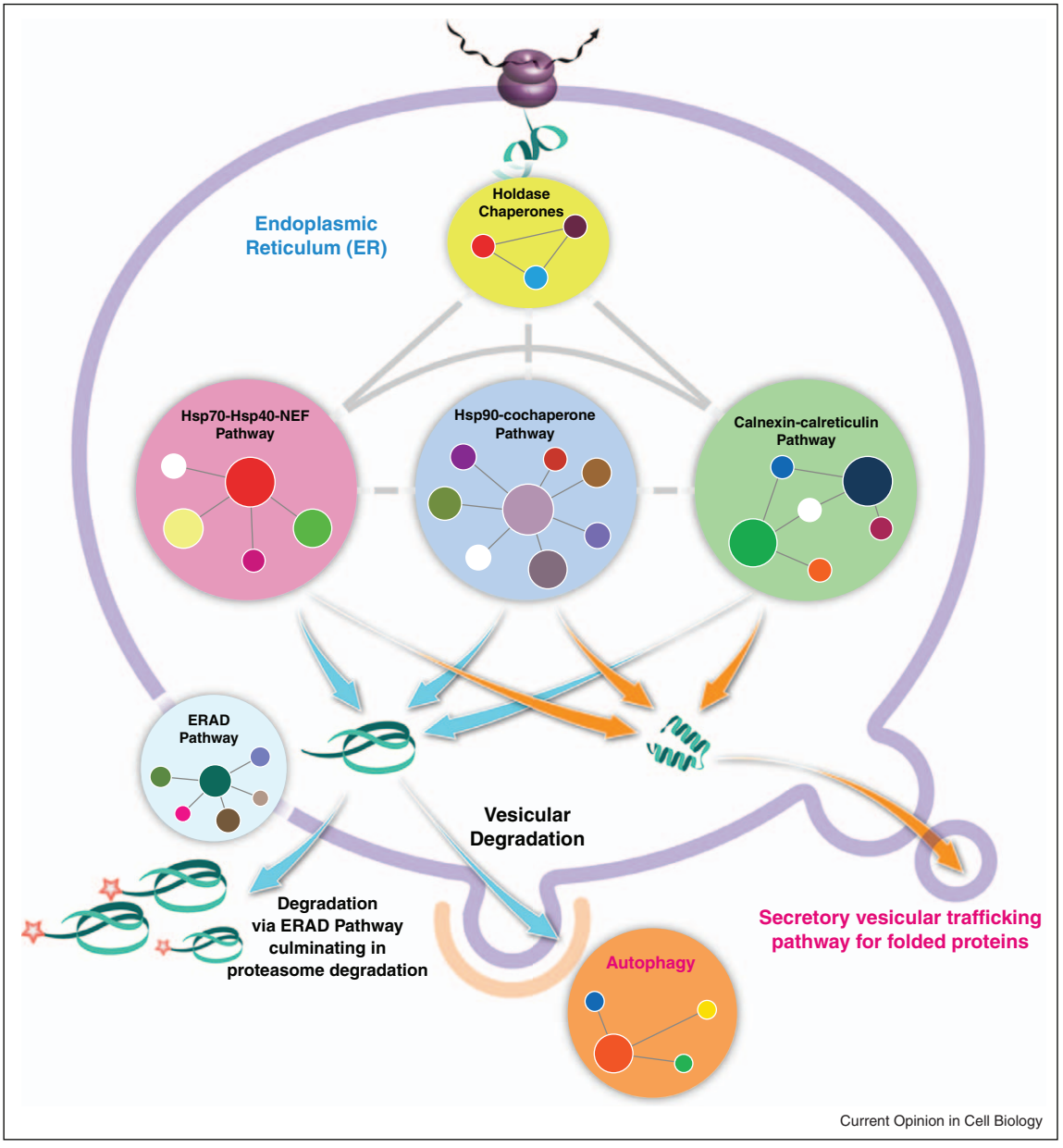
The folding efficiency of proteins that have evolved to function in destination environments that are chemically distinct from the endoplasmic reticulum (ER), such as in the lysosome that has an operating pH of 5, can be dramatically reduced by a mutation in the neutral pH environment of the ER. Many of the mutant lysosomal enzymes that cause lysosomal storage diseases (LSDs) do not fold efficiently in the ER, resulting in excessive ER-associated degradation (ERAD) (Figure 1) [13]. Nonetheless, if the properly folded mutant enzymes can be trafficked to the lysosome, they are actually quite stable and sufficiently active at pH 5 to degrade their substrate [14,15]. One way to increase ER folding competency is to lower the growth temperature of patient-derived fibroblasts. Lowering the growth temperature to 25 °C also makes the cytosol more folding competent, strikingly improving the folding, trafficking and function of the misfolding-prone ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) [16]. Thus, loss of function, due to excessive degradation, can be minimized by increasing the folding capacity of the proteostasis network in the compartment where it is deficient (the ER in LSDs and the cytosol in ΔF508 CFTR).

Herein, we review emerging chemical and biological approaches for improving defective proteostasis leading to the lysosomal storage diseases and cystic fibrosis. These therapeutic strategies are applicable to numerous loss-of-function diseases associated with excessive misfolding and degradation and have important implications for how medicine will probably be practiced in the future.

A chemical approach to stabilize and increase the folded population of a misfolding-prone protein

Pharmacologic chaperones represent a promising therapeutic strategy to ameliorate loss-of-function diseases and

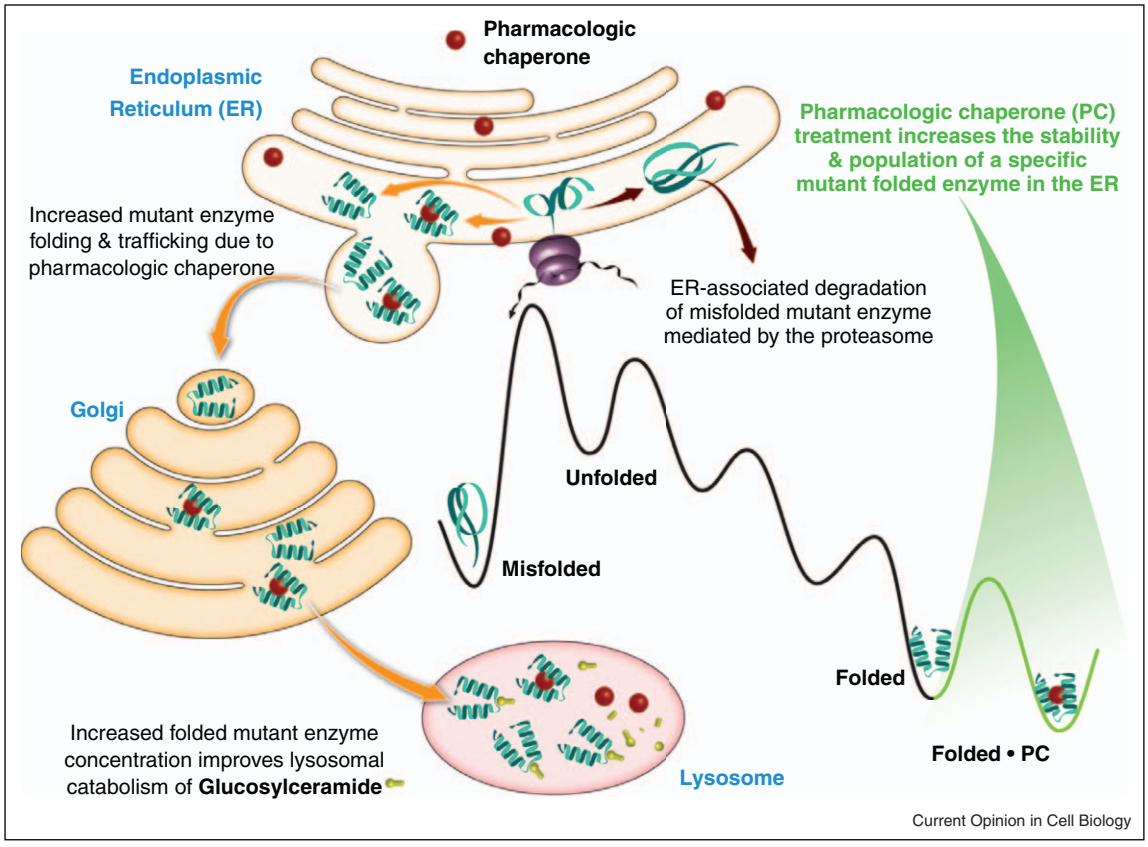
Figure 1



The Proteostasis Network Schematic of the endoplasmic reticulum (ER) proteostasis network in mammalian cells depicting the components and the connections in simplified format. A holdase chaperone delivers the nascent chain to the Hsp-70-40-nucleotide exchange factor (NEF) pathway and/or the Hsp90-cochaperone pathway and/or the Calnexin-calreticulin pathway that can lead to folding and vesicular trafficking or degradation mediated by ER-associated degradation (proteasome) and/or by autophagy.

are currently being evaluated in clinical trials for LSDs and for cystic fibrosis. Pharmacologic chaperones are small molecules that bind to and stabilize the folded state of a specific misfolding-prone protein, thereby increasing the concentration of the folded mutant protein that can engage its trafficking receptor and proceed to its destination environment, resulting in increased function (Figure 2). Fan *et al.* [17•] first demonstrated that a potent inhibitor of α-galactosidase A, 1-deoxy-galactonojirimycin, rescues the cellular misfolding and degradation of α-galactosidase A mutants associated with Fabry disease. Since then, pharmacologic chaperones have been developed for Gaucher's [18] infantile Batten diseases [19], and others employing patient-derived cellular models. The downside of the pharmacological chaperone approach is that a specific small molecule has to be tailored for each non-homologous protein linked to a loss-of-function misfolding disease, which number in excess of 50.

Figure 2



Pharmacologic Chaperoning A small molecule pharmacologic chaperone binds to the folded ensemble of a specific mutant misfolding-prone lysosomal storage disease-associated enzyme in the endoplasmic reticulum (ER) and stabilizes the mutant enzyme. This increases the fraction of folded enzyme that can bind to the trafficking receptor and be trafficked to the lysosome to degrade its substrate.

Biological approaches to match proteostasis network capacity with demand to ameliorate misfolding diseases

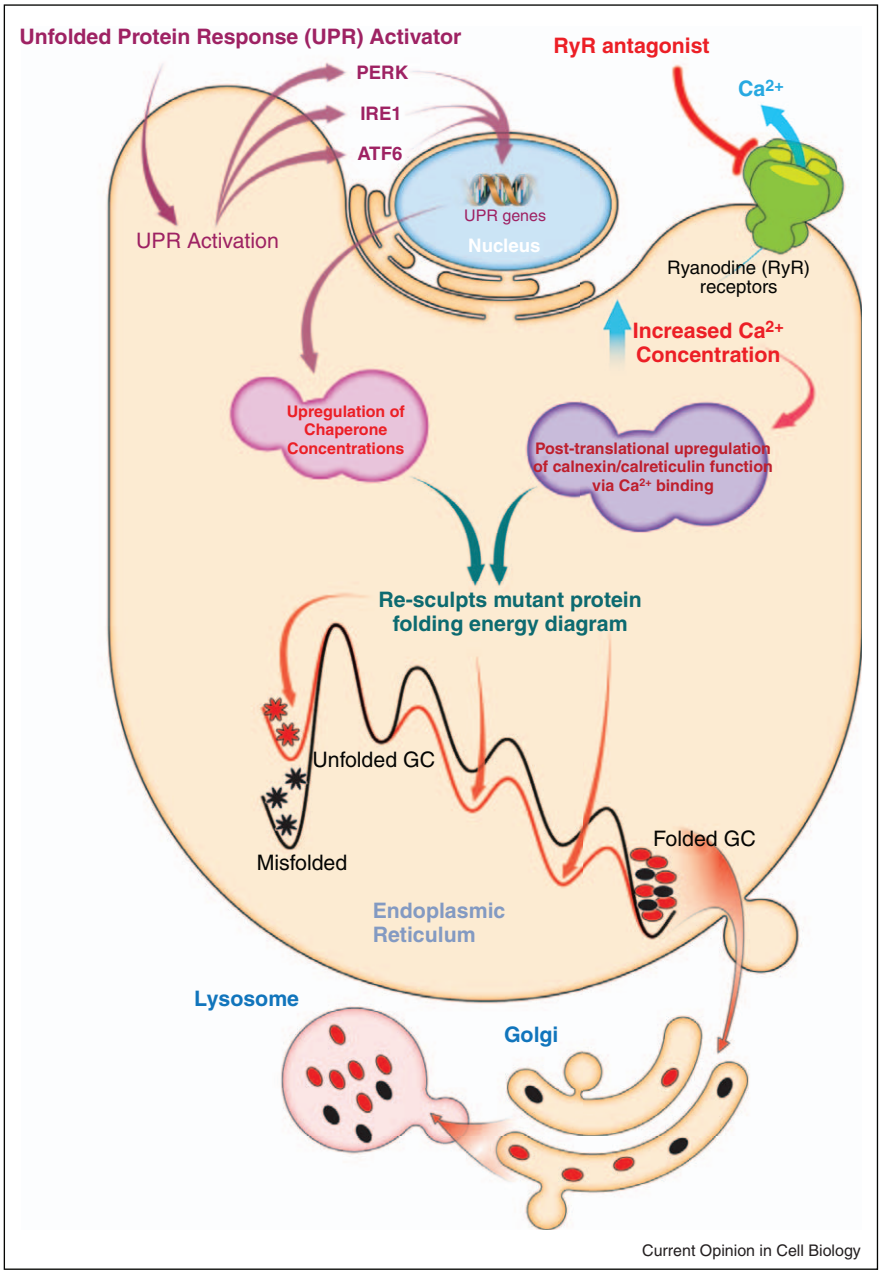
A more general therapeutic strategy to alleviate loss-of-function diseases uses proteostasis regulators, small molecules or biologics (e.g. siRNA) that enhance proteostasis network capacity in the deficient compartment [1••]. Proteostasis regulators can function through many different mechanisms, including activation of unfolded protein response (UPR) signaling, whereby hundreds of ER proteostasis network proteins are upregulated in a coordinated fashion through a transcriptional program [6,7]. Proteostasis regulators can also enhance proteostasis capacity through post-translational and epigenetic mechanisms [8•,9,20•].

Proteostasis regulators upregulate proteostasis network capacity

Celastrol is a proteostasis regulator that enhances ER proteostasis capacity by inducing all three arms of the UPR [21•]. Treatment of Gaucher's patient-derived fibroblasts with celastrol demonstrated that the IRE1-XBP and PERK arms of the UPR appear to be functionally important for enhancing mutant enzyme proteostasis [21•]. This strategy also increases the efficiency of folding, trafficking, and function of nonhomologous enzymes associated with a spectrum of LSDs [21•]. That the IRE1-XBP arm is important in LSDs was expected, since this is the only arm of the UPR that is conserved across eukaryotes [6,7]. Stress-responsive UPR signaling increases chaperone and folding enzyme levels in the ER, which resculpts the mutant enzyme's folding free energy diagram 'pushing' more protein toward the folded state at the expense of mutant enzyme misfolding, degradation, and aggregation (Figure 3).

A second class of proteostasis regulators increases ER Ca^{2+} levels. These small molecules function by inhibition of ryanodine receptor Ca^{2+} efflux channels in the ER membrane (Figure 3) [9] and probably L-type Ca^{2+} channels in the plasma membrane [8•]. Increased ER Ca^{2+} levels appear to upregulate the chaperoning capacity of calnexin (and probably other Ca^{2+}-regulated ER chaperones) through Ca^{2+} binding, without increasing chaperone concentrations. Enhanced chaperone activity resculpts folding free energy diagrams, increasing the

Figure 3



Adapting the Proteostasis Network Small molecule proteostasis regulators can induce the unfolded protein response (UPR) transcriptional program leading to the coordinated upregulation of endoplasmic reticulum (ER) proteostasis network capacity. The chaperones, co-chaperones and folding enzymes can resculpt the folding free energy diagram of mutant enzymes, 'pushing' more protein toward the native state by lowering the energy of intermediate and transition states and thus minimizing misfolding. Small molecule proteostasis regulators can also function by post-translational regulation mechanisms. One example are the Ryanodine receptor antagonists that increase the ER Ca^{2+} concentration leading to more Ca^{2+} binding by the chaperones, including calnexin and calreticulin, increasing their activity and ability to resculpt the folding free energy diagram of mutant lysosomal enzymes by 'pushing' more protein toward the native state at the expense of misfolding.

population of folded mutant lysosomal enzymes that are capable of engaging their trafficking receptors, at the expense of ERAD (Figure 3) [9].

Chaperone activity is also crucial for CFTR proteostasis, since siRNA depletion of the Hsp90 co-chaperone, Aha1, partially restores the folding, trafficking, and Cl^- conductance of ΔF508 CFTR. In this case, cytosolic chaperones are crucial because the misfolding-prone nucleotide binding domain I is in the cytosol. Presumably, the Hsp90 ATPase cycle was slowed to match the slowed folding kinetics of the cytosolic domain harboring the ΔF508

mutation, although other mechanisms are possible [22•,23].

Consistent with cytosol proteostasis being important, Marozkina *et al.* [24] found that the signaling molecule S-nitrosoglutathione (GSNO) enhances ΔF508 CFTR proteostasis by S-nitrosylating the Hsp70/Hsp90 organizing protein, Hop. GSNO decreased the Hop concentration, decreasing Hop binding to CFTR that leads to CFTR degradation. Cell growth at a permissive temperature in the presence of GSNO exhibited a synergistic rescue of ΔF508 CFTR proteostasis, consistent with combined approaches that are mechanistically distinct.

Proteostasis regulators that influence intracellular trafficking pathways

Upregulating a misfolding-prone protein's specific trafficking receptor can also restore proteostasis, as demonstrated in a Gaucher's disease cellular model. Overexpression of LIMP-2, the glucocerebrosidase trafficking receptor [25•], in COS7 cells was sufficient to restore the lysosomal localization of L444P glucocerebrosidase, a severe neuropathic Gaucher's disease mutant. Thus, small molecules that upregulate LIMP-2 expression levels may function as proteostasis regulators for Gaucher's disease. Similarly, overexpression of the mannose-6-phosphate receptor used by other LSD-associated enzymes to traffic to the lysosome may also prove useful for ameliorating other LSDs.

Regulating intracellular vesicular trafficking can also influence lysosomal enzyme proteostasis. Reducing intracellular cholesterol levels in Gaucher's patient-derived fibroblasts improved mutant glucocerebrosidase trafficking and function [26]. This observation and reports of autophagic defects in the lysosomal storage diseases [27–30] support the idea that manipulating the intracellular vesicular trafficking pathways offers value for treating these diseases. An activity-based screen identified ubiquitin specific protease-10 (USP-10), a deubiquitinating enzyme of ΔF508 CFTR, as a potential therapeutic target for cystic fibrosis [31]. USP-10 is localized in early endosomes and regulates the post-endocytic sorting of CFTR. Overexpressing USP-10 decreased the amount of ubiquitinated CFTR and increased its abundance in the plasma membrane of human airway epithelial cells [31].

Proteostasis regulators that influence protein degradation decisions

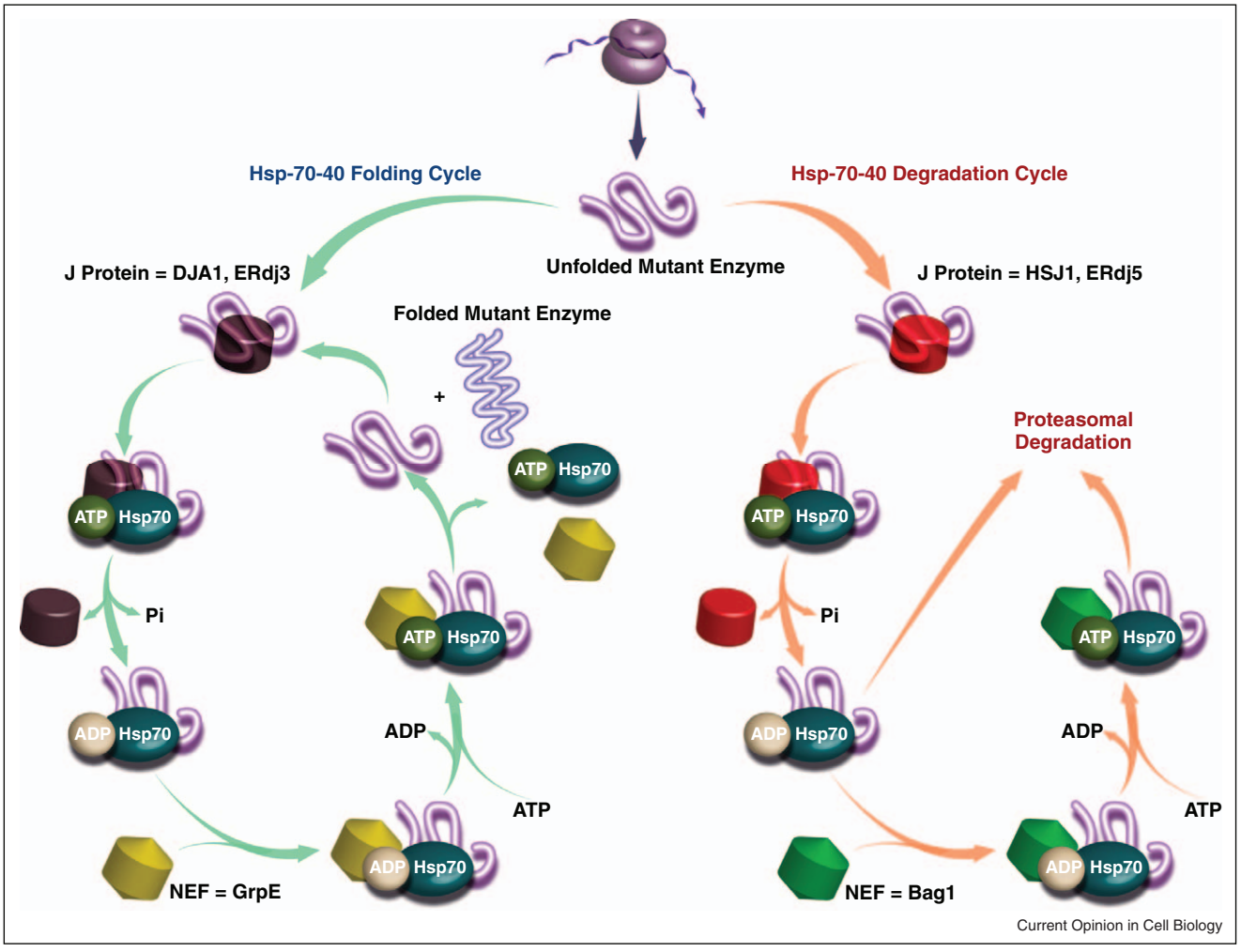
Since competition between protein folding and degradation is a key feature of maintaining proteostasis, proteostasis regulators that enhance folding and trafficking at the expense of degradation offer promising therapeutic strategies. For instance, antagonizing the Hsp70-40 degradation pathway or activating the Hsp70-40 folding pathway is expected to rescue misfolding-prone and degradation-prone proteins (Figure 4). Park *et al.* [32] demonstrated that a soluble sulfogalactosyl ceramide mimic selectively inhibited Hsp40-activated Hsc70 ATP hydrolysis, reducing Hsc70 chaperone function. This increases the immature form (band b) of ΔF508 CFTR, suggesting increased escape from ERAD, leading to increased maturation and iodide efflux from ΔF508 CFTR in transfected BHK cells. In contrast, Schmidt *et al.* [33] found that a J-domain containing protein, the cysteine string protein (Csp), inhibited CFTR ER exit and facilitated its degradation. Overexpression of Csp enhanced CFTR-Hsc70 and CFTR-CHIP interactions, promoting CFTR ubiquitination and degradation, suggesting that antagonizing these interactions is a possible therapeutic strategy.

Interfering with the ubiquitin-mediated proteasomal degradation process is yet another means to counter extensive protein degradation in favor of folding, trafficking, and function. Hassink *et al.* [34] showed that an ER-resident transmembrane deubiquitinating enzyme, ubiquitin specific protease (USP)-19, rescues ΔF508 CFTR from proteasomal degradation. USP-19 is a UPR-regulated deubiquitinating enzyme that appears to be involved in a late step of protein quality control, by rescuing ERAD substrates that have been retrotranslocated to the cytosol. Tcherpakov *et al.* [35] found that the ER ubiquitin ligase, RNF5, associates with and ubiquitinates the JNK-associated membrane protein (JAMP). JAMP ubiquitination inhibits its association with the Rpt5 ATPase and p97, leading to an inefficient clearance of ΔF508 CFTR, suggesting an opportunity could exist for enhancing CFTR proteostasis. Consistent with this hypothesis, Ye *et al.* [36] demonstrated that the siRNA knockdown of c-Cbl, a multifunctional protein with ubiquitin ligase activity and protein adaptor function, increased CFTR surface expression and CFTR-mediated Cl^- function. c-Cbl first facilitates CFTR endocytosis by a ubiquitin-independent mechanism, and subsequently ubiquitinates CFTR in early endosomes, promoting CFTR lysosomal degradation in human airway epithelial cells.

Degradation of CFTR can also occur as a consequence of proteostasis network decisions made in the Golgi. Cheng *et al.* [37] found that silencing the SNARE protein, STX6, increases both wild type and ΔF508 CFTR protein levels and Cl^- ion channel function. STX6 interacts with the PDZ domain containing protein CAL that is localized to the Golgi and in Golgi-derived vesicles. The effect of STX6 on CFTR proteostasis and function is dependent on CAL. Interestingly, silencing STX6 enhances the low temperature effect in rescuing ΔF508 CFTR, which again suggests the utility of combining mechanistically distinct therapeutic strategies for cystic fibrosis treatment.

Recent elegant work from Okiyoneda *et al.* [38••], using siRNA-mediated functional screens, takes advantage of

Figure 4



Competition between Protein Folding and Degradation is a Central Feature of Proteostasis Network Function Depicted is an Hsp-70-40-nucleotide exchange factor (NEF) proteostasis pathway that is intentionally generic (not organelle specific), illustrating how the competition between folding and degradation of a foldable protein is affected. As can be discerned from this representative pathway, the concentrations and activity of many components (many not shown) influence the ratio of folding to degradation for a particular client protein.

the fact that the ΔF508 CFTR is degraded from the plasma membrane when shifted from 26 °C to 37 °C. They identified chaperones, co-chaperones, and ubiquitin-conjugating and ubiquitin-ligating enzymes (that make up ''the CFTR peripheral protein quality control system'') that eliminate unfolded CFTR from the cell surface. An unanticipated finding was that several cytosolic proteins, including the ubiquitin-conjugating enzyme UbcH5, the ubiquitin ligase CHIP, and Hsp70/Hsp90 proteins that influence CFTR proteostasis during nascent protein synthesis, also play similar roles at the plasma membrane or in the peripheral protein quality control system. This observation suggests that the fate of proteins throughout their functional life cycle is influenced by members of the proteostasis network whose effect may not be easily anticipated.

Proteostasis regulators that impact proteostasis at the genomic and epigenomic levels

A novel strategy to restore proteostasis comes from a study by Sardiello *et al.* [39], who discovered that the transcription factor EB coordinates the transcriptional behavior of most lysosomal genes. Overexpression of EB in cultured cells upregulated lysosomal genes and genes related to lysosomal biogenesis and function. This finding underlines the therapeutic potential of small molecule transcription factor activators that regulate lysosomal genes for treating multiple lysosomal storage diseases.

Manipulating the proteostasis network at the epigenetic level is another promising approach for treating loss-of-function diseases. Hutt *et al.* [20•] demonstrated that

reducing the activities of histone deacetylases (HDACs), in particular HDAC7, using suberoylanilide hydroxamic acid or siRNA, partially restored the surface channel activity of the ΔF508 CFTR in human primary airway epithelia. Reducing HDAC7 activity increased mRNA levels encoding the CFTR interactome, suggesting that epigenetic upregulation of the CFTR proteostasis network is responsible for the increased CFTR Cl^- channel activity. It is anticipated that epigenetic manipulation of the proteostasis network will ameliorate other loss-of-function diseases.

Combining pharmacologic chaperones and proteostasis regulators yields a synergistic rescue of proteostasis

Because pharmacologic chaperones increase the population of the folded state by direct binding and stabilization (''pulling protein toward the folded state''), whereas proteostasis regulators increase the folded state population by upregulating the proteostasis network that resculpts the folding free energy diagram of the mutant enzyme (''pushing protein toward the folded state''), it is not surprising that combination of a proteostasis regulator and a pharmacologic chaperone synergize to rescue mutant enzyme folding, trafficking, and function in LSDs. Mu *et al.* [21•] showed that treatment of Gaucher's fibroblasts harboring the L444P glucocerebrosidase mutant with the pharmacologic chaperone, NN-DNJ, and a proteostasis regulator, MG-132, or celastrol, resulted in a 4–6 fold increase in the lysosomal enzyme activity, greater than the sum of the effect with each compound alone. L444P glucocerebrosidase is usually not amenable to pharmacologic chaperoning because of the very low steady state concentration of the folded state that exists in patient-derived fibroblasts. Proteostasis regulators increase the folded state population of L444P glucocerebrosidase and that, in turn, renders pharmacologic chaperones more efficacious. A similar synergistic rescue of lysosomal enzyme proteostasis is predicted when two mechanistically distinct proteostasis regulators are co-applied to cells.

Conclusions and perspective

Adapting the proteostasis network, by utilizing small molecules or RNAi, has emerged as a promising strategy to treat a wide variety of loss-of-function diseases. The concern that increasing proteostasis network capacity will increase the concentration of much of the proteome maturing in that compartment is an important one. However, quantitative whole cell proteomics carried out on proteostasis regulator-treated cells reveal that the vast majority of the proteome remains unchanged because it exhibits sufficiently fast folding, high enough thermodynamic stability, and slow enough misfolding or aggregation kinetics to fold very efficiently at the basal level of proteostasis network capacity [21•]. The ability of proteostasis regulators to resculpt the folding free energy diagram of mutant proteins enables one proteostasis regulator to be used for multiple misfolding diseases, assuming that the mutant proteins depend on common proteostasis network components. This transforms the economics of drug development for rare diseases; for example, numerous lysosomal storage diseases can in principle be treated with a single proteostasis regulator that upregulates proteostasis network components capable of folding many of the LSD-associated mutant enzymes. Moreover, the synergistic effect realized by combining two mechanistically distinct proteostasis regulators, or a proteostasis regulator and a pharmacologic chaperone, suggests that misfolding diseases will be treated in the future with at least two agents capable of restoring proteostasis through distinct mechanisms of action.

Acknowledgements

We thank Dr. Colleen Fearns for critical feedback on this review. This work was supported by the NIH (DK75295), the Skaggs Institute for Chemical Biology, and the Lita Annenberg Hazen Foundation.

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- •• of outstanding interest

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