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Differential Scales of Protein Quality Control

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Proteins are notorious for their unpleasant behavior—continually at risk of misfolding, collecting damage, aggregating, and causing toxicity and disease. To counter these challenges, cells have evolved elaborate chaperone and quality control networks that can resolve damage at the level of the protein, organelle, cell, or tissue. On the smallest scale, the integrity of individual proteins is monitored during their synthesis. On a larger scale, cells use compartmentalized defenses and networks of communication, capable sometimes of signaling between cells, to respond to changes in the proteome's health. Together, these layered defenses help protect cells from damaged proteins.

Introduction

The human cell contains several billion protein molecules at total concentrations ranging from 50 to 300 mg/ml, approaching the saturation found in protein crystals (Asherie, 2004; Finka and Goloubinoff, 2013). To synthesize a proteome of this magnitude, ~3 million ribosomes work to translate codons at a rate of close to six amino acids per second (Duncan and Hershey, 1983; Ingolia et al., 2011). The synthesis of a typical protein requires 3,000 molecules of ATP, making translation of proteins the most energetically expensive process that a cell undertakes and one that consumes up to 75% of its total energy budget (Lane and Martin, 2010; Piques et al., 2009).

This is a massive number of macromolecules. Despite the amount of work undertaken during translation, the chemical synthesis of proteins remains remarkably efficient: the vast majority of polypeptides that are produced from a single mRNA are perfect chemical copies of one other. By contrast, both the folding and maintenance of proteins in their functional, native, 3D conformations frequently fails. Quality control of the proteome is made more difficult by the high degree of heterogeneity present across populations of proteins, which prevents them from fitting into standardized categories of size, shape, or stability. For example, the mean size of a human protein is around 500 amino acids, but polypeptides can reach extreme sizes; the longest isoform of titin, the largest protein in the human body, is 3.7 MDa (Krüger and Linke, 2011; Siwiak and Zielenkiewicz, 2013) (Figure 1). This dwarfs Complex I of the mitochondrial electron transport chain, a supercomplex of ~1 MDa in size, which is composed of more than 40 different proteins (Calvaruso et al., 2012). More recently, researchers have begun to catalog examples of the opposite extreme: translated short ORFs (sORFs) that encode for only a few amino acids (Brar et al., 2012; Hashimoto et al., 2008; Ingolia et al., 2011).

Proteins also vary greatly in their lifespans (Toyama et al., 2013). The shortest of proteins, such as HIF-1α, live only a few minutes, whereas the longest, such as collagen, are stable over the life of an animal (Salceda and Caro, 1997; Verzijl et al., 2000). Proteins are often modular; every hundred or so amino acids, the polypeptide sequence typically arrives at a different, independently folding domain, which will have distinct stability and susceptibility to degradation (Xu and Nussinov, 1998). Secondary structures of proteins, α helices and β sheets, exhibit highly different propensities toward aggregate formation and thus have differential burdens on the quality control of the cell. Some proteins contain entire domains that are designed to have no order; these intrinsically disordered proteins (IDPs), notably, still have important physiological or regulatory roles within the cell. Posttranslational modifications, of which scientists have now identified almost 300 different types, can considerably alter the folding and stability of the protein (Prabakaran et al., 2012). Finally, with age, proteins accumulate damaging modifications that dramatically affect their function. As many as 35 different types of oxidative damage and a dozen different types of carbonylations—and no doubt many other modifications—have been found to accumulate with age (Madian and Regnier, 2010). Hypothetically, the diversity of both the protein and the types of damage it may accrue may be part of the reason why a hierarchy of quality control mechanisms is necessary to survey and react to changes in proteome health.

For the past 40 years, there have been tremendous advances toward deciphering the processes that make sure that the proteome is well behaved: to understand the quality control checkpoints, the stress responses, the degradation machineries, and the systems of communication that are activated during times of stress. Recent advances highlight the importance of many of these individual systems, including those associated with chaperone networks (Kim et al., 2013), lysosome-mediated degradation (Kroemer et al., 2010), and proteasome function (Varshavsky, 2012), which have recently been reviewed elsewhere. Here, we begin with a close look at the quality control

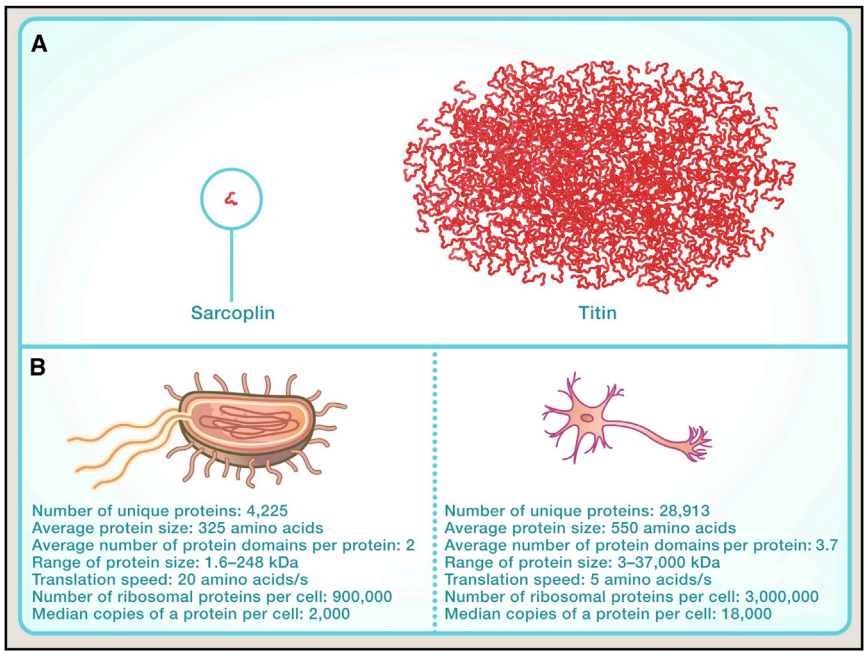

Figure 1. The Heterogeneity of the Proteome

This figure provides an illustration of the relative difference in the variation in the proteomes that has developed both within a species and between species.

(A) Sarcolipin is one of the smallest proteins in the human proteome at just 31 amino acids in length. It functions in the ER, binding to the Ca^{2+}/ATP SERCA pump to regulate its function (Bal et al., 2012). In contrast, the human protein titin is more than 1,000× its size, containing 34,350 amino acids. Titin has both regulatory and structural roles within the sarcomeres (Krüger and Linke, 2011).

(B) A comparison between prokaryotes and eukaryotes reveals extreme differences in protein numbers, size, translational speed, and ribosomal machinery. Numbers listed were obtained from Milo et al. (2010).

mechanisms that monitor individual proteins as they are synthesized at the ribosome; we then move to examine the methods by which aberrant proteins are sequestered into compartments within the cell as it tries to shield them from negatively affecting its function. Finally, we take a more distant look at how changes in the proteome are sensed and reacted to across cells and tissues within a metazoan organism.

Part One: Quality Control at the Ribosome

Before and during Synthesis

The most direct way for a cell to avoid the accumulation of damaged proteins is to avoid creating flawed proteins, which it does by recognizing and controlling for these errors as the polypeptides are being synthesized. Translational errors have large biophysical effects on the stability of the polypeptide; a single mutation, for example, can cause a destabilizing change in free energy of the protein of 0.5–5 kcal/mol (DePristo et al., 2005), whereas truncation is likely to prevent folding or lead to proteins missing functional domains. Based upon the average number of proteins in a human cell and their median size, we could predict that hundreds of billions of translated amino acids compose the polypeptides in a given cell at any given moment. If the error rate of translation were not kept in check, the proteome as a whole would readily face destabilization.

Ribosome complexes thus include both scaffolding proteins and proteins that assist in quality control of the nascent chain. In metazoans, an estimated 5% of the total mass of proteins comes from ribosomal proteins themselves (Wilson and Nierhaus, 2007). Even the simplest of ribosomes, found in archaea, bacteria, and mitochondria, are composed of ~50 different proteins and their associated rRNAs (Noller, 2012). Despite the large proportion of the proteome that is composed of ribosomal subunits, rRNA is responsible for the catalytic functions of the ribosome during translation (Noller, 2012; Poole et al., 1998). The nearly 300 proteins characterized as participants in the process of eukaryotic translation primarily function to ensure the efficiency, quality, and accuracy of the nascent polypeptide. Because of these scaffolding and quality control proteins, only one in every 10,000 amino acids is misincorporated (Zaher and Green, 2009). However, given the huge number of amino acids in the proteome, tens of millions of aberrant proteins are still produced. The importance of proper protein synthesis is underscored by the fact that mutations that increase the rate of misincorporation of amino acids or prevent the proper cotranslational degradation of anomalous polypeptides lead to neurodegenerative disorders (Chu et al., 2009; Lee et al., 2006).

Recent work has suggested that ribosomes employ multiple, elegant mechanisms in order to reduce error rate and increase translation fidelity. These machineries are often actively regulated and adaptively deployed. Alterations in the rate of translation, for example, allow for more accurate translation and folding (Gingold and Pilpel, 2011). Rate of translation is dependent upon the composition of the ribosome and the amount and type of accessory factors present. First and foremost, however, efficiency and accuracy in translation will rely upon the ribosome's substrate, the mRNA.

The quality of the mRNA and its codon usage affects translation rates, and translation rate, in turn, alters chaperone binding to the nascent chain (Gloge et al., 2014). Traditionally, the use of codons that slow translation has been hypothesized to increase the proportion of nascent chains that are properly folded (Komar et al., 1999; Siller et al., 2010; Spencer et al., 2012). This phenomenon has been elegantly demonstrated by engineering luciferase sequences containing wobble codons (Spencer et al., 2012). Alternatively, rapidly translating mRNAs may decrease the probability of intermediate state folding and thus increase the final proportion of protein reaching a native state (O'Brien et al., 2014). In either case, the effects of translation rate on nascent chain folding can increase or decrease both the enzymatic activity and the structural stability of a protein (Zhou et al., 2013), suggesting that translation itself may affect the kinetic partitioning between native states (Sinclair et al., 1994).

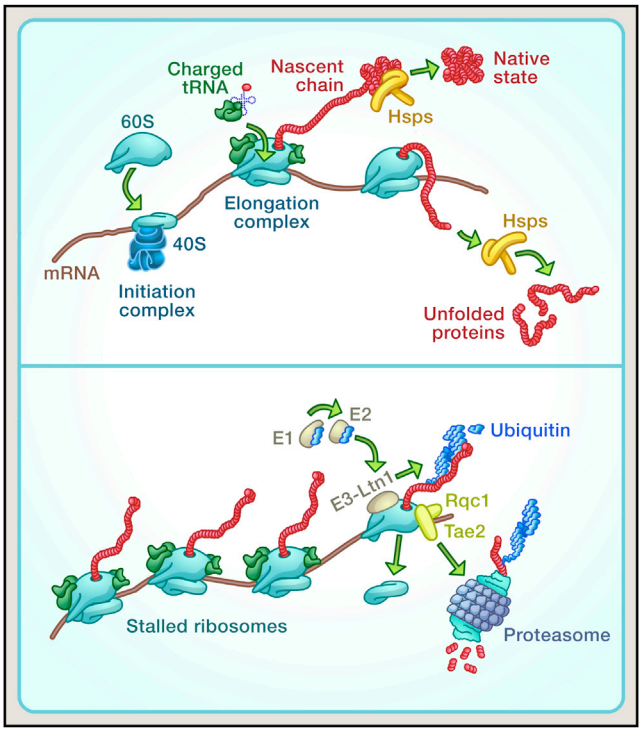

Figure 2. Quality Control at the Ribosome

Translation processes require the participation of a large number of both ribosomal subunits, as well as accessory proteins. To initiate synthesis, the initiation complex forms at the 40S ribosome and recruits the 60S, after which time the initiation complex is released. The elongation complex binds during elongation to help ensure the efficiency of the process and ensure that the tRNA is charged with the correct amino acid. Multiple Hsps bind to the nascent chain and assist with protein folding. Nascent chains compete with misfolded proteins in the cytosol for Hsps, and cotranslational folding can be affected by the elevation of proteotoxic stress elsewhere. Stalled ribosomes elicit the recruitment or participation of surveillance complexes (not pictured), followed by the recruitment of the RQC and the E3 ligase, Ltn1.

For proteins containing hydrophobic stretches or aggregation prone sequences, slowing of translation specifically during the synthesis of aggregation-prone stretches of amino acids allows for the increased binding of chaperones prior to its release, preventing it from forming aggregates. In keeping with this idea, structured proteins found within cellular aggregates are often sequestered there as nascent polypeptides, suggesting that their cotranslational folding chaperones failed to fold them in time to avoid aggregation (Olzscha et al., 2011). Intriguingly, however, the opposite effect is seen with IDPs, which form metastable and, often, multidomain proteins that increase in aggregation propensity upon changes in the cellular environment. Nascent chain IDPs are not found in aggregates, and instead, only older IDPs are sequestered, suggesting that this class of proteins becomes aggregation prone only after their translation is complete (Olzscha et al., 2011).

During times of stress, cells can transiently reduce the rate of translation globally, a perturbation that allows for the continued, targeted synthesis of a small group of chaperones. The implementation of translational arrest is a strategy used by subcellular compartments in response to organelle stress or changes in nutrient and energy availability through kinases that include GCN2 and PERK (Baird and Wek, 2012) as sensors of homeostasis in the mitochondria and endoplasmic reticulum (ER), respectively. More recent evidence indicates that translation elongation is paused in response to acute cytoplasmic heat shock, in part through competition between the nascent chains and cytoplasmic unfolded proteins for chaperones (Shalgi et al., 2013). Translation elongation is also blocked upon the application of drugs that induce protein-folding stress in combination with those that simultaneously block proteasome activity (Liu et al., 2013). This suggests that proteotoxic stressors and the increase in misfolded proteins cause an escalation in the initiation of measures that protect both the ribosome and its products.

A second point of quality control occurs at the step of proofreading tRNA. tRNA synthetases must distinguish between amino acids present in the cellular pool to reduce the rate at which smaller amino acids are misincorporated into the active site of the tRNA and thus have an additional domain distinct from that which acetylates that is capable of editing out the incorrectly incorporated amino acids (Guo and Schimmel, 2013). A loss of this proofreading domain is detrimental to cytosolic ribosomal synthesis, promoting striking proteotoxic effects that can end in neurodegenerative disease (Lee et al., 2006). Similarly, mutations in the tRNA recognition site of the mitochondria alanyl-tRNA synthetase that hinder proofreading can cause infantile cardiomyopathy (Götz et al., 2011).

The increased diversity and complexity in the proteins of eukaryotic lineages has resulted in the need for a third mechanism of quality control, cotranslation folding. Prokaryotes and eukaryotes differ fundamentally in the length and structure of their proteins, as the percentage of longer polypeptides nearly doubles in eukaryotes (Netzer and Hartl, 1997). Cotranslational folding of these long polypeptides helps to circumvent the formation of folding intermediates that could otherwise undermine the capacity for the protein to reach its native state (Netzer and Hartl, 1997). In eukaryotes, cotranslational folding is assisted by the binding of a subset of heat shock proteins (the Hsp40 and Hsp70) to the nascent chain (Gloge et al., 2014). Importantly, unfolded nascent chains compete with unfolded proteins in the cytosol for these chaperones, and perturbations of the cell that increase general levels of unfolded proteins necessarily also affect the cotranslational folding (Figure 2). Cotranslational folding in eukaryotes is essential, and the mechanism of cotranslational folding has been comprehensively reviewed recently (Gloge et al., 2014).

Fourth, the quality of the nascent chain depends upon the integrity and possibly the composition of the ribosome. Stalled or damaged ribosomes can be identified by the no-go mediated mRNA decay (NGD) and nonfunctional rRNA decay (NRD) pathways, respectively (Karbstein, 2013; Moore and Sauer, 2007). Additional pathways exist to identify improperly assembled ribosomes during maturation in a process that involves surveillance both within the nucleolus and cytoplasm. For example, as they are synthesized, unassembled ribosomal proteins are commonly degraded within the nucleoplasm prior to their export, helping to ensure the integrity of ribosomal proteins (Lam et al., 2007). Haploinsufficiency of subunits of the ribosome cause a nucleolar stress response, suggesting that the ribosome is checked for

its integrity prior to export to the cytoplasm (Jones et al., 2008). Intriguingly, haploinsufficiency of specific ribosomal subunits causes distinct diseases in mammals, including developmental failures (Kondrashov et al., 2011), asplenia (Bolze et al., 2013), and anemia (Boria et al., 2010). Degradation of whole ribosomes can also occur upon starvation through the ubiquitin tagging of the ribosome followed by selective autophagy, a process called ribophagy (Kraft et al., 2008).

Compositional alternations in the structure of the ribosome may play an additional role in the ribosomal efficiency. Beyond the accessory factors involved with translational assembly, initiation, elongation, and termination—all of which affect ribosome function during stress—the diversity and complexity of the composition of the ribosome, as its subunits come together in differential ways, will likely influence the instantaneous proteome that is being produced (Barna, 2013). Importantly, diseases associated with mutations in ribosomal components are often associated with tissue-specific phenotypes, and ribosomal mRNA levels vary between tissues, suggesting alternate requirements for ribosomal subunits in specific organs (McCann and Baserga, 2013). The changing composition of a ribosome has been suggested to form a ''ribosomal code'' capable of preferentially translating subset of mRNAs (Komili et al., 2007). Given such a hypothesis, the composition of the ribosome subunits in itself may alter under conditions of stress, and this may promote the translation of specific, stress-responsive targets in response to unfolded proteins. We do not yet fully understand how or why these changes occur and to which specific stresses they might respond.

After the Completion of Synthesis

Recently, it has become clear that a fraction of proteins is tagged for immediate degradation during translation (cotranslational ubiquitination, CTU). Currently, it is estimated that up to 15% of nascent chains in human cells are cotranslationally tagged for degradation, emphasizing the importance of cotranslational degradation in quality control at the ribosome (Duttler et al., 2013; Wang et al., 2013). The proteolytic degradation of newly translated proteins is also thought to provide a source of MHC-I-presented antigenic peptides (the defective ribosomal products [DRIP] hypothesis) (Schubert et al., 2000), although more recent evidence suggests that peptides generated from mature proteins may play a significant role in MHC I presentation (Rock et al., 2014). Importantly, ubiquitinated nascent chains are associated with both stalled and active complexes. This suggests not only that ubiquitination occurs prior to the release of the nascent chain from the ribosome but also that the UPS system plays an important part in mediating the detrimental effects of nonsense mRNAs.

In eukaryotes, truncated mRNAs or difficult to translate sequences lead to ribosome stalling. Stalled ribosomes are recognized by a poorly understood mechanism that depends on the integral ribosomal protein Rack1 (Asc1 in yeast) (Kuroha et al., 2010), a ribosome-associated E3 ligase, Hel2 (Brandman et al., 2012), and potentially the Dom34-Hbs1 surveillance complex, which can split ribosomes into their 60S and 40S subunits. The nascent polypeptide on the 60S ribosome is then degraded by the ribosome quality control (RQC) complex, which contains a second E3 ligase, Ltn1 (Bengtson and Joazeiro, 2010; Brandman et al., 2012; Defenouillère et al., 2013; Inada, 2013; Verma et al., 2013; Vernace et al., 2007). A parallel but mechanistically distinct mechanism for sensing truncated mRNA has been identified in bacteria. Here, the transfer-messenger RNA (tmRNA), which has dual tRNA and mRNA properties, leads to C-terminal tagging of the nascent polypeptide with a degradation signal (Moore and Sauer, 2007).

Disassembly of the stalled ribosome also allows for the reuse of ribosome subunits when possible (Shoemaker et al., 2010). Intriguingly, when activated, the RQC also appears to induce the heat shock pathway using a mechanism that is distinct from the pathways by which cytosolic misfolding is sensed (Brandman et al., 2012). Although the threshold for activation of the heat shock pathway is not known, this suggests that a small number of ribosomes (or even a single stalled ribosome) may be capable of communicating its stress to a more global audience of ribosomes, quite possibly influencing their behavior.

Although up to a quarter of ribosomes are stalled, less than 15% of those become ubiquitinated, suggesting the existence of an additional regulatory mechanism by which a particular stalled complex is recognized. Indeed, multiple different ribosome-associated E3 ligases can impact cotranslational degradation in yeast (Duttler et al., 2013). A remarkable cotranslational quality control system has recently been elucidated in bacteria. Here, accidental incorporation of an incorrect amino acid leads to transition of ribosome to a state that is highly error prone and eventually terminates before completing synthesis (Shoemaker and Green, 2012).

Part Two: The Compartmentalization of Quality Control

A view of protein quality control from beyond that of the individual protein suggests that proteins are shepherded into subcellular compartments capable of sequestering, folding, and/or degrading groups of aberrant proteins. The localization of misfolded or aggregating proteins into compartments that are distinct from the organelles has added a secondary layer to our knowledge of quality control. From what we know now, the sole function of many of these compartments is to handle misfolded proteins, but it is likely that the function of many of these will prove more complex. By sequestering the misfolded proteins, aggregates, or amyloid within appropriate and specific subcellular compartments, the cell minimizes the capacity for these species to cause cellular damage or to nucleate the aggregation of additional proteins. Additionally, the cell can concentrate its efforts to solubilize and fold these proteins, when possible, prior to degradation. Here, we highlight emergent findings behind the mechanisms by which the cell compartmentalizes its aberrant proteins.

Sequestration of Ubiquitinated Proteins: JUNQ

Recent work has revealed a conserved sequestration of ubiquitinated proteins into membrane-enclosed, juxtanuclear compartments (the juxtanuclear quality control compartment, JUNQ) (Kaganovich et al., 2008) (Figure 3). JUNQ is the cellular quality control center in which soluble misfolded proteins or aggregates from the cytosol accumulate for proteasomal degradation and refolding (Kaganovich et al., 2008). In a cell, only a single JUNQ will be found under proteotoxic conditions. The determinants of its formation and localization are unknown, as well

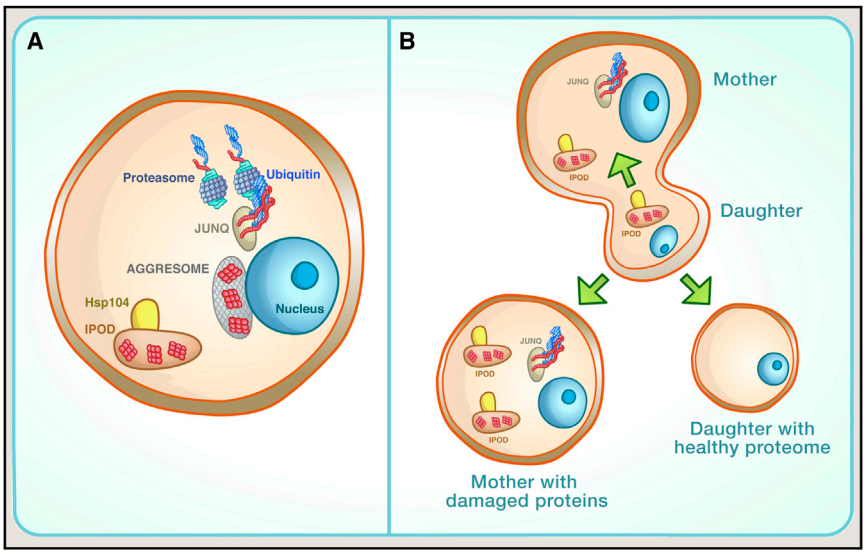

Figure 3. Compartmentalization of Proteins
(A) Misfolded, aggregated, or amyloidogenic proteins are targeted for sequestration into one of several different subcellular compartments. Soluble and ubiquitinated proteins are transported via the cytoskeleton to JUNQ, whereas aggregated, insoluble or amyloid proteins are targeted for the cytoplasmic iPOD, to which the disaggregase Hsp104 is also recruited. When proteasomes are inhibited, aggregating proteins can also accumulate inside of aggresomes.
(B) During replication, mother cells protect daughter cells by retaining damaged aggregates in a mechanism that requires cytoskeletal participation.

as how its boundaries are defined. However, its appearance is evident after the application of heat shock or proteasome inhibition (Sontag et al., 2014). It is possible that JUNQ is the mature form of coalesced, smaller compartments of soluble, ubiquitinated proteins, called Q bodies (Escusa-Toret et al., 2013). Proteins are targeted to JUNQ by their ubiquitination (Kaganovich et al., 2008), and substrate transport to JUNQ requires the actin cytoskeleton (Specht et al., 2011). Many chaperones and proteasome complexes can be found surrounding JUNQ, suggesting, possibly, that JUNQ serves the beneficial purpose of concentrating a misfolded protein with its chaperones, increasing its probability of refolding in addition to simple degradation. Consistent with this idea, fluorescence recovery after photobleaching (FRAP) analysis of fluorescently tagged proteins that aggregate and move to these locations has found their diffusion to be consistent with rapid movement in and out of JUNQ (Kaganovich et al., 2008). Although JUNQ formation is evidenced in response to aggregates, it is not known whether there are genetic requirements for its upregulation in response to proteasomal stress. Moreover, we do not understand the extent to which this determines proteasome location in the cell and how it biases degradation between the nucleus and cytoplasm.

Sequestration of Insoluble Proteins: IPODs

At the same time that JUNQ was first reported, researchers observed large, highly insoluble aggregates or amyloid segregate into a distinct cytoplasmic structures, spatially distant from JUNQ, which they named IPODs (insoluble protein deposits) (Kaganovich et al., 2008). Substrates targeted to JUNQ are primarily soluble, exchanging rapidly with the surrounding cytoplasm. JUNQ substrates are ubiquitinated and recruit the proteasome and chaperones to assist its attempts to refold or become degraded. In contrast, proteins targeted to IPODs are insoluble aggregates or amyloid. Whereas only a single JUNQ is found within each cell, multiple IPODS can exist in a given cytoplasm.

The proteins found within IPOD are highly terminal. Importantly, however, both the yeast disaggregase Hsp104 and the autophagy protein Atg8 colocalize with IPODs, indicating a strong effort by the cell to degrade these toxic species. Once disaggregated by Hsp104, the protein may be refolded with the assistance of additional chaperones also found near IPOD, including Hsp70 and Hsp40 (Glover and Lindquist, 1998).

Degradation of Proteins in the Nucleus: San1

A hallmark of eukaryotic cells is the enclosure of chromosomal DNA by a double-leafed membrane: the nuclear membrane. Whereas small proteins can passively diffuse through nuclear pores, larger aggregates cannot. Thus, ensuring the integrity of the nuclear proteins requires distinct quality control mechanisms. Intriguing insights into how this is accomplished have been revealed by studies in the yeast *Saccharomyces cerevisiae* (Gardner et al., 2005) examining the machinery responsible for the degradation of misfolded nuclear proteins. These studies revealed a critical nuclear localized E3 ligase termed San1.

San1 has exquisite specificity for misfolded proteins, allowing it to discriminate between folded and misfolded forms of the same protein. San1 is an unusually large E3 ligase—far larger than is required for its catalytic activity. Exploring the properties of the nonligase domains of San1 has revealed insight into how San1 is able to recognize misfolded proteins. Specifically, it was found that San1 has short substrate recognition motifs interspersed among flexible, disordered regions, which appear to provide San1 with a plasticity that allows it to conform to a wide variety of misfolded substrates (Rosenbaum et al., 2011).

Additional Methods of Sequestration: Aggresomes, ERAD Vesicles, and ALISs

Large, juxtanuclear inclusions that colocalize with the microtubule organizing center (MTOC), called the aggresomes, sequester aggregated proteins, chaperones, and proteasome subunits (Kopito, 2000). Aggresome inclusions are confined by a cage of vimentin, intermediate filaments that may represent the remnants of a filament collapse in the cell (Johnston et al., 1998). Aggregates are transported to the aggresome via dynein microtubules and HDAC6 (Kawaguchi et al., 2003). Aggresomes are generally considered stable, although some evidence suggests their autophagic degradation (Takalo et al., 2013).

Additional compartments used for the sequestration of soluble substrates have been described. For example, small

compartments filled with soluble, ER resident proteins and chaperones such as calnexin and calreticulin are found near the centrosome upon proteasome inhibition (Kamhi-Nesher et al., 2001). These ER-associated degradation (ERAD)-associated vesicles are hypothesized to confine ERAD substrates prior to their degradation by the cytosolic ubiquitin proteasome system (Kamhi-Nesher et al., 2001). This sequestration prior to processing would reduce potentially toxic effects of unfolded or damaged ERAD proteins with cytosolic proteins. Researchers have additionally proposed the sequestration of defective nascent proteins. Defective ribosomal substrates (DRiPs) have been observed in aggresome-like structures, or ALISs, that could be induced after the application of nutrient or oxidative stress (Szeto et al., 2006). ALISs are induced by the inhibition of autophagy and colocalize with autophagic markers such as GFP-LC3, suggesting that they may represent a type of undeveloped autophagic vesicle (Szeto et al., 2006).

The variety and prevalence of all of these types of compartments, from JUNQ to aggresomes, underscore their essential function in sequestering proteins and triaging them for repair or degradation. Importantly, these examples may only represent a portion of the types of inclusions and compartments with the cell. Furthermore, we are just at the beginnings of understanding their regulation: these compartments may exhibit a dynamic and fluid relationship between damaged and aggregative proteins, potentially signaling to and from stress response pathways, and affecting the synthesis and function of the chaperone and degradation pathways ultimately responsible for their processing.

Asymmetric Partitioning of Damaged Proteins

When all of these mechanisms collectively fail to destroy the damaged proteins or elicit cell death, the cell is faced with one last decision: as it divides, should it hold on to its damaged proteins? Does it give them to its daughter cells? And how can it achieve asymmetric division of its proteins, even if damaged? Although these options are not available to a terminally differentiated cell, such as a neuron, asymmetric inheritance of damaged proteins presents a critical opportunity essential to cells such as our germline and stem cells. How this opportunity is exploited necessarily depends upon the cell type being studied and the organism in which it occurs. For many cells, the answer appears to be to keep the damaged proteins in the older cell, thus protecting the newer generation. An asymmetric inheritance of protein damage is seen in *Drosophila*, yeast, and *E. coli*, for example (Aguilaniu et al., 2003; Rujano et al., 2006; Stewart et al., 2005) (Figure 3). In germline cells, likewise, during the asymmetric divisions invoked by murine stem cells, the passage of damaged proteins to daughter cells appears to be avoided through the asymmetric retention of damaged proteins in the ESC upon division (Hernebring et al., 2006). This act would protect the daughter cell from damaged proteins that otherwise would risk destroying the resulting lineage of cells.

Retention of damage proteins by the stem cell also represents an obvious challenge to a cell that must retain its functions for an indefinite amount of time. The accumulation of damaged proteins within the stem cell might soon lead to diminished stem cell function and possibly stem cell failure and death. In human patients expressing the polyglutamine aggregates of axatin-3, for example, stem cells in both the small intestine and skin appear devoid of the large aggregates found in differentiated cells, despite the fact that they express the aggregation-prone protein (Rujano et al., 2006). Thus, the long-lived stem cell may have mechanisms by which it rids itself of damaged proteins that are not present in its daughter cells, presumably working through the differential regulation of its protein degradation machinery. Consistent with this idea, human ESC cells have almost six times higher proteasomal activity (Buckley et al., 2012; Vilchez et al., 2012).

The compartmentalization of aggregates thus confers an additional advantage to the cell: during division, it allows for the specific retention of damage in the desired lineage. This model may be extended beyond the proteome: it is possible that other macromolecules such as lipids are portioned differentially in order to reduce burdens on younger cells. Going forward, we will continue to learn more about the mechanisms behind the decision to asymmetrically distribute damage and understand more fully the consequences that functional losses in compartmentalization may have on future generations of cells and within the aging organism.

Part Three: Systems of Communication

Within the cell, organelles mount large subcellular defenses that often include a global change in gene expression; organelles communicate with each other and affect each other's homeostasis; and organelles may convey information on their state of health to distal cells and tissues. This can result in a coordinated reaction throughout the organism. Below, we discuss the communication of stress and initiation of protein quality control mechanisms within organelles and across organisms.

The Endoplasmic Reticulum

The most ancient ER isolated is perhaps that which was found inside one of the deepest and most primitive branches in eukaryotic evolution, in the single-celled parasite *Giardia lamblia*. Using immunogold labeling, researchers identified and characterized a *Giardia* homolog of the ER chaperone BiP that colocalizes with the endomembrane and comes complete with a KDEL motif for retention with the ER (Soltys et al., 1996), a finding that confirmed traditional models on the evolution of the eukaryotic cell. In *Giardia*, mitochondria and peroxisomes have been lost or are now absent, and the Golgi has only a rudimentary presence, but the ER remains (Soltys et al., 1996). This may also represent the earliest known origin of ER-based chaperones now involved with protein folding.

The presence of the Hsp70 family chaperone BiP is conserved throughout eukaryotic cells, where it remains integral for the function of the ER. The mammalian ER is responsible for the folding and maturation of almost a third of the total proteome, including almost all polypeptides destined for secretion or insertion into the plasma membrane. In addition, the ER houses the enzymes responsible for synthesizing the majority of steroids and lipids secreted in cell-to-cell communications or in the biogenesis of membranes, and disruption of ER function can cause insulin resistance and chronic inflammation (Gregor and Hotamisligil, 2007; Hotamisligil, 2010). Preventing misfolding or aggregation within this environment is extraordinarily challenging, as the ER must manage to fold and modify proteins

that are sometimes in excess of 100 mg/ml (Snapp, 2005). Moreover, folding in the ER is often particularly challenging, as it is slow and coupled to covalent disulfide formation and transmembrane insertion. To protect this essential folding environment, a specific stress response system, the unfolded protein response (UPR^{ER}), responds to the presence of misfolded proteins in the ER (Ron and Walter, 2007).

UPR^{ER} activation can be presented as the coordinated effort between three distinct pathways, each of which transmits information from the cytosol to the ER by way of proximal transmembrane receptors—PERK, ATF6, or IRE1—that are activated in response to misfolded polypeptides. Each of these three receptors initiates a distinct downstream signal transduction pathway, culminating in a program that either reduces newly synthesized protein load (as is seen when PERK activation increases levels of eIF2α phosphorylation, causing translational arrest) or by increasing the ER capacity for folding or degradation (as is seen with IRE1 and ATF6). More specifically, activation of the IRE1 pathway leads to the regulated splicing of a transcription factor, XBP1, by the IRE1 endoribonuclease (Calfon et al., 2002; Yoshida et al., 2001). Spliced XBP1 (XBP1s) is translated and regulates a range of transcriptional targets that include the chaperones necessary for ER assisted folding (Acosta-Alvear et al., 2007; Shen et al., 2005), such as BiP/GRP78, GRP94, calreticulin, and calnexin; the protein disulfide isomerases PDI and ERP57; and proteins involved with ERAD, including *hrd-1* and *sel-1*. The UPR^{ER} stress responses intersect with inflammatory pathways in the context of metabolic homeostasis (Ron and Walter, 2007). In addition to the activation of XBP-1s by IRE1, IRE1 can locally degrade mRNAs destined for cotranslational insertion into the ER, a function termed RIDD (Hollien and Weissman, 2006), that reduces the burden of new proteins entering into the ER.

How does the aberrant protein initiate an ER stress response? Traditionally, two models for the activation of the UPR^{ER} have existed: in the first model, the unfolded proteins bind directly to the transmembrane receptor, causing dimerization or oligomerization and subsequent activation of the pathway. In a second model, the chaperone BiP is bound to the transmembrane receptor until the level of misfolded proteins in the ER surpasses a threshold, after which BiP is titrated away from the receptors. The loss of BiP binding to the receptors causes receptor activation, and the UPR^{ER} is initiated.

In actuality, these models may not be exclusive. Recently, Gardner and Walter (2011) have provided evidence for the direct binding of yeast IRE1 to hydrophobic and basic residues of peptides from unfolded proteins in yeast. Alternatively, in mammals, a direct interaction of Ire1α with BiP and Ire1β with unfolded proteins has been proposed (Oikawa et al., 2012). Intriguingly, the interaction with BiP may be required for differential UPR^{ER} responses; yeast Ire1 that is still bound to BiP is capable of initiating a sustained but weakened UPR^{ER} signal (Ishiwata-Kimata et al., 2013). This suggests the possibility that a chronic (as opposed to acute) UPR^{ER} activation may be propagated differentially and has differential consequences upon the survivorship of the cell. This hypothesis is especially intriguing given the prolonged, chronic activation caused by genetic UPR^{ER} activation that is also capable of increasing lifespan in model organisms (Taylor and Dillin, 2013).

During aging, many ER chaperones, including BiP and calnexin, are downregulated, and activation of the UPR^{ER} upon ER stress is abrogated with age (Ben-Zvi et al., 2009; Naidoo, 2009; Salminen and Kaarniranta, 2010; Taylor and Dillin, 2013). Similarly, ectopic induction of the UPR^{ER} is sufficient to prevent age-onset decline of proteostasis and delay age-related pathologies (Naidoo, 2009; Salminen and Kaarniranta, 2010; Taylor and Dillin, 2013).

Degradation and Quality Control of the Mitochondrial Proteome

Derived from a proteobacterium, the mitochondrion probably came into existence already containing its own stress responsive pathways, many of which were absorbed eventually into the proteome of the host cell. For example, a variant of the bacterial chaperonin GroEL, mitochondrial Hsp60, is still present in mammalian mitochondria today (Leroux and Hartl, 2000).

The study of stress responses associated with the mitochondria traditionally focused on those that respond to the loss of mitochondrial function, usually in the form of reduced membrane potential, which resulted in apoptosis. Alternatively, damaged mitochondria are removed from the cell through the initiation of autophagy-like processes called mitophagy (Kim et al., 2007). Mitophagy is intimately linked with defective mitochondria found in Parkinson's disease through the combined action of Pink1 and Parkin (Vives-Bauza et al., 2010). Importantly, mitophagy entails the specific removal of damaged mitochondria, rather than a nonspecific engulfment of a portion of the cytosol.

In response to protein misfolding challenges that do not necessarily result in an overt loss of membrane potential, the cell may initiate the mitochondrial UPR (UPR^{mt}). During this inducible and acute reaction, mitochondrial stress is communicated to the nucleus and results in the increased expression of mitochondrial-associated protein chaperones, including HSP-6 (mitochondrial hsp-70 heat shock protein family member) and HSP-60 (mitochondrial GroE/hsp60/hsp10 chaperonin) (Benedetti et al., 2006; Yoneda et al., 2004; Zhao et al., 2002). Disrupting subunits of ETC complexes by either RNAi or mutation activates the mitochondrial stress response (Benedetti et al., 2006; Yoneda et al., 2004). Furthermore, because the mitochondrial electron transport chain is made of subunits encoded by both the nuclear and mitochondrial genomes, the stoichiometry and assembly of these massive complexes is hypothesized to create proteotoxic stress. Consistently, either genetic or pharmacological perturbation of the mitochondrial translation machinery results in stoichiometric imbalance and induction of the UPR^{mt}.

Much like the UPR of the ER, the UPR^{mt} has a dedicated system in place to monitor its proteome and communicate stress to the nucleus to increase expression of mitochondrial chaperones. Primary activation of the UPR^{mt} occurs upon the reshuffling of the transcription factor ATFS-1 from the mitochondria to the nucleus (Haynes et al., 2010; Nargund et al., 2012). ATFS-1 has both mitochondrial and nuclear localization sequences. Under nonstressed conditions, ATFS-1 is imported into the mitochondria, where it is degraded by active proteases (Nargund et al., 2012). During mitochondrial stress, the relative proportion of mitochondrial to nuclear localized ATFS-1 adjusts, most likely because of deficiencies in the import capacity of the

mitochondria. The nuclear action of ATFS-1 then results in the increased expression of mitochondrial chaperones, including the mitochondrial chaperonin HSP-60 and mitochondrial HSP70 (HSP-6) (Benedetti et al., 2006; Haynes et al., 2007, 2010; Nargund et al., 2012). To date, ATFS-1 is the primary transcription factor characterized as having a role in UPR^{mt} activation.

Although the identity of the core circuitry of the UPR^{mt} is under intense study, it remains unclear whether and what type of unfolded proteins can signal to the UPR^{mt}, what the full range is of downstream targets affected by ATFS-1, and how the heterogeneity of mitochondrial health in each cell is evaluated during UPR^{mt} activation. This is especially relevant in cell types that contain hundreds of mitochondria, such as neurons.

Inducible Quality Control in the Cytoplasm

It is now clear that the response to protein misfolding in the cytoplasm mirrors responses observed in its intracellular, membrane-bound cousins of the cytoplasm. The initial discovery of a cytoplasmic stress response came more than 50 years ago when Ferruccio Ritossa first observed changes to the morphology of the chromatin after heat shock in *Drosophila* salivary glands caused by massive transcriptional upregulation at specific loci (Ritossa, 1962). More than 20 years later, a single *Drosophila* transcription factor, Hsf1, was identified as being capable of regulating this response (Parker and Topol, 1984; Wu, 1984). The network of genes that were induced by heat treatment was later coined the "heat shock response" and included a large number of the chaperones (Hsp70s, Hsp90s, and small heat shock proteins) capable of binding to and assisting in the folding of proteins. Under most cases of heat shock, HSP70 transcript levels can be increased more than 20,000 times above background. With increased temperature, the probability of correctly folding and maintaining proteins in their native confirmation decreases. By deductive reasoning, heat shock induces thermal folding of proteins, resulting in unfolded proteins that somehow signal to HSF1 to activate the heat shock response. Consistent with this hypothesis, injection of frog oocytes with heat-denatured (boiled) BSA results in chromatin-bound HSF1 (Ananthan et al., 1986). Additionally, a thermounstable allele of actin can ectopically induce the heat shock response in *Drosophila* (Hiromi and Hotta, 1985; Hiromi et al., 1986). Alternatively, mild heat shock may actually stimulate the rate of translational elongation, titrating chaperones away from the cytoplasm and to the nascent chain and triggering a heat shock response. This model is supported by evidence in bacteria for a completion between nascent chains and cytoplasmic proteins for the pool of chaperones (Kim et al., 2013).

These studies suggested a direct relationship in which protein misfolding in the cytoplasm is communicated to HSF-1 to induce a subset of chaperones to help refold the misfolded proteins. Consistent with this hypothesis, ectopic overexpression of HSF-1 is sufficient to confer resistance to thermal stress and increase lifespan in the nematode *C. elegans* (Hsu et al., 2003) and alleviate the toxicity associated with expression of diseases associated with misfolding or aggregating proteins (Fujimoto et al., 2005). The same, albeit to a lesser magnitude, is true for ectopic overexpression of one of HSF1's major targets, HSP70. These effects are widely attributed to HSF-1's capacity to upregulate chaperones that can protect the stability of the proteins during the aging process (Ben-Zvi et al., 2009; Hsu et al., 2003; Morimoto, 2008).

How does HSF1 sense misfolded proteins in the cytoplasm? Under nonstressed conditions, HSF1 can be found in tight complex with HSP90, much like many nuclear hormone transcription factors (Zou et al., 1998). Under protein denaturing stress, HSP90 is thought to release HSF1 and help refold the proteome that is under challenge, allowing HSF-1 to interact with accessory factors in transit to the nucleus. In fact, chemical inhibitors of HSP90 can induce the heat shock response in an HSF1-dependent manner (Westerheide and Morimoto, 2005). It is not clear, however, if this is due to HSF1 being free of HSP90 or due to the loss of protein homeostasis that will occur when HSP90 is inhibited.

Although the heat shock response is celebrating its 50th and HSF-1 its 30th anniversary, there is still much to be learned about how the cytoplasm registers and responds to proteotoxic stress. Besides denatured BSA and a mutant actin, a direct demonstration of the effect of misfolded proteins on HSF-1 activation has not been shown in eukaryotes. Nor do we know the extent of protein misfolding required to trigger this response. Moreover, the specific classes of misfolding and stressors capable of activating HSF-1 have not been fully been identified. Finally, it is clear that a wide range of others stresses, including cancer, also induce a heat shock response, although the spectrum of proteins that are induced differs depending on the stress (Mendillo et al., 2012).

Interorganelle Communication

Communication between organelles has come into light, especially given the tight link between mitochondrial function and the ER (Kornmann et al., 2009). However, a new appreciation of the interconnections between organelles during times of stress and aging is just beginning to be realized. If the subsystems of the cell are broken into their individual parts akin to the organ systems of a complex organism, then one organelle may be more susceptible to stress than another, causing a catastrophic effect on cellular function. Alternatively, each organelle may work autonomously to promote both its own health and the health of the cell.

Work in the budding yeast *Saccharomyces cerevisiae* is providing experimental results that suggest that there is an orchestrated hierarchy of organelle quality control that dictates cellular homeostasis, especially under the stress of aging (Dimitrov et al., 2009). As most dividing cells age, the rate of DNA mutations in their nuclear genome increases, allowing once heterozygous mutations to be revealed by mutations in the corresponding copy. Driving this process appears to be the proper coupling of the mitochondrial electron transport chain to ensure membrane potential, a coupling that is lost as cells age. As membrane potential is reduced, so too is import efficiency of key enzymes localized in the mitochondria.

Many biochemical reactions are housed in the mitochondria besides just those required for energy production. With a loss of import efficiency is also lost the import of key enzymes for iron-sulfur biogenesis, a key component of enzymes for DNA repair processes. Therefore, a clear picture emerges whereby the reduction of mitochondrial membrane potential results in

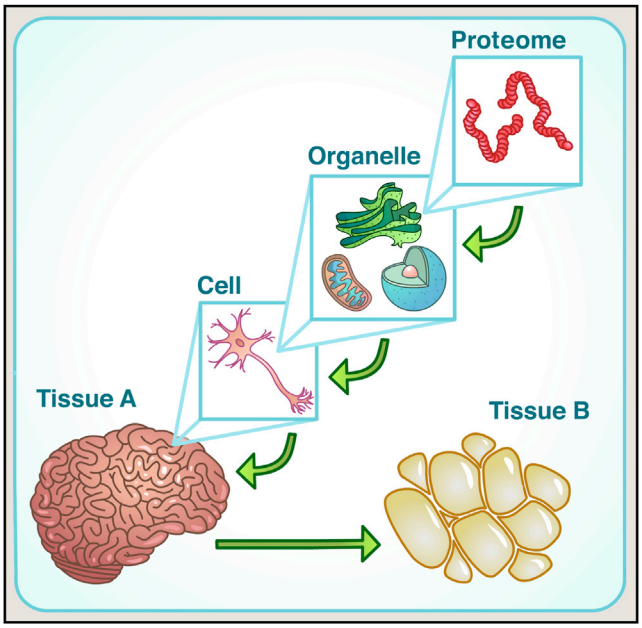

Figure 4. The Communication of Proteome Health
Changes in the protein folding state of the individual protein can be escalated, creating organelle-specific responses, cell-wide defense mechanisms, tissue-specific responses, and communication among tissues of the organism.

fewer Fe-S complexes formed, thereby further compromising DNA repair fidelity (Veatch et al., 2009). However, this example only highlights one sequence in the interactions of organelles during aging.

Also coincident with increased nuclear DNA mutations and reduced membrane potential for mitochondria during the aging process is the loss of pH regulation in the lysosome (in yeast, the lysosome is the vacuole) (Hughes and Gottschling, 2012). The inability to maintain pH regulation in the vacuole has profound effects upon the import and export of amino acids, one of the major storage depots for amino acids in the cells. Particularly affected are the neutral amino acids, which are key for mitochondrial bioenergetics. Therefore, the connected nature of each subsystem becomes more complex with the lysosome now affecting the mitochondria, which then ultimately affects genomic quality control.

The Cell-Nonautonomous Nature of Stress Responses

As life moved from the first eukaryote to form a metazoan, how did the stress responses that maintain the proteome evolve? Did they remain in their primitive cell-autonomous mode? Hypothetically, it would seem advantageous for the evolving organism for one cell to signal and help to control the protein folding environment of its neighboring cell. This suggests the possibility of coordinated activity to control the folding environment across tissues or organism. Could something as basic as the protein fold be communicated across cells?

One of the earliest drivers for metazoan evolution was probably the ability to coordinate the utilization of limited metabolic resources across the entire organism. With the recently acquired metabolic factory of the mitochondria, there was probably considerable pressure to control mitochondrial function in a uniform manner across cell types to allocate limited metabolic currencies. As mentioned earlier, the UPR^{mt} provides the core machinery, mitochondrial chaperones, to ensure that the mitochondrial proteome remains intact and functional. Therefore, it was not too surprising that the UPR^{mt} not only acts cell autonomously but can also function at a distance (Durieux et al., 2011).

Stoichiometric imbalance of the mitochondrial electron transport chain by RNAi inhibition of a subunit of Complex IV results in upregulation of the UPR^{mt} in distal tissues and increased longevity of the worm *C. elegans* (Durieux et al., 2011) (Figure 4). Likewise, RNAi inactivation against ETC components in the fly also increases longevity and induces the UPR^{mt} (Copeland et al., 2009; Owusu-Ansah et al., 2013). In the fly, this also acts in a cell-nonautonomous fashion. Likewise, genetic or pharmacological manipulation of the mitochondrial ribosome also results in increased longevity and induction of the UPR^{mt} in worms and mice, suggesting that this is a conserved mechanism across phyla (Houtkooper et al., 2013). However, there has been no demonstration that induction of the UPR^{mt} is sufficient to alter the aging process.

The nonautonomous nature of protein homeostatic stress pathways is not confined to the UPR^{mt}. The UPR^{ER} also functions in a cell-nonautonomous manner to protect entire organisms from ER stress and promote longevity (Taylor and Dillin, 2013). The cytoplasmic heat shock response is organized in such a manner that neurons responsible for thermosensation are essential for disseminating the heat shock response across the entire organism in the worm (Prahlad and Morimoto, 2011). Furthermore, conditions that create proteostatic imbalance, such as ectopic overexpression of HSP90, in a tissue-specific manner can induce the heat shock response in naive cells and tissue types (van Oosten-Hawle et al., 2013).

The communication of stress across organelles and across tissues is possible for other types of subcellular components as well. For example, it is likely that ribosomes are aware of the state of health of those ribosomes around them and perhaps even those in distal cells. Indeed, in addition to mediating the degradation of nascent chains on stalled ribosomes, the RQC also activates an Hsf1-mediated stress response that may in turn affect global stress responses, including rates of translation (Brandman et al., 2012). Critical open questions remain regarding how these components recognize stalled ribosome and how the RQC transmits signals to Hsf1. Similarly, it is likely that recognition of translational arrest in a single tissue is conveyed to other tissues as well. For example, attenuation of translation in the somatic cells of *C. elegans* is sufficient to elicit a global reduction in translation and extension in lifespan across the organism (Syntichaki et al., 2007). Overexpression of dTor, S6kinase, or Tsc2 in the fat cells of *Drosophila* extends lifespan, most probably by initiating a global response to the reduction in translation initiation and changes in metabolism (Kapahi et al., 2004). We thus are poised to understand more about the tissue-specific mechanisms by which ribosomes and translational arrest can affect longevity.

It then seems possible that the canonically autonomous parts of a cell—the suborganelles and subcompartments—may communicate both with each other and with the organism as a whole in response to aging. A better understanding of the

patterns, mechanisms, and consequences of endocrine-based stress responses will allow us to productively target the source and signal causing the distal effects seen in many age-onset diseases.

Perspectives

A dichotomy is emerging in which folded proteins increase the fitness of the organism and unfolded proteins decrease its fitness. The developing picture indicates that nearly all large, subcellular processes, from the organelles to ribosomes, may have specific ways of sensing the proteome and reacting to proteotoxic stress. Key players in the core machineries of the stress-responsive pathways of the cytoplasm, ER, and mitochondria have now been identified in detail. However, it remains less clear how the protein folding stress pathways are activated and what the key determinants of activation are. Although it has largely been assumed that misfolded proteins trigger the initial response, this seems unlikely given that fact that the majority of the proteome is rarely in its native conformation. More likely, nodes of key networks required for cellular function are monitored. The lessons learned from ribosome quality control are already revealing that the central hub of protein translation, the enigmatically large ribosome, is under close scrutiny by surveillance factors at almost every step of synthesis of new proteins.

In addition to the large machinery of the ribosome serving as a beacon to stress response pathways, what other large, networked machines could serve a similar function? The appropriate function of the cytoskeleton is an absolute prerequisite for the proper function of a wide range of essential cellular processes, including autophagy, axonal transport, organelle integrity and dynamics, the transport of aggregates, chaperone localization, the regulation of macromolecular crowding, mRNA transport, endocytosis, and exocytosis. This strongly suggests that an upstream network of protective proteins may have evolved to regulate cytoskeletal form and function. A tight control of this network would be absolutely essential in any kind of a protective response during times of cytoskeletal stress. We hypothesize that cells have an additional, specific stress response to protect their cytoskeletons. Consistent with this idea, as mentioned above, mutant forms of actin can induce the heat shock response in *Drosophila*, suggesting that, perhaps, the heat shock response is, in itself, a cytoskeletal stress response (Hiromi and Hotta, 1985).

In the future, will we find that other large complexes with central positions in the network of the cell are key checkpoints for protein quality control of the cell as a whole? Will there be a quality control for the proteasome, nucleopore complex, centrioles, intermediate filament, and tubulin fibrils, for example?

Caution should also be taken against thinking of stress responses and chaperones as being binary in their actions. Although stress responsive transcription factors may activate or repress as many as hundreds of genes at a time, there is more likely a toll on the fitness of the cell from the indiscriminate upregulation of such a large network of genes. Given the complexity in systems within the cell, this would indicate a surprisingly strong and unequivocal response to a variety of environmental perturbations. More likely, the cell is capable of tuning these stress responses in ways most appropriate to the given situation. Our capacity to detect these changes has been hampered by our reliance upon reporter expression as an indication for the upregulation of a global stress response and by our inability to manipulate stress responses using techniques that mimic levels of physiological stress. With advances in technology, we will come to a better understanding of the gradient of responses that more probably occur within a cell. Finally, although much discussion centers on the activation of stress responses, we still have much to learn about the specific thresholds required for their activation and the mechanisms by which these responses may be turned off again once homeostasis has been restored.

The proteome is a complicated and constantly evolving entity. As we consider its course throughout evolution—from its beginnings in a pool of free amino acids, to the gradual origin of translation and degradation machineries, to the creation of chaperones and stress responsive mechanisms, to the need to develop systems to communicate proteotoxic stress across the organism—we still are only touching the surface of the probable complexity of its interactions and functions within the cell. We should continue to recognize the importance of the macromolecules upon which our proteome relies—and which rely upon it—to function, and we overlook the coevolution of our proteome that has occurred in synchrony with the thousands of different species of bacteria and their byproducts that exist in a state of symbiosis within us. In the next decades, our understanding of the causative factors that determine protein homeostasis will continue to evolve as well.

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