Protein degradation and protection against misfolded or damaged proteins

Alfred L. Goldberg

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA
(e-mail: alfred_goldberg@hms.harvard.edu)

The ultimate mechanism that cells use to ensure the quality of intracellular proteins is the selective destruction of misfolded or damaged polypeptides. In eukaryotic cells, the large ATP-dependent proteolytic machine, the 26S proteasome, prevents the accumulation of non-functional, potentially toxic proteins. This process is of particular importance in protecting cells against harsh conditions (for example, heat shock or oxidative stress) and in a variety of diseases (for example, cystic fibrosis and the major neurodegenerative diseases). A full understanding of the pathogenesis of the protein-folding diseases will require greater knowledge of how misfolded proteins are recognized and selectively degraded.

Proteins within cells are continually being degraded to amino acids and replaced by newly synthesized proteins. This process is highly selective and precisely regulated[1], and individual proteins are destroyed at widely different rates, with half-lives ranging from several minutes to many days. In eukaryotic cells, most proteins destined for degradation are labelled first by ubiquitin in an energy-requiring process and then digested to small peptides by the large proteolytic complex, the 26S proteasome. Indicative of the complexity and importance of this system is the large number of gene products (perhaps a thousand) that function in the degradation of different proteins in mammalian cells. In the past decade, there has been an explosion of interest in the ubiquitin–proteasome pathway, due largely to the general recognition of its importance in the regulation of cell division, gene expression and other key processes[1]. However, the cell's degradative machinery must have evolved initially to serve a more fundamental homeostatic function — to serve as a quality-control system that rapidly eliminates misfolded or damaged proteins whose accumulation would interfere with normal cell function and viability[2–4].

Long before the appearance of the ubiquitin–proteasome pathway in eukaryotes, prokaryotes had evolved elaborate proteolytic machinery to destroy misfolded proteins rapidly[4,5]. These intracellular proteolytic systems are energy dependent and differ markedly in size and complexity from the simple proteases that function in the extracellular milieu, such as the pancreatic proteases that digest dietary proteins. If such typical extracellular proteases were free in the cytosol, they would quickly convert the cell into a bag of amino acids. The fundamental problem that evolution had to solve was how to provide cells with the capacity to destroy misfolded or damaged proteins rapidly without the non-specific destruction of essential cell constituents.

Here, I outline the key features of the degradative process, its importance for cell viability and the research that led to its elucidation. As discussed here and elsewhere in this Insight, misfolded proteins can arise in cells by mutation and through various postsynthetic events; they can be highly toxic, mainly because of their tendency to form intracellular aggregates. Greater knowledge about this proteolytic pathway will be crucial for understanding the pathogenesis of diseases resulting from failures in correct protein folding and in developing rational therapies.

Structural alterations lead to rapid degradation

The ability of cells to degrade abnormally folded proteins selectively was first demonstrated more than 30 years ago[4] with the discovery that various treatments that perturb the proper folding of proteins caused their rapid hydrolysis. In fact, these findings led to the initial recognition that a protein's structure determines not only its catalytic properties but also its intracellular stability[2]. However, the precise changes in conformation that are recognized by the cell's degradative machinery and that trigger rapid hydrolysis are not clear and have still not been studied systematically.

Perhaps most illustrative are the early findings about degradation of abnormal globins in reticulocytes[6,7], which produce one very well-characterized protein, haemoglobin, almost exclusively. Because the powerful tools that are now available to perturb protein structures (such as site-directed mutagenesis) were undreamt of then, those early studies followed the fate of proteins that had incorporated, in place of the natural residues, amino-acid analogues that prevented the protein from assuming its normal tertiary conformation[2,4]. Normally, haemoglobin is the most stable intracellular protein, lasting the lifespan of red cells (about 110 days). However, after the incorporation of a synthetic valine analogue, the abnormal globin had a half-life of 10 min (refs 6, 7). These short-lived globins failed to bind haem or form tetramers, and instead formed amorphous aggregates before degradation (see below). Very similar events have since been observed in several human diseases, for example, the unstable haemoglobinopathies, in which mutations that prevent the binding of haem cause complete degradation within minutes of translation, leading to a severe anaemia[8].

Wider recognition of the importance of this degradative process occurred with the advent of recombinant DNA technology in the 1980s and the discovery that many foreign proteins expressed in bacteria fail to accumulate owing to their rapid degradation. Many proteins of major medical importance (such as insulin) cannot fold properly in *Escherichia coli*, are recognized as abnormal and are rapidly degraded[9]. Successful expression of such proteins required the development of mutant bacteria with reduced degradative capacity or improved expression systems that overwhelm the proteolytic systems[9].

Although most of our knowledge about misfolded proteins comes from mutated proteins, cells also rapidly degrades abnormal proteins that result from errors in transcription or translation. Accordingly, ribosomal mutations

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Table 1 Abnormal proteins rapidly degraded in cells

Type of abnormality	Cause
Incomplete proteins	Nonsense mutations, incorporation of puromycin, premature termination, proteolytic cleavage
Missense proteins	Mutations, incorporation of amino-acid analogues, biosynthetic errors
Free subunits of multimeric complexes	Excess synthesized subunits
Postsynthetic damage	Oxygen radicals, intracellular denaturation
Genetic engineering	Gene fusions, frame-shifts, incorrect localization
Protein misfolding	

in *E. coli* or treatment with streptomycin, each of which causes translational errors and the production of error-laden proteins, leads to an overall increase in proteolysis[4,11]. In fact, because of this rapid degradation, the actual error rate in gene expression *in vivo* is impossible to determine with precision.

Incomplete proteins, such as can arise through mistakes in RNA splicing, the incorporation of puromycin[2,4,6] or nonsense mutations, are rapidly degraded in all cells. Such fragments fail to assume their proper conformation. Bacteria also have a specialized mechanism to target for destruction incomplete polypeptides while they are still on stalled ribosomes[11], but no similar mechanism has yet been found in eukaryotes.

The quality-control systems in bacteria and eukaryotic cells do not degrade all, or even most, mutated proteins: only those mutations that markedly perturb protein folding trigger rapid hydrolysis (such as mutations of key residues or large deletions) (see Table 1). For example, only about one-fifth of the hundreds of human haemoglobin point mutations generate proteins that undergo rapid degradation (as suggested by the low levels of the abnormal protein in mature red cells)[8]. A major recent development has been the discovery that many abnormally folded proteins in the secretory pathway (for example, the cystic fibrosis transmembrane conductance regulator) are translocated back into the cytosol, where they undergo rapid degradation (see the article by Sitia and Braakman in this issue p. 891, and ref. 10).

Degradation of newly synthesized proteins

The folding of newly synthesized proteins to their proper conformations involves the sequential actions of multiple molecular chaperones[13,14], and this process can take many minutes or even longer, and may often be unsuccessful. A large fraction of newly synthesized proteins is rapidly degraded[2,15,16]. As many as 30% of newly synthesized proteins in eukaryotes might undergo degradation within minutes of synthesis[15]. Although initially attributed to mistakes in ribosomal function (and named DRIPS, for 'defective ribosomal products'), it seems more likely that a large fraction of these short-lived species are products of unsuccessful folding or failures of multimer assembly. However, the actual fraction of short-lived species that are genuinely aberrant proteins is controversial and extremely difficult to determine rigorously for multiple reasons. For example, a number of important regulatory proteins have very short half-lives (as short as 2–20 min)[1]; also fragments of all secretory and membrane proteins (signal peptides) are degraded as part of the secretory process. Nevertheless, it seems likely that many newly synthesized polypeptides are destroyed because of the inherent inefficiency of protein folding[15] and that, after release from the ribosome, polypeptides face a life–death kinetic competition between successful folding and rapid hydrolysis of unfolded species.

Particularly critical steps in the folding process are the binding of cofactors and the association of different subunits to form multimeric complexes[13,14]. In the absence of cofactors or complementary subunits, free subunits generally fail to achieve native conformations and have exposed hydrophobic surfaces. Such free subunits tend to be rapidly degraded[2]. For example, ribosomal subunits, when not in ribosomes, are very unstable in all cells. In human thalassaemia, there is an excess of α- and β-globin subunits not in tetramers that are rapidly degraded within reticulocytes[8]. However, their destruction is not sufficiently rapid to prevent free chains from precipitating and distorting erythrocyte shape. This defect in protein assembly leads eventually to anaemia because these misshapen red cells are rapidly destroyed by the spleen (which functions as a quality-control system to ensure that circulating cells have the correct shape)[8]. The degradation of free subunits of multimeric enzymes thus not only protects cells against accumulation of denatured, potentially toxic polypeptides but also functions as a quality-control mechanism that ensures that subunits of multimeric complexes are present in the proper stoichiometry.

Postsynthetic damage to cell proteins

For a protein, the cell can hardly be a hospitable environment, especially in warm-blooded species. Within cells, proteins are constantly exposed to highly reactive molecules and to conditions that favour denaturation (see Box 1) and are under constant surveillance by the proteolytic systems, which continually monitor mature proteins for postsynthetic denaturation or chemical damage. Consequently, a protein's life within the cell is likely to be 'nasty, brutish and short'.

To maintain the activity of most proteins in the laboratory, it is essential to store them at low temperatures in pure solutions or even freeze-dry them. A competent biochemist would never store his favourite protein at 37 °C in a highly reactive environment such as the intracellular milieu, where reactive oxygen species (for example hydroxyl radicals) are continually being generated that can destroy amino acids in proteins[17,18]. It is also an environment where reactive sugars abound that can glycate proteins, where many enzymes are found that modify or destroy proteins (for example proteases), and where there are fatty acids that can function as detergents, and partially folded nascent chains that act as nuclei for aggregation. These protein-modifying processes must continually cause stochastic damage to well-formed cellular proteins, triggering their degradation[2]. Even in pure solutions, multiple changes in primary sequence (deamidation of glutamines, and residue isomerization) occur over time (termed 'protein ageing'), leading to rapid degradation by proteasomes[19]. Although it generally functions as part of the ubiquitin–proteasome pathway, the 26S proteasome has the capacity to degrade certain unfolded or damaged proteins, including 'aged' or denatured proteins, without initial marking them by ubiquitylation. The frequency of such modifications and denaturing events must be high, because the free energy for stabilizing proteins is not great. Furthermore, in certain environments, the frequency of damage to cell proteins increases — for example after heat shock, in which many proteins are unfolded[3], or during oxidative stress or inflammation, when oxygen radicals are generated in increased amounts[17].

Box 1
Intracellular conditions that damage cell proteins

Temperature of 37 °C or higher (denaturing conditions).
Many reactive small molecules — these cause oxidation, deamidation, glycation or nitrosylation.
Many enzymes that modify proteins, for example proteases or kinases.
High salt concentrations (which favour dissociation of multimers)
Many fatty acids, which act like detergents.
Other unfolded proteins — nascent polypeptides, damaged or mutant polypeptides and insoluble inclusions are sticky.

Conclusion: to maintain a protein's function, avoid the intracellular milieu.

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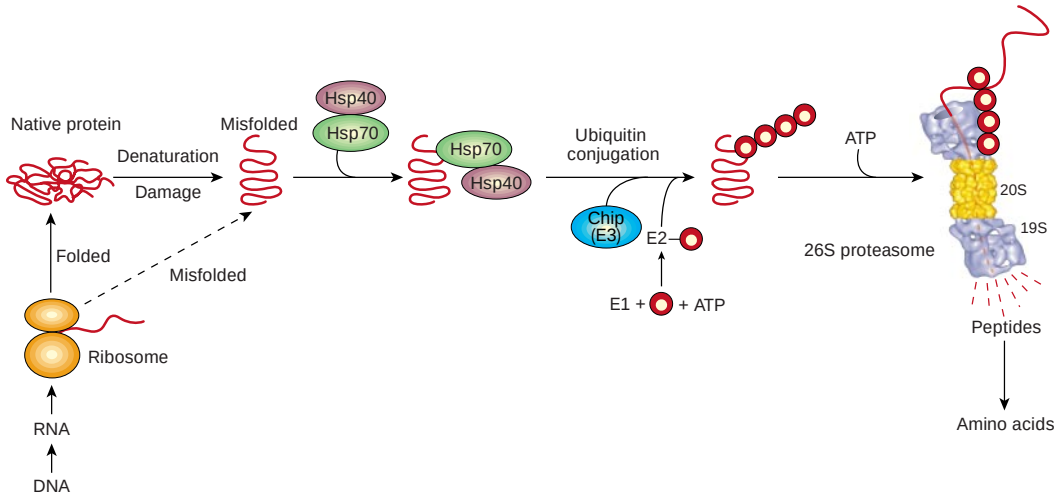

Figure 1 The ubiquitin–proteasome pathway. Molecular chaperones may function in protein folding and in the degradation of misfolded species. By associating with exposed hydrophobic domains, chaperones Hsp70/40 promote the folding of newly synthesized proteins and favours their refolding. Alternatively, they can facilitate the recognition of abnormal proteins, leading to their ubiquitylation by CHIP, the E3, and their degradation by the 26S proteasome. The red circles represent ubiquitin.

Although many proteins are short-lived, the bulk of cell proteins are 'long-lived', and 1–2% of them are degraded per hour in mammals[16]. Even though these rates are physiologically regulated, the degradation of these long-lived proteins follows exponential decay kinetics, suggesting that a stochastic event triggers their destruction. Thus, for many — and perhaps most — proteins, a random event, spontaneous denaturation or chemical modification leading to unfolding might be the critical event triggering degradation[2].

Abnormal proteins may aggregate before degradation

A characteristic feature of denatured proteins is that they aggregate and leave solution (because of the tendency of normally buried hydrophobic domains to associate with one another). Similarly, when the production of misfolded proteins exceeds the cell's degradative capacity, these polypeptides often form intracellular aggregates before their rapid degradation[3,7,20]. Indeed, the finding that many cloned, misfolded proteins also aggregate in the cytosol of bacteria has sometimes proved to be of major benefit to researchers, allowing the rapid isolation of the foreign polypeptides. The formation of such inclusions occurs when the cell's proteolytic systems and molecular chaperones, which normally prevent aggregation (for example Hsp70/40)[13,14] or resolubilize microaggregates (Hsp104)[21], cannot keep up with the rate of production of unfolded molecules.

Similar inclusions are found in various inherited and neurodegenerative diseases, in which these inclusions are ubiquitylated and associated with proteasomes[3] (see also the article in this issue by Selkoe, p. 891), strongly suggesting a failure of the cell's degradative machinery[3]. When mammalian cells are treated with proteasome inhibitors, such inclusions appear rapidly, thus indicating that many substrates of the proteasome in normal cells are misfolded proteins[3]. As abnormal proteins accumulate, larger inclusions are found near the centrosome as a result of an active microtubule-dependent process that isolates the unfolded proteins in amorphous structures, which are often termed 'aggresomes'[22]. If the production of such abnormal proteins ceases and degradation is allowed to proceed, all cells, from bacteria[20] to neurons[23], can eliminate the proteinaceous inclusions. The mechanisms in mammalian cells for solubilizing and digesting these proteins are unknown, and further understanding of this process could have therapeutic applications for various protein-folding diseases.

The ubiquitin–proteasome pathway

A fundamental feature of protein degradation in all cells, and even mitochondria, is that it requires metabolic energy. This requirement for ATP is unexpected on thermodynamic grounds. Consequently, it was initially viewed as evidence for an ATP requirement for the function of lysosomes, then believed to be the only site of protein breakdown in cells. However, the selective degradation of abnormal proteins also requires ATP in cells that lack lysosomes (for example, bacteria)[4,6]. This insight led to the development of cell-free preparations that catalyse the ATP-dependent degradation of abnormal proteins[6]. Attempts to understand this mysterious role for ATP led to the discovery of the involvement of ubiquitin conjugation in marking proteins for degradation[24] and of the 26S proteasome[25,26]. In eukaryotes, ATP is initially essential to activate ubiquitin, which is then transferred to one of the cell's 20–40 different ubiquitin-carrier proteins (E2s). The exquisite selectivity of this pathway resides in the ubiquitin ligases, or E3s, which are specific for different protein substrates[1]. Mammalian cells contain hundreds of different ubiquitin ligases, which, together with a specific E2, catalyse the formation of the ubiquitin chain on a limited number of protein substrates, triggering their rapid degradation by the 26S proteasome[27] (see below and Fig. 1).

Which E2s and E3s are involved in this cellular quality-control system and how they recognize misfolded proteins are still open questions. Several E3s are essential for the degradation of abnormal proteins in the secretory pathway (see the progress in this issue by Sitia and Braakman p. 891, and ref. 10), and certain E2s (Ubc4 and Ubc5) are necessary for the rapid degradation of cytosolic abnormal proteins and are induced as part of the heat-shock response[11]. This transcriptional response is triggered in all cells by the appearance of unfolded proteins (for example, after exposure to increased temperatures or 'oxidants') and leads to an enhanced cellular content of chaperones and increased degradative capacity[3,11,28].

Recently, one E3, CHIP (for carboxy terminus of Hsp70-interacting protein)[29,30], has been identified in mammals that seems to ubiquitylate certain mutant proteins selectively in a process requiring the molecular chaperones Hsp70 or Hsp90, which bind to unfolded or hydrophobic domains[13,14] and seem to facilitate substrate recognition by CHIP[29,30]. These chaperones are normally among the most abundant cell proteins, and their levels rise further in harsh conditions that damage cell proteins[3,11,28]. By binding to unfolded domains

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of nascent proteins, they prevent their aggregation and facilitate normal folding in stressed cells, and promote the refolding of denatured molecules[13,14]. The discovery that these chaperones also have a function in the selective degradation of abnormal proteins has important implications. Chaperone involvement in proteolysis, as well as folding, would provide an efficient quality-control mechanism, because unsuccessful folding would mean prolonged association of the substrate with the chaperones, leading to rapid degradation[29–32]. However, it remains unclear whether CHIP catalyses the ubiquitylation of abnormal proteins generally or whether many E3s function in the degradation of different types of aberrant protein. It is also unclear whether ubiquitylation is essential for the proteasomal elimination of all abnormal proteins *in vivo*, because this process occurs efficiently in bacteria and archaea, which lack ubiquitin. In addition, *in vitro* eukaryotic 26S proteasomes degrade many unfolded proteins (for example, 'aged' calmodulin) without ubiquitin conjugation[19].

ATP-dependent proteolytic machines

A very different and more general explanation of the requirement of ATP for the degradation of abnormal proteins emerged from our studies of this process in *E. coli* and mitochondria[33]. Large proteolytic complexes were discovered whose activity was coupled to ATP hydrolysis[33–35]. For example, the Lon (La) protease is a 600 kDa ATPase complex that rapidly degrades most abnormal proteins in *E. coli*[33–35]. The Lon homologue in mitochondria is necessary for the viability of eukaryotes, presumably because it helps to prevent the build-up of aberrant proteins within this organelle[36,37]. In addition, bacteria and mitochondria contain several other proteolytic complexes (namely ClpAP, ClpXP, HslUV and FtsH)[5,33–36] that are composed of distinct ATPase and proteolytic subcomplexes. Like the proteasome, these enzymes are large complexes (at least 20–30-fold larger than typical proteases) that processively degrade proteins to oligopeptides. In each case, ATP hydrolysis appears necessary for the unfolding of substrates and for their delivery into a proteolytic subcompartment, where the substrates are digested, thus avoiding the non-specific destruction of other cell constituents. These proteases are all heat-shock proteins[3,5,10,33], which means that they are coordinately induced together with molecular chaperones whenever cells generate large amounts of unfolded proteins (Table 1)[3,10,28]. For example, they are often induced in bacteria that express cloned foreign proteins[11].

In all cells and mitochondria, these proteases function together with molecular chaperones in protein degradation[3]; for example, the rapid hydrolysis of certain abnormal proteins in *E. coli* requires the homologues of chaperones Hsp70/40 (ref. 32), whereas the degradation of others requires GroEL/ES chaperonins (also called Hsp60/10) (ref. 38). These large particles create an isolation chamber within which proteins can fold[13,14], yet they also seem to facilitate the proteolytic digestion of proteins that are hard to degrade[38].

How the cell's two major defence systems, molecular chaperones and proteasomes, function together in the elimination of abnormal proteins is not clear. Prolonged chaperone binding can help in the identification of proteins with unfolded domains[30,32], but the chaperones might also function as cofactors in maintaining the substrate in a soluble, unfolded state that facilitates proteolytic attack[31,38]. An attractive ('triage') model is that a failure of the chaperones to perform protein folding or the refolding of damaged proteins leads to the recruitment of proteases or ubiquitylation enzymes to eliminate the potentially dangerous polypeptides[3,30–32].

The 26S proteasome

Whereas proteolysis in bacteria and mitochondria involves several specialized ATP-hydrolysing proteases, eukaryotic cells in their cytosol and nucleus contain only one such enzyme of enormous size (namely 50 subunits totalling 2.4 MDa) that degrades various types of substrate fed to it by multiple ubiquitin ligases. Understanding of the functioning of this proteolytic complex has advanced markedly in recent years[27]. The 26S proteasome is composed of a core 20S particle, in which proteins are digested to short peptides, and one or two 19S regulatory particles, responsible for substrate recognition and transport into the core particle[27]. The 20S particle is composed of four stacked rings, whose subunits surround a central cavity. Its two inner β-rings form a central chamber containing the proteolytic sites, which face the central cavity. This organization ensures that protein digestion is isolated from the surrounding cytosol. Eukaryotic proteasomes contain two sites that cleave after hydrophobic residues, two after acidic residues and two after basic residues; thus they can cut most types of peptide bond[5,27]. Substrates can enter the 20S particle only through a gated channel in the centre of the α-ring[39], which is normally maintained in a closed state, and access is controlled by the associated ATPases in the 19S particle[40].

The entry channel is quite narrow, and even in its open state it allows entry of only unfolded proteins[39,40]. While excluding normal globular proteins, this architecture requires complex ATP-dependent mechanisms to recognize, unfold and linearize substrates and to inject them into the 20S proteasome[40]. It is still unclear where in the 19S is located the initial binding site for ubiquitin chain and the enzymatic machinery for disassembling these chains[1,27]. The base of the 19S contains a ring of six ATPases that provide added selectivity and somehow unfold proteins, translocate them and trigger gate-opening into the 20S particle[40]. This elegant mechanism uses the energy in a considerable number of ATP molecules in degrading a protein, perhaps one-third of the ATP used by the ribosome in their synthesis[40]. The essential features of this process emerged early in evolution; the proteasomes of Archaea function together with a very similar ATPase ring (termed PAN) that selects, unfolds and translocates substrates[5,40]. With the emergence of eukaryotes, this process became linked to ubiquitin conjugation, which, like the architecture of the proteasome and its linkage to ATP hydrolysis, should be viewed as mechanisms to ensure that only unwanted proteins are selectively degraded.

Key questions and future prospects

The selective degradation of abnormal proteins has been known for about 30 years, but many fundamental questions remain unanswered. Although many adverse events during gene expression and protein folding, and many untoward postsynthetic events, trigger degradation, it is unclear to what extent this quality-control process contributes to overall protein turnover in normal cells. In addition, the mechanisms by which various types of misfolded polypeptides are recognized remain a mystery that has received only limited study. One important clue is that their degradation in all cells requires molecular chaperones, which probably function in substrate recognition[3,30,32] but perhaps also in maintaining substrates in a soluble, easily digestible state[31,38].

This lack of mechanistic understanding is surprising and regrettable, because this process is of fundamental biological importance. The genetic deletion of virtually any subunit of the 26S proteasome in yeast prevents viability[27], and various point mutations leave the cells viable but very sensitive to conditions that increase the production of abnormal proteins (such as heat shock)[27]. When unfolded proteins build up and exceed the cell's degradative capacity (for example, in the presence of proteasome inhibitors), cells activate the heat-shock response[41,42] and induce the synthesis of more proteasomes[43]. The continued accumulation of such proteins eventually triggers the activation of Jnk kinases and apoptosis[3,44]. It is now well established that damage to cell proteins (just like damage to DNA or chromosomal organization) can activate the cell-death programme. However, the ability of unfolded proteins to activate apoptosis has received little attention, yet it probably has an important function in diseases where unfolded proteins accumulate and are associated with neurodegeneration. Importantly, certain cancers, especially multiple myeloma, are particularly sensitive to apoptosis induced by proteasome inhibitors, and one such inhibitor (Velcade) has recently been

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approved for treatment of myeloma[42,45]. These cancer cells generate very large amounts of abnormal immunoglobins and consequently are probably the cells that are most dependent on proteasome function for the continual elimination of abnormal proteins.

Evolution has provided multiple levels of protection against abnormal proteins — from proteasomes and chaperones to induction of the heat-shock genes and apoptosis. To what extent these mechanisms protect us against various human diseases is still uncertain. It is clear that in many diseases these proteolytic systems fail to prevent the accumulation of the damaging proteins, perhaps because of defects in this degradative machinery. Greater understanding of this quality-control process is therefore not only of scientific interest but might lead to new therapies. □

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