



Editorial

Introduction and overview

Between the 1960s and 1980s, most life scientists focused their attention on studies of nucleic acids and the translation of the coded information. Protein degradation was a neglected area, considered to be a non-specific, dead-end process. While it was known that proteins turn over, the large extent and high specificity of the process—whereby distinct proteins have half-lives that range from a few minutes to several days—was not appreciated. The discovery of the lysosome by Christian de Duve did not change this view significantly, as it became clear that this organelle is involved mostly in the degradation of extracellular proteins, and their proteases are not substrate specific. While certain stimuli could increase or decrease lysosomal degradation of intracellular proteins, the process involved the bulk of the proteins and not targeting of specific substrates. The discovery of the complex cascade of the ubiquitin pathway revolutionized the field. It is clear now that degradation of cellular proteins is a highly complex, temporally controlled, and tightly regulated process that plays major roles in a variety of basic pathways during cell life and death, and in health and disease. With the multitude of substrates targeted, and the myriad processes involved, it is not surprising that aberrations in the pathway are implicated in the pathogenesis of many diseases, certain malignancies and neurodegenerative disorders among them.

Several observations lead to the prediction that degradation of intracellular proteins must be carried out by a nonlysosomal mechanism(s). The process is highly specific and different proteins have half-life times that vary from a few minutes (e.g. the tumor suppressor p53) to several days (e.g. the muscle proteins, actin and myosin) and up to a few years (crystalline). Furthermore, inhibitors of lysosomal degradation—weak bases such as chloroquine, for example—do not have any effect on degradation of intracellular proteins under basal metabolic conditions. These compounds titrate the normal acidic intralysosomal pH and bring it to a point that does not allow activity of the lysosomal proteases. These findings led to the hypothesis that degradation of intracellular proteins must be carried out by a non-lysosomal proteolytic system that is endowed with a high degree of specificity towards its substrates. Also, the fact that the proteolytic enzymes and their substrates reside in the same cellular compartment predicted a requirement for tightly regulated machinery that uses metabolic energy

for control. The discovery of the ubiquitin-proteasome proteolytic pathway has resolved these enigmas.

Degradation of a protein via the ubiquitin-proteasome pathway involves two successive steps: (a) conjugation of multiple ubiquitin moieties to the substrate, and (b) degradation of the tagged protein by the downstream 26S proteasome complex, with the release of free and reusable ubiquitin. This last process is mediated by ubiquitin recycling enzymes—deubiquitinating enzymes, DUBs (for a scheme of the ubiquitin proteasome pathway, see Fig. 1).

Conjugation of ubiquitin, a highly evolutionarily conserved 76 residue polypeptide, to the protein substrate proceeds via a three-step cascade mechanism. Initially, the ubiquitin-activating enzyme, E1, activates ubiquitin in an ATP-requiring reaction to generate a high-energy thiolester intermediate, E1-S~ubiquitin. One of several E2 enzymes (ubiquitin-carrier proteins or *Ubiquitin-Conjugating enzymes*, UBCs) transfers the activated ubiquitin moiety from E1, via an additional high-energy thiolester intermediate, E2-S~ubiquitin, to the substrate that is specifically bound to a member of the ubiquitin–protein ligase family, E3. There are a number of different classes of E3 enzymes. For the HECT (*H*omologous to the *E6-AP C-Terminus*) domain E3s, the ubiquitin is transferred once again from the E2 enzyme to an active site Cys residue on the E3, to generate a third high-energy thiolester intermediate, E3-S~ubiquitin, prior to its transfer to the ligase-bound substrate. RING finger-containing E3s catalyze direct transfer of the activated ubiquitin moiety from E2 (that is bound to the RING finger) to the E3-bound substrate. The mode of action of other ubiquitin ligases, such as U-box and PHD domain-containing enzymes has not been elucidated yet. E3s catalyze the last step in the conjugation process: covalent attachment of ubiquitin to the substrate. The ubiquitin molecule is generally transferred to an ϵ -NH₂ group of an internal Lys residue in the substrate to generate a covalent isopeptide bond. In some cases, however, ubiquitin is conjugated to the N-terminal amino group of the substrate. By successively adding activated ubiquitin moieties to internal Lys residues on the previously conjugated ubiquitin molecule, a polyubiquitin chain is synthesized. The chain is recognized by the downstream 26S proteasome complex. Thus, E3s play a key role in the ubiquitin-mediated proteolytic cascade since they serve as the specific recognition factors of the system.

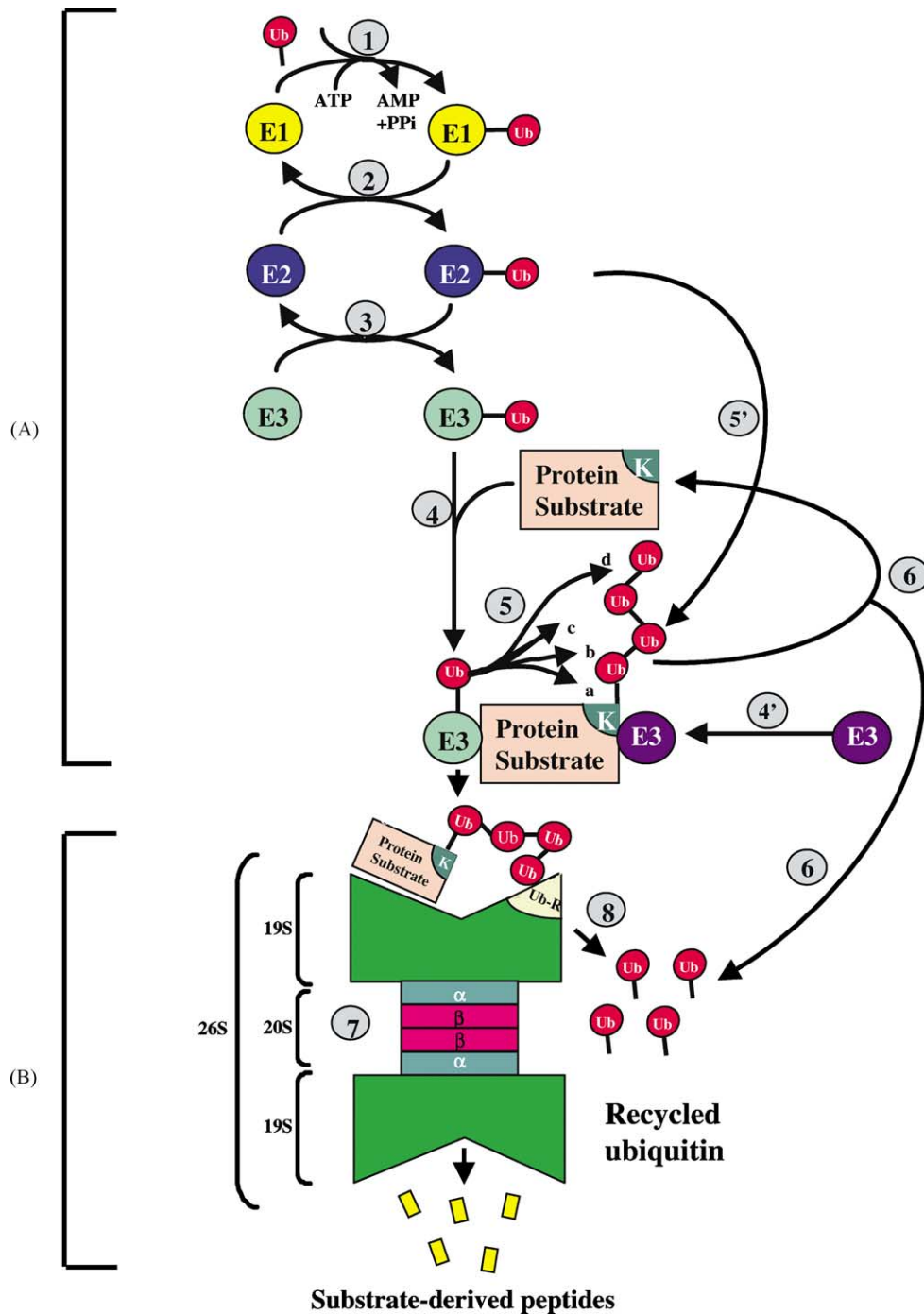


Fig. 1. The ubiquitin proteolytic pathway. **(A)** Conjugation of ubiquitin to the target substrate. **(1)** Activation of ubiquitin by the ubiquitin-activating enzyme, E1, to generate an E1-S~ubiquitin high-energy thioester. **(2)** Transfer of the activated ubiquitin from E1 to a ubiquitin-carrier protein, E2 (ubiquitin-conjugating enzyme, UBC), with release of free E1. An E2-S~ubiquitin high-energy thioester intermediate is generated. **(3)** Transfer of the activated ubiquitin from E2 to a ubiquitin protein ligase, E3, to generate an E3-S~ubiquitin high-energy thioester. This reaction occurs in the case of HECT (*H*omologous to the *E6-AP C-T*erminal domain E3s). **(4)** A HECT domain E3 binds to the protein substrate that has an internal Lys (K) residue to which the polyubiquitin chain will be anchored. **(5)** Successive conjugation (a, b, c, and d) of activated ubiquitin moieties to the target substrate and synthesis of a polyubiquitin chain on the HECT domain-bound substrate. **(4')** Binding of a protein substrate to a RING finger domain E3. **(5')** Transfer of the activated ubiquitin moiety from E2 directly to the E3 (RING finger domain)-bound substrate and formation of the polyubiquitin chain. **(6)** Deubiquitination of protein substrates by deubiquitinating enzymes (DUBs). "Correction" of erroneously conjugated proteins. **(B)** Degradation of the polyubiquitinated protein substrate by the 26S proteasome complex. **(7)** Binding of the polyubiquitinated substrate to the 26S proteasome complex (probably via a ubiquitin receptor subunit(s) in the 19S complex) followed by degradation of the substrate into short peptides by the 20S proteasome. **(8)** Ubiquitin is recycled via the activity of deubiquitinating enzymes (DUBs).

The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides. It is composed of two subcomplexes: a 20S core particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure composed of four stacked rings, two identical outer α rings and two identical inner β rings. The eukaryotic α and β rings are composed each of seven distinct subunits, giving the 20S complex the general structure of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The catalytic sites are localized to some of the β subunits. Each extremity of the 20S barrel is typically capped by a 19S RP. One important function of the 19S RP is to recognize ubiquitinated proteins and other potential substrates of the proteasome. Several ubiquitin binding subunits of the 19S RP, as well as cytosolic, have been identified; however, their importance and mode of action have not been discerned. A second function of the 19S RP is to open an orifice in the α ring that will allow entry of the substrate into the proteolytic chamber. Also, since a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel opening function and the unfolding of the substrate require metabolic energy, and indeed, the 19S RP contains six different ATPase subunits. Following degradation of the substrate, short peptides derived from the substrate are released, as well as free and reusable ubiquitin.

It should be noted that in the case of many cell surface membrane proteins, such as the EGF receptor, engagement of the ligand results in ubiquitination which routes the ligand–receptor complex to degradation in the lysosome. The mechanisms involved in this process have been delineated only partially, but it is clear that modification by a single ubiquitin moiety may be sufficient to target the modified protein.

A major unresolved question is how does the system achieve its high specificity and selectivity. Why are certain proteins extremely stable in the cell, while others are extremely short lived? Why are certain proteins degraded only at a particular time point during the cell cycle or only following specific extracellular stimuli, yet they are stable under most other conditions? It appears that specificity of the ubiquitin system is determined by two distinct and unrelated groups of proteins: (i) E3s and (ii) ancillary proteins. First, within the ubiquitin system, substrates must be specifically recognized by an appropriate E3 as a prerequisite to their ubiquitination. In most cases however, substrates are not recognized in a constitutive manner, and are not recognized directly by the E3. In some instances, the E3 must ‘be switched on’ by undergoing post-translational modification in order to yield an active form that recognizes the substrate. In many other cases, it is the substrate that must undergo a certain change that renders it susceptible for recognition. The stability of additional proteins depends on association with ancillary proteins such as molecular chaperones that act as recognition elements in *trans* and serve as a link to

the appropriate ligase. Others, such as certain transcription factors, have to dissociate from the specific DNA sequence to which they bind in order to be recognized by the system. Stability of yet other proteins depends on oligomerization. Thus, in addition to the E3s themselves, modifying enzymes (such as kinases), ancillary proteins, or DNA sequences to which substrates bind, also play an important role in the recognition process.

Ubiquitin-mediated proteolysis of a variety of cellular proteins plays an important role in many basic cellular processes. Among these are regulation of cell cycle and division, differentiation and development, involvement in the cellular response to stress and extracellular effectors, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels and the secretory pathway, DNA repair, transcriptional regulation, transcriptional silencing, long-term memory, circadian rhythms, regulation of the immune and inflammatory responses, and biogenesis of organelles. The list of cellular proteins that are targeted by ubiquitin is growing rapidly. Among them are cell cycle regulators such as cyclins, cyclin-dependent kinase inhibitors, and proteins involved in sister chromatid separation, tumor suppressors, and transcriptional activators and their inhibitors. Cell surface receptors and endoplasmic reticulum (ER) proteins are also targeted by the system. Finally, mutated and denatured/misfolded proteins are recognized specifically, and are removed efficiently. In this capacity, the system is a key player in the cellular quality control and defense mechanisms.

With the many processes and substrates targeted by the ubiquitin pathway, it is not surprising to find that aberrations in the system underlie, directly or indirectly, the pathogenesis of many diseases, many malignancies among them. While inactivation of a major enzyme such as E1 is obviously lethal, mutations in enzymes or in recognition motifs in substrates that do not affect vital pathways or that affect the involved process only partially, can result in a broad array of phenotypes. The pathological states associated with the ubiquitin system can be classified into two groups: (a) those that result from loss of function—mutation in a ubiquitin system enzyme or target substrate that result in stabilization of certain proteins, and (b) those that result from gain of function—abnormal or accelerated degradation of the protein target.

In general, specific cancers can result from *stabilization* of growth promoting factors and oncoproteins or *destabilization* of tumor suppressor gene products. Some of the natural substrates for degradation by the proteasome are growth promoting factors and oncoproteins that if not properly removed from the cell can promote cancer. For instance, ubiquitin targets N-myc, c-myc, c-fos, c-jun, Src, β -catenin, the hypoxia-inducible factor 1 α (HIF-1 α), and the adenovirus E1A proteins. It also targets many receptors for growth promoting factors such as the EGF receptor. Stabilization of these proteins may result in uncontrolled growth signaling, activation of transcription, and anti-apoptotic

effects, for example, that may all lead to malignant transformation. Destabilization of tumor suppressor proteins such as p53 and p27^{Kip1} has also been implicated in the pathogenesis of malignancies. This is due to activation—constitutive or viral protein-induced of specific ubiquitin ligases, E3s.

Because of the central role the ubiquitin system plays in such a broad array of basic cellular processes, development of drugs that modulate the activity of the system may provide a highly specific, target-oriented therapeutic approach to many diseases. Inhibition of enzymes common to the entire pathway, such as the proteasome, may affect many processes non-specifically, although a narrow window between beneficial effects and toxicity can be identified for a short-term treatment. Recent experimental evidence strongly suggests that such inhibitors may indeed be beneficial in certain pathologies, such as in cancer, asthma, brain infarct and autoimmune encephalomyelitis. In malignancies, the drugs may act via inhibition of degradation of different cell cycle regulators or inhibition of activation of the pro-apoptotic transcriptional regulator NF- κ B. A completely different approach to drug targeting can be the development of small molecules that bind and inhibit specific E3s. For example, specific phospho-peptide derivatives that span the phosphorylation targeting domains in different substrates can serve as “baits” to the respective E3s. Thus, a peptide or a molecule that spans the recognition domain of p27^{Kip1} and I κ B α , can be used to inhibit their degradation. This will lead to an increase in their steady-state level, enabling the cell to resume control of cell cycle regulation and increase its sensitivity to apoptotic stimuli, respectively. Thus, compounds that exert such activity can be thought of as potential drugs for the treatment of certain forms of malignancies. Yet, the similarity between the phosphorylation sites of I κ B α and β -catenin may also lead to stabilization of β -catenin, which is an activator, and its excessive transcriptional activity can result in an opposite effect—stimulating malignant transformation. A

better approach may be the development of small molecules that are substrate specific and bind, preferably, to specific substrates or to their ancillary proteins rather than to an E3. When accelerated degradation of a tumor suppressor results in exposure of cells to malignant transformation, selective inhibition of the recognition machinery can potentially reverse the malignant phenotype. Peptides that bind specifically to HPV-E6 and prevent its association with p53, interfere with p53 targeting. They were able to induce p53 in HPV-transformed cells with subsequent reversal of certain malignant characteristics and induction of apoptosis. Likewise, treatment directed at increasing the level of p27^{Kip1} resulted in regression of the malignant phenotype in experimental cancer models. Apoptosis can also be targeted. Thus, one can think of stimulating the self-ubiquitinating and auto-destructive activity of inhibitors of apoptosis proteins (IAPs), that in many cases are RING-finger-containing E3s, involved in targeting of caspases and other pro-apoptotic factors. This can result in re-sensitization of cancerous cells, that in many cases have acquired resistance to apoptosis, to death-inducing stimuli. This can be achieved by high expression of their respective stimulatory proteins—Smac/Diablo, Reaper, Hid and Grim, for example, or active peptides that are derived from them.

This collection of articles highlights the role played by the ubiquitin system in pathogenesis of malignant transformation, and shed light on potential, extremely powerful and specific, therapeutic modalities that can be developed now, based on the knowledge we acquired, to treat these aberrations.

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