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Basic Medical Research Award

The ubiquitin system

AVRAM HERSHKO, AARON CIECHANOVER & ALEXANDER VARSHAVSKY

Ubiquitin-mediated protein degradation: The early days

AVRAM HERSHKO

It has been often stated that until recently the ubiquitin system was thought to be mainly a 'garbage disposal' for the removal of abnormal or damaged proteins. This statement is certainly not true for those who have been interested in the selective and regulated degradation of proteins in cells. The dynamic turnover of cellular proteins was discovered in the pioneering studies of Rudolf Schoenheimer in the 1930s, when he first used isotopically labeled compounds for biological studies[1]. Between 1960 and1970 it became evident that protein degradation in animal cells is highly selective, and is important in the control of specific enzyme concentrations[2]. The molecular mechanisms responsible for this process, however, remained unknown. Some imaginative models have been proposed to account for the selectivity of protein degradation, such as one suggesting that all cellular proteins are rapidly engulfed into the lysosome, but only short-lived proteins are degraded in the lysosome, whereas long-lived proteins escape back to the cytosol[3].

I became interested in the mechanisms of intracellular protein breakdown when I was a post-doctoral fellow in the laboratory of Gordon Tomkins 30 years ago (1969–1971). At that time, the main subject in that laboratory was the mechanism by which corticosteroid hormones cause the increased synthesis of the enzyme tyrosine aminotransferase. I found this subject a bit crowded, so I chose to study a different process that also regulates tyrosine aminotransferase concentration: the degradation of this enzyme. I found that the degradation of tyrosine aminotransferase in cultured hepatoma cells is completely arrested by inhibitors of cellular energy production, such as fluoride or azide[4]. These results confirmed and extended the previous observations of Simpson on the energy dependence of the release of amino acids from liver slices[5]. Similar energy requirements for the degradation of many other cellular proteins were subsequently found in a variety of experimental systems[6].

I was very impressed by the energy dependence of intracellular protein breakdown, because proteolysis itself is an exergonic process that does not require energy. I assumed that there was an as-yet-unknown proteolytic system that uses energy for the highly selective degradation of proteins. After returning to Israel in 1971 and setting up my laboratory at the Technion, my main goal was to identify the energy-dependent system responsible for the degradation of cellular proteins. It took a bit of faith to base my entire research project on the effects of energy 'poisons', because these inhibitors could affect protein breakdown rather indirectly. For example, I remember that when Racker, a great biochemist, visited my laboratory in Haifa in the mid-1970s, he dismissed these observations as being secondary to the inhibition of the proton pump, which maintains the acidic environment in lysosomes. I was convinced, however, that lysosomal autophagy cannot account for the selectivity and regulation of intracellular protein breakdown. I was also convinced that the best way to identify a new system was that of classical biochemistry: to reproduce ATP-dependent protein breakdown in a cell-free system and then to fractionate such a system and to find the mode of action of its components.

An ATP-dependent proteolytic system from reticulocytes was first described by Etlinger and Goldberg[7], and then was analyzed by our biochemical fractionation–reconstitution studies. In this work, I was greatly helped by Aaron Ciechanover, who was then my graduate student. Substantial support and advice were provided by Irwin Rose, who hosted me in his laboratory in Fox Chase Cancer Center for a sabbatical year in 1977–1978 and many times afterwards. Initially, reticulocyte lysates were fractionated on DEAE-cellulose into two crude fractions: fraction 1, which was not adsorbed, and fraction 2, which contained all proteins adsorbed to the resin and eluted with high salt. The original aim of his fractionation had been to remove hemoglobin (present in fraction 1), but we found that fraction 2 lost most of ATP-dependent proteolytic activity. Activity could be restored by combining fractions 1 and 2. The active component in fraction 1 was a small protein that we purified

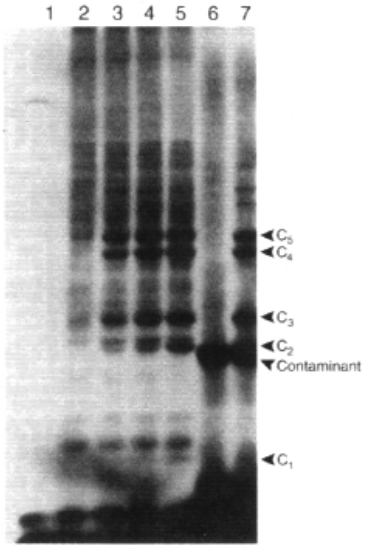

Fig. 1. Discovery of the ligation of ubiquitin to lysozyme, a substrate of the proteolytic system. Reaction products were separated by SDS–PAGE. Lane 1, incubation of ^{125}I-labeled ubiquitin with fraction 1 in the absence of ATP; ubiquitin remains free and migrates at the front. Lanes 2–5, incubation of ^{125}I-labeled ubiquitin with fraction 1 in the presence of ATP. Lane 2, ubiquitin becomes covalently linked to many high-molecular-weight derivatives, presumably endogenous protein substrates present in fraction 2. Lanes 3–5, several new labeled bands appear (C1–C5), which increase with increasing concentrations of lysozyme. Lanes 6 and 7, incubation of ^{125}I-labeled lysozyme with fraction 2 in the absence of ATP (lane 6) or with ATP and unlabeled ubiquitin (lane 7); bands C1–C5 contain the label of ^{125}I-labeled lysozyme and consist of increasing numbers of ubiquitin molecules ligated to lysozyme. Reproduced from ref. 13, with permission.

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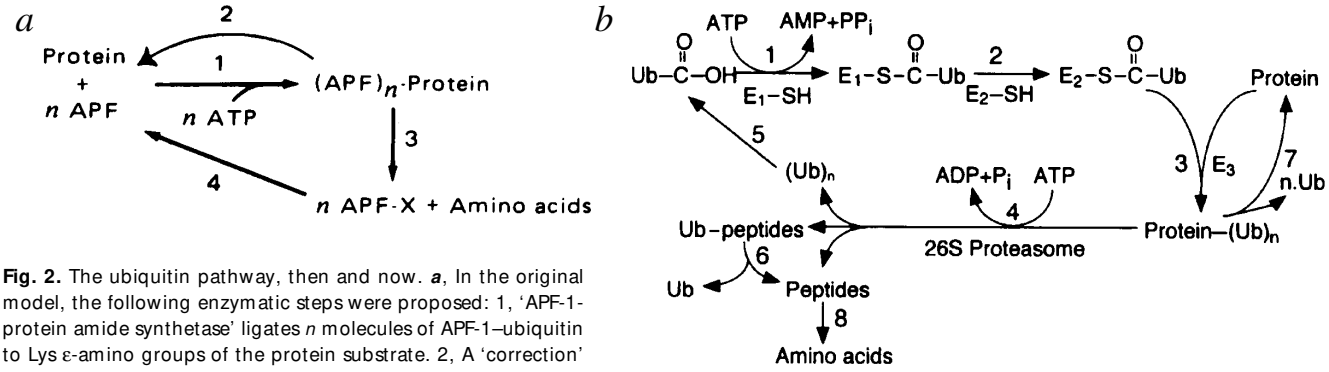

Fig. 2. The ubiquitin pathway, then and now. ***a***, In the original model, the following enzymatic steps were proposed: 1, 'APF-1-protein amide synthetase' ligates *n* molecules of APF-1–ubiquitin to Lys ε-amino groups of the protein substrate. 2, A 'correction' amidase (isopeptidase) releases free protein and APF-1–ubiquitin from erroneous ligation products. 3, An endopeptidase (protease) specifically acts on proteins ligated to several molecules of APF-1 and cleaves peptide bonds with the liberation of APF-1 still linked to Lys or a Lys-containing peptide (APF-1-X). 4, Amidase (isopeptidase) cleaves the bond between APF-1 and the ε-amino group of Lys residues and thus liberates reusable APF-1–ubiquitin. Reproduced from ref. 13, with permission. ***b***, Current information on the enzymatic reactions of the ubiquitin system. Steps 1, 2 and 3, accomplished by E1, E2 and E3, correspond to step 1 of the original model. Step 4, accomplished by the 26S proteasome, corresponds to step 3 of the original model. Steps 5, 6 and 7, accomplished by ubiquitin–carboxy-terminal hydrolases (isopeptidases), correspond to steps 2 and 4 of the original hypothesis. Reproduced from ref. 15, with permission.

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by taking advantage of its remarkable stability to heat treatment[8]. It was first called APF-1, for ATP-dependent proteolysis factor 1. The identification of APF-1 as ubiquitin was later made by Wilkinson and co-workers[9], after our discovery of its ligation to proteins. Ubiquitin was first thought to be a thymic hormone, but subsequently was found to be present in many tissues and organisms, hence its name[10]. It was found to be conjugated to histone 2A (ref. 11), but its functions remained unknown. Although we did not know at that time that APF-1 was ubiquitin, I will use the term ubiquitin here to facilitate the discussion.

The purification of ubiquitin from fraction 1 was the key to the elucidation of the mode of its action in the proteolytic system. At first I thought that it could be an activator, or a regulatory subunit of a protease or other enzyme component of the system present in fraction 2. To examine this possibility, purified ubiquitin was radioiodinated and incubated with crude faction 2 in the presence or absence of ATP. There was substantial ATP-dependent binding of ^{125}I-labeled ubiquitin to high-molecular-weight proteins by gel filtration chromatography[12]. However, a covalent amide linkage was unexpectedly formed, as shown by the stability of the 'complex' to treatment with acid, alkali, hydroxylamine or boiling with SDS and mercaptoethanol[12]. Analysis of the reaction products by SDS–PAGE showed that ubiquitin was ligated to many high-molecular-weight proteins. Because crude fraction 2 from reticulocytes contains not only enzymes but also endogenous substrates of the proteolytic system, we began to suspect that ubiquitin might be linked to protein substrates, rather than to an enzyme. In support of this interpretation, we found that proteins that are good (although artificial) substrates for ATP-dependent proteolysis, such as lysozyme, form several conjugates with ubiquitin[13]. In the original experiment that convinced us that ubiquitin is ligated to the protein substrate, similar high-molecular-weight derivatives were formed when ^{125}I-labeled ubiquitin was incubated with unlabeled lysozyme (Fig. 1, lanes 3–5), and when ^{125}I-labeled lysozyme was incubated with unlabeled ubiquitin (Fig. 1, lane 7). Analysis of the ratio of radioactivity in ubiquitin and lysozyme indicated that the various derivatives consisted of increasing numbers of ubiquitin molecules linked to one molecule of lysozyme. On the basis of these findings, we proposed a model in 1980 (Fig. 2*a*) in which several molecules of APF-1–ubiquitin are linked to Lys ε-amino groups of the protein substrate by an 'APF-1-protein amide synthetase' (Fig. 2*a*, step 1). We proposed that proteins ligated to several ubiquitins were broken down by a specific protease that recognizes such conjugates (Fig. 2*a*, step 3). Thus, the protein would be broken down to free amino acids and to APF-1–ubiquitin still linked by isopeptide linkage to Lys or a small peptide (APF-1-X). Finally, free APF-1–ubiquitin is released for re-use by the action of a specific amidase/isopeptidase (Fig. 2*a*, step 4). Based on a suggestion by Ernie Rose, we added a hypothetical 'correcting' isopeptidase to this scheme, which would release free ubiquitin and substrate protein from products of erroneous ubiquitin–protein ligation (Fig. 2*a*, step 2). An isopeptidase that may have such correction function was described recently[14].

Comparison of the original model with our current knowledge of the reactions of the ubiquitin pathway[15] (Fig. 2*b*) shows that the original model was essentially correct, but much further detail provides explanation for the high selectivity of ubiquitin-mediated protein degradation. Thus, we have found that 'APF-1-protein amide synthetase' is actually composed of three types of enzymes: a ubiquitin-activating enzyme E1, a ubiquitin-carrier protein E2 and a ubiquitin-protein ligase E3 (ref. 16). Specific E3 enzymes recognize specific structural features in specific protein substrates, and thus account for substrate selectivity[17]. Proteins ligated to multi-ubiquitin chains are degraded by a 26S proteasome complex discovered by Rechsteiner and co-workers[18]. ATP is needed not only for the ubiquitin–protein ligation reaction, as originally proposed, but also for the action of the 26S proteasome[18]. Finally, free and reusable ubiquitin is released by the action of a large variety of ubiquitin C-terminal hydrolases[15].

As indicated before[19], the main lesson from our story is the continued importance of the use of biochemistry in modern biomedical research. Without biochemistry, it is doubtful whether an entirely new system could have been discovered. On the other hand, molecular genetics has been essential in discovering the many functions of this system in processes such as cell cycle control, signal transduction and the immune response[15].

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The ubiquitin–proteolytic pathway: From obscurity to the patient bed

AARON CIECHANOVER

Traditionally, researchers in the field of proteolysis have tried to characterize and purify a single protease while pursuing the activity they are studying. I used a similar approach at the beginning of my studies as a graduate student with Avram Hershko, when we initiated our efforts to purify the non-lysosomal, ATP-dependent proteolytic activity that Etlinger and Goldberg[7] and we[20] had characterized earlier in reticulocyte lysate. An obvious first step during the purification of proteins from red blood cells is to remove hemoglobin, the main protein in the extract, by anion exchange chromatography. When we fractionated the lysate, we could not recover the proteolytic activity in either the flow-through material that contained hemoglobin (fraction I) or in the adsorbed, high-salt-desorbed material (fraction II). The proteolytic activity could be recovered, however, after the addition of both fractions I and II to the reaction mixture[8] (Table 1). This was a critical experiment, as it taught us that this complex system contained at least two (and if two, possibly more, as was later shown to be true) complementary factors, and not a single, 'traditional' protease that was also ATP-dependent. From then on, we used the power of 'classical' biochemistry and a 'complementing-add-and-subtract' approach to initially purify from fraction I ATP-dependent proteolytic factor-1 (APF-1; ref. 21). We called the protein APF-1, as it was the first factor we characterized. At the same time we started to identify additional complementing factors, and looked for a simple terminology to enable convenient communication in the laboratory.

APF-1 was later identified as ubiquitin[22], a known protein of previously unknown function. Keith Wilkinson and Arthur Haas were post-doctoral fellows with Irwin A. Rose at the Fox Chase Cancer Center in Philadelphia, where Avram Hershko spent a sabbatical in 1977–1978 and, later, his summers. I joined him during the summers of 1978–1981. Wilkinson and Haas were fascinated by the new type of post-translational modification by APF-1 and, along with Michael Urban, who worked in a neighboring laboratory studying histones, identified APF-1 as ubiquitin. It was known that ubiquitin generates a single modified adduct with histones H2A and H2B; however, the function of these adducts has unexpectedly remained obscure to these very days. We adopted the existing terminology, and APF-1 became ubiquitin. In parallel, we showed that multiple molecules of the protein are conjugated covalently to the target substrate and suggested that they serve as a degradation signal[12,13]. Initially we thought that each ubiquitin moiety attaches to a single internal Lys in the target molecule. Later Hershko[23] and then Varshavsky[24] and their colleagues demonstrated the formation of a poly-ubiquitin chain anchored to a single internal Lys residue. In the conjugation reaction, the C-terminal group of ubiquitin (Gly) generates a high-energy, isopeptide bond with an ε-amino group of an internal Lys residue of the substrate. Later we showed that the first conjugation event can also involve the N-terminus residue of the protein. We then went on to dissect the mechanism that underlies the activation of ubiquitin that must precede the generation of the high-energy bond with the target protein. Here, we made use of the known mechanism of amino-acid activation during protein synthesis, but mostly of the mechanism shown by Fritz Lipmann and his colleagues when they reconstituted a cell-free, non-ribosomal biosynthesis of the bacterial deca-peptide antibiotic gramicidin S (ref. 25). The similarities between the three mechanisms of activation of animo acids for protein and peptide synthesis and ubiquitin were strikingly similar. Dissection of the activation mechanism of ubiquitin paved the road to purification, by reaction-based, 'covalent' affinity chromatography over immobilized ubiquitin, of the three sequentially acting, conjugating enzymes: the ubiquitin-activating enzyme E1, the ubiquitin carrier protein E2 and the ubiquitin protein ligase E3, which accomplishes the last and most-essential step in the conjugation reaction, specific ligation of ubiquitin to the target protein[16,26]. In 1980, before the identification of APF-1 as ubiquitin and the dissection of the conjugation mechanism, we had already proposed a model[13] (Fig. 2a) according to which conjugation of multiple APF-1 molecules targets the substrate to degradation by an unknown, yet conjugate-specific, downstream protease. Intact APF-1 that can be re-used is recycled through the activity of isopeptidases. The protease, the 26S proteasome complex, was characterized and purified later by Martin Rechsteiner and colleagues[18]. In 1982, a more-detailed model was described, and the system began to be placed in its appropriate biological context[6]. In particular, its functions were analyzed and compared to those of the lysosomes involved mostly in the degradation of extracellular proteins taken up through pinocytosis and receptor-mediated endocytosis[6].

The physiological relevance of our initial findings remained unknown, as until that time (1981) we did all our studies in a cell-free reconstituted system, using secretory (and not intracellular; as these were available in large amounts and low cost) proteins as model substrates. The first evidence that the system is involved in degradation of proteins *in vivo* came from immunochemical analysis of ubiquitin adducts in cells[27]. Using antibodies raised against ubiquitin, we showed that, after incubation of cells in the presence of amino-acid analogs, the resulting abnormal proteins are short-lived. Their rapid degradation is accompanied by a transient, yet substantial increase in the level of ubiquitin adducts, strongly indicating that they serve as essential intermediates in the proteolytic process. Later, stronger and more-direct proof of the involvement of the ubiquitin system in the degradation cellular proteins came from the observation Alexander Varshavsky, Daniel Finely and I made that a cell-cycle arrest mutant that contains a thermolabile E1 enzyme is also defective in the degradation of short-lived abnormal proteins at the non-permissive temperature[28,29].

An important yet unresolved problem at that time involved the identification of the specific signaling motifs that target proteins for degradation. My initial entry into this fascinating area

Table 1 Anion exchange chromatographical resolution of reticulocyte lysate into unadsorbed (fraction I), and adsorbed, high-salt-desorbed (fraction II) complementing proteolytic activities.

Enzyme fraction used	Percent degradation of labeled globin	
	(–) ATP	(+) ATP
Complete lysate	1.5	10.0
Fraction I (flow-through material)	0	0
Fraction II (high salt desorbed eluate)	1.5	2.7
Fraction I + Fraction II	1.6	10.6

Adapted with modifications from ref. 8.

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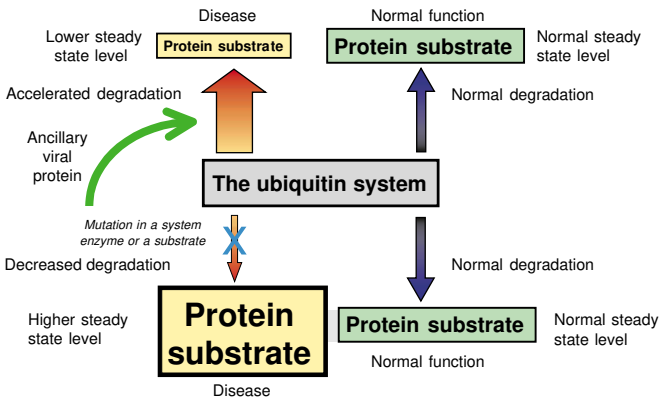

Fig. 3 Accelerated or decreased rates of ubiquitin-mediated proteolysis can underlie the pathogenesis of human diseases. Protein substrates are degraded each at a distinct and specific rate that may vary in different conditions (blue arrows), and maintain a steady-state level (green boxes) that enables them to function properly. Accelerated degradation (thick red arrow), as occurs in HPV E6-targeted degradation of p53, results in a low steady-state level of the target substrate (small beige box) and exposure of the cell to malignant transformation. Decreased degradation (narrow red arrow with a blue X) can occur when the signaling motif in the substrate is mutated (mutations in the phosphorylation sites in β-catenin in certain cases of malignant melanoma or colorectal carcinoma, or mutations in the NEDD4 E3 recognition motif of the kidney epithelial sodium channel in Liddle hypertension syndrome); or when E3 is mutated (as in Angelman syndrome, in which E6-AP is mutated). In all these examples, the excess accumulated substrate (large beige box) is 'toxic'.

was unanticipated. A large-scale purification of APF-1 carried out in Haifa in 1980 showed a substantial discrepancy between its high dry weight and low protein content, as determined by a Lowry assay. Hershko and I thought that the protein was probably a ribonucleoprotein complex, and the excess non-protein mass was due to the RNA component. Treatment of the APF-1 preparation with RNase A led to abrogation of its stimulatory activity towards bovine serum albumin (BSA), but not lysozyme. Not appreciating at that time the extent of the high specificity of the system toward its different substrates, we could not explain the 'selective' RNase effect, and did not pursue the study. The discrepancy between weight and protein measurement was resolved later with the finding that ubiquitin has a low content of the aromatic amino acids that are the basis of every known method for protein measurement. Obviously, ubiquitin is a pure protein and not a ribonucleoprotein complex. Later, our studies showed that nuclease added to the ubiquitin preparation destroyed $tRNA^{Arg}$ that was necessary, along with arginyl–tRNA protein transferase, to convert the N-terminal acidic (Asp) residue of BSA to Arg (refs. 30,31). Only the modified BSA and not the wild-type BSA can bind to the 'basic' N-terminal binding site of E3α, the ubiquitin ligase involved in recognition via the N-terminal residue (the N-end rule pathway ligase; ref. 15 and see below commentary by A. Varshavsky). Lysozyme, with a Lys at the N-terminal residue, does not undergo this post-translational modification and is recognized directly by E3α, and its degradation, therefore, is not sensitive to RNase. This finding became part of the more thorough and systematic mode of recognition identified through genetic tools by Alexander Varshavsky and his colleagues, and is known as the N-end-rule pathway[32]. Earlier, Hershko also noted the importance of an exposed N-terminal residue in targeting certain model proteins for degradation[33], but at that time, the mechanistic relevance of this finding was not apparent. Although most known substrates of the ubiquitin system are targeted through different recognition motifs[34], the N-end-rule pathway was the first well-defined signal of substrate recognition (see commentary by A. Varshavsky).

As for the myriad cellular substrates and regulatory functions of the ubiquitin system now known, it was only in the early 1990s that scientists started to unravel these secrets. The discovery of oncoproteins, tumor suppressors, transcriptional factors and cell-cycle regulators have shown that all these proteins are short-lived and their stability is tightly regulated. This previously unknown mode of regulation through destruction, which, unlike phosphorylation, is irreversible and may have evolved to secure directionality, has attracted many scientists to study the systems and signals that govern the stability (and hence the activity) of many different proteins[35–38]. Without even knowing the underlying mechanisms, Varshavsky, Finley and I predicted that the ubiquitin system may be involved in the regulation of the cell cycle[28]. The prediction was based on the observation that the ts85 mutant cell is defective in both E1 that inactivates the ubiquitin system and in transition along the S/G2 boundary of the cell cycle. This prediction has been corroborated by many studies demonstrating involvement of the ubiquitin system in programmed degradation of a broad array of cell cycle regulators.

Given the many processes and substrates involved, recent indications of the involvement of the system in the pathogenesis of many inherited as well as acquired diseases are not unexpected. Drug companies are trying to target the aberrations in the system that underlie these pathologies. The common denominator shared by all these diseases is a change in the steady-state level of a particular protein substrate or set of protein substrates. In general, the diseases belong to two classes, resulting from accelerated or decreased rates of degradation of different substrates (Fig. 3). The second class can be further divided into diseases due to mutations in enzymes of the system or to mutations in recognition motifs of substrates (Fig. 3). Although it is not possible to systematically review here all these diseases (reviewed recently in ref. 39), a few salient examples follow. An example of accelerated degradation involves the degradation of p53 induced by the human papillomavirus (HPV) E6 oncoprotein, which probably underlies the pathogenesis of human uterine cervical carcinoma, a very prevalent and severe malignant disease. E6 associates with p53 and targets it for rapid degradation mediated by the ubiquitin ligase E6-associated protein (E6-AP), which does not recognize the free tumor suppressor. Inactivation of the cellular DNA damage-control machinery exposes the cell to malignant transformation. Mutations in E6-AP lead to Angelman syndrome, an inherited disease associated with severe mental retardation and motor disorders. Here, accumulation of unidentified native substrate(s) of E6-AP (p53 is not the native substrate of the enzyme and is normally targeted by Mdm2) is probably toxic to the developing brain. In another example, mutations in the phosphorylation-targeting motif of the transcription factor β-catenin, or in adenomatosis polyposis coli (APC) that is part of the β-catenin degradation complex, lead to stabilization and accumulation of the protein, accompanied by its uncontrolled activity. These mutations may be involved in the pathogenesis of many forms of colorectal carcinomas and malignant melanomas. Viruses such as HPV have evolved different mechanism that enable them to evade the normal mode of

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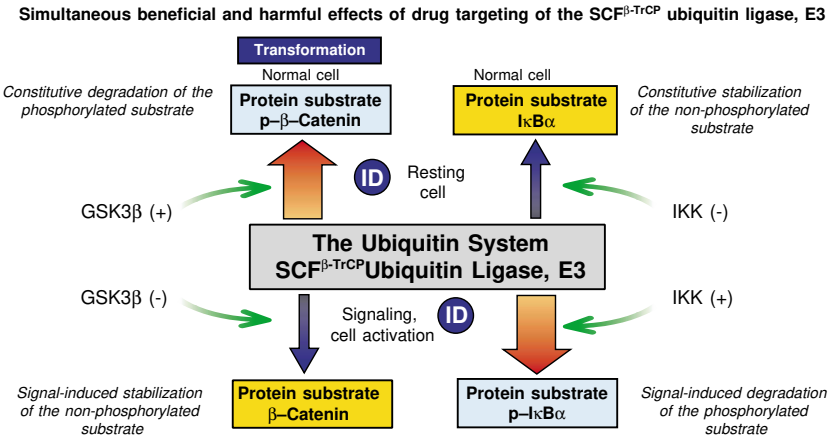

Fig. 4 Drug targeting of an E3 enzyme can become a 'double-edged sword'. In the resting cell (top), IκBα (blue arrow and beige box, upper right) is not phosphorylated and degraded slowly, whereas β-catenin is phosphorylated constitutively by GSK3β and is degraded rapidly following ubiquitination by the SCF$^{β\text{-}TrCP}$–E3–ubiquitin–ligase complex (heavy red arrow and light blue box, upper left). In the signaled cell (bottom), IκBα is phosphorylated by IκB kinase (IKK), rapidly ubiquitinated by the same SCF–TrCP complex and degraded (thick red arrow and light blue box, lower right). Also, in signaled cells, GSK3β is inhibited, and the non-phosphorylated β-catenin is stabilized, translocated into the nucleus and stimulates transcription (blue arrow and beige box, lower left). In general, IκB-containing cells are distinct from β-catenin-containing cells and so are the signals that activate the two pathways. However, the phosphorylated recognition motifs of the two proteins are similar and they seem to be targeted by the same TrCP ubiquitin ligase. An E3 inhibitor (inhibitory drug, ID) can lead to inhibition of degradation of IκB in stimulated cells, and consequently to suppression (beneficial effect) of NF-κB-induced inflammatory processes that may occur in autoimmune diseases, for example. At the same time, ID treatment will result in suppression of degradation of phosphorylated β-catenin in resting cells, with resultant accumulation of the transcription factor and possible subsequent malignant transformation (harmful effect).

activity of the ubiquitin system and allow them to continue their replication and propagation. Epstein Barr nuclear antigen 1 (EBNA-1) persists in healthy carriers for life, and its persistence contributes to some of the virus-related pathologies. Unlike all other Epstein Barr viral proteins, EBNA-1 cannot elicit a cytotoxic T lymphocyte (CTL) response. A long, C-terminal Gly-Ala repeat inhibits ubiquitin-mediated degradation and subsequent major histocompatibility complex (MHC) class I antigen presentation of EBNA-1. The human cytomegalovirus (CMV) encodes two endoplasmic reticulum (ER) resident proteins, US2 and US11, that bind to MHC class I molecules in the ER and escort them to the translocation machinery. After retrograde transport to the cytoplasm, they are ubiquitinated and degraded by the proteasome. Removal of the MHC molecules enables the virus to evade the immune system. A completely different case involves Liddle syndrome. In this disorder, a mutation in the recognition motif that targets the kidney epithelial sodium channel (ENaC). to ubiquitination by the Nedd4 E3 leads to accumulation of the channel, excessive reabsorption of sodium and water, with resulting severe hypertension.

Because of the central function of the ubiquitin system in many basic cellular processes, development of drugs that modulate the system may be difficult. Inhibition of enzymes common to the entire pathway, such as the proteasome, may affect many processes nonspecifically, although a narrow 'window' between beneficial effects and toxicity can be identified for a short-term treatment. An attractive possibility is the development of small molecules that inhibit specific E3 molecules. For example, specific phospho-peptide derivatives can inhibit the β-TrCP ubiquitin ligase, E3 complex (β-Transducin repeat-Containing Protein[40]). However, this approach can turn into a 'double-edged sword' (Fig. 4). Ideally, small molecules should be developed that bind to specific substrates or to their ancillary proteins, and thus inhibit a specific process. Peptide aptamers (small molecules/peptides that bind to active/association sites of proteins and inhibit their native interactions) that bind specifically to HPV E6 and probably prevent its association with p53, have been shown to induce apoptosis and reverse certain malignant characteristics in HPV-transformed cells, probably by interfering with p53 targeting[41]. Unfortunately, because of the rarity of proteins targeted by similar mechanisms, this approach may be currently limited to a small number of cases.

Discovering the functions and degrons of the ubiquitin system

ALEXANDER VARSHAVSKY

Through preparation, help from friends and a lot of luck I was able to leave the former Soviet Union in the fall of 1977, and ended up in Boston. A month later I was a faculty member of the Biology Department of the Massachusetts Institute of Technology (MIT), before I knew what exactly grants were (and before the colleagues who hired me became aware of that fact). In Moscow, I studied chromosome structure and regulation of gene expression, and looked forward to continuing this work.

There were few similarities between my earlier milieu and the astonishing new life. The libraries were one of them. They were just as quiet and pleasant in Cambridge as in Moscow, and a library at MIT soon became my second home. Reading there I came across a curious 1977 paper by Harris Busch, Ira Godknopf and their colleagues. They found a DNA-associated protein that had one C-terminus but two N-termini, an unprecedented structure. The short arm of that Y-shaped protein was joined, through its C-terminus, to an internal Lys of histone H2A. The short arm was soon identified, by Margaret Dayhoff, as ubiquitin, a 76-residue protein of unknown function that was described (as a free protein) by Gideon Goldstein and colleagues in 1975 (ref. 10).

I became interested in this first ubiquitin conjugate, UbH2A. Back in Russia, I had begun to develop a method for high-resolution analysis of nucleosomes. These DNA–protein complexes were subjected to electrophoresis in a low-ionic-strength polyacrylamide gel (a forerunner of the gel-shift assay), followed by second-dimension electrophoresis of either DNA or proteins. We located UbH2A in a subset of the nucleosomes, succeeded in separating these nucleosomes from those lacking UbH2A, and eventually showed that UbH2A-containing nucleosomes were enriched in transcribed genes and excluded from the inactive (heterochromatic) parts of the chromosomes[9].

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Meanwhile, Avram Hershko, his graduate student Aaron Ciechanover and their colleagues at the Technion (Haifa, Israel) were studying ATP-dependent protein degradation in extracts from rabbit reticulocytes. In 1978–1980 they demonstrated that a small protein, which they called APF-1 (ATP-dependent proteolytic factor 1), was covalently conjugated to proteins that were about to be degraded in the extract. They suggested that a protein-linked APF-1 served as a signal for a downstream protease, and began the analysis of enzymology of APF-1 conjugation. In 1980, Keith Wilkinson, Michael Urban and Arthur Haas showed that APF-1 and ubiquitin were the same protein[9].

When I saw that 1980 paper, two seemingly independent realms, protein degradation and chromosomes, came together. I realized that we were dealing with a proteolytic system of immense complexity and exceptionally broad, still to be discovered, range of functions. I decided to find genetic approaches to this entire problem, because a system of such complexity was unlikely to be understood through biochemistry alone. In 1980, reverse-genetic techniques were about to become feasible with the yeast *Saccharomyces cerevisiae*, but were still a decade away from mammalian genetics. I continued to read, as widely as I could. On a fateful day at the end of 1980, I came across a paper by Yamada and colleagues that described a conditionally lethal, temperature-sensitive mouse cell line called ts85. The researchers showed that a specific nuclear protein disappeared from ts85 cells at increased temperatures, and suggested that this protein might be UbH2A. When I saw their data, I had to calm down to continue reading, because I knew that this protein was UbH2A. (In the preceding two years we had learned much about the electrophoretic properties of UbH2A.)

Daniel Finley had just joined my lab to study regulation of gene expression, but soon switched to ts85 cells. A few months into the project, Finley and I made the crucial observation that ubiquitin conjugation in an extract from ts85 cells was temperature-sensitive, in contrast to an extract from parental cells. Soon afterward, I invited Ciechanover, who came from the Hershko laboratory for a postdoctoral stint at another MIT lab, to join Finley and me in the continuing study of ts85 cells. He did, and we published two papers in 1984 that demonstrated two main results: that mouse ts85 cells have a temperature-sensitive, ubiquitin-activating (E1) enzyme, and that these cells stop degrading the bulk of their normally short-lived proteins at the nonpermissive temperature[28,29]. This was the first evidence that ubiquitin conjugation was required for protein degradation *in vivo*. These findings[28,29] also indicated that ubiquitin conjugation was essential for cell viability. In addition, ts85 cells tended to be arrested at the G2 phase of the cell cycle, and the synthesis of heat-shock proteins was strongly induced in these cells at the nonpermissive temperature, indicating that ubiquitin-dependent proteolysis is involved in the cell-cycle progression and stress response[28]. In 1983, Tim Hunt and colleagues discovered unusual proteins in rapidly dividing fertilized clam eggs. These proteins, which they called cyclins, were degraded at the exit from mitosis. We suggested in 1984 that cyclins were destroyed by the ubiquitin system[29], a hypothesis shown to be correct by Michael Glotzer, Andrew Murray and Marc Kirschner in 1991.

The ts85 results[28,29] left little doubt, among the optimists, about the importance of the ubiquitin system in cellular physiology. Unfortunately, these findings could not be deepened and made more rigorous, because of limitations of mammalian somatic cell genetics, which was still hampered at that time by the impossibility of altering genes at will. Therefore, in 1983 we began systematic analysis of the ubiquitin system in *S. cerevisiae* (Fig. 5). In 1984, Finley and Engin Özkaynak cloned the first ubiquitin gene, and found that it encoded a polyubiquitin precursor protein. By 1987, they showed that this gene, *UBI4*, was strongly induced by different stresses. Moreover, deletion of *UBI4* resulted in cells that were hypersensitive to every noxious treatment we tried, including heat and oxidative stress[42]. These results validated and extended an inference from the 1984 findings with ts85 cells, thereby establishing one broad and essential function for the ubiquitin system.

In a parallel 1987 study, Stefan Jentsch and John McGrath isolated ubiquitin-conjugating (E2) enzymes from *S. cerevisiae*. One evening, a phone call from an excited Stefan Jentsch marked the discovery of yet another function of the ubiquitin system: a partially sequenced yeast E2 enzyme was found to be RAD6, a protein known to yeast geneticists for years as an essential component of DNA repair pathways[43]. RAD6 was the first enzyme of the ubiquitin system that was shown to mediate a specific physiological function. The sequence of RAD6 was weakly similar

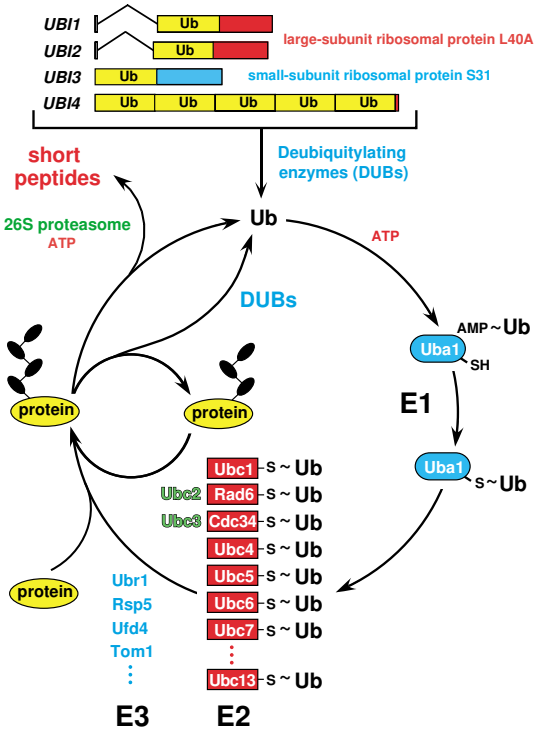

Fig. 5 The ubiquitin system of *S. cerevisiae*[51]. The yeast ubiquitin genes, two of which (*UBI1* and *UBI2*) contain introns, encode fusion proteins of ubiquitin (yellow rectangles) to itself (*UBI4*) or to one of the two specific ribosomal proteins (*UBI1–UBI3*) (red and blue rectangles). These fusion proteins are cleaved by deubiquitinating enzymes, yielding mature ubiquitin. ~, Thioester bonds between ubiquitin and the active-site Cys residues of ubiquitin-specific enzymes. The conjugation of ubiquitin to other proteins involves a preliminary ATP-dependent step, in which the last residue of ubiquitin (Gly76) is joined, through a thioester bond, to a Cys residue in the ubiquitin-activating (E1) enzyme encoded by *UBA1*. The activated ubiquitin is transferred to a Cys residue in one of at least 13 distinct ubiquitin-conjugating (E2) enzymes encoded by the *UBC* family genes, and from there to a Lys residue of an ultimate acceptor protein (yellow oval). This last step and the formation of a multi-ubiquitin chain (black ovals) require participation of another component, called E3 (the names of some of the yeast E3 proteins are included). A targeted, ubiquitinated protein substrate is processively degraded to short peptides by the ATP-dependent 26S proteasome.

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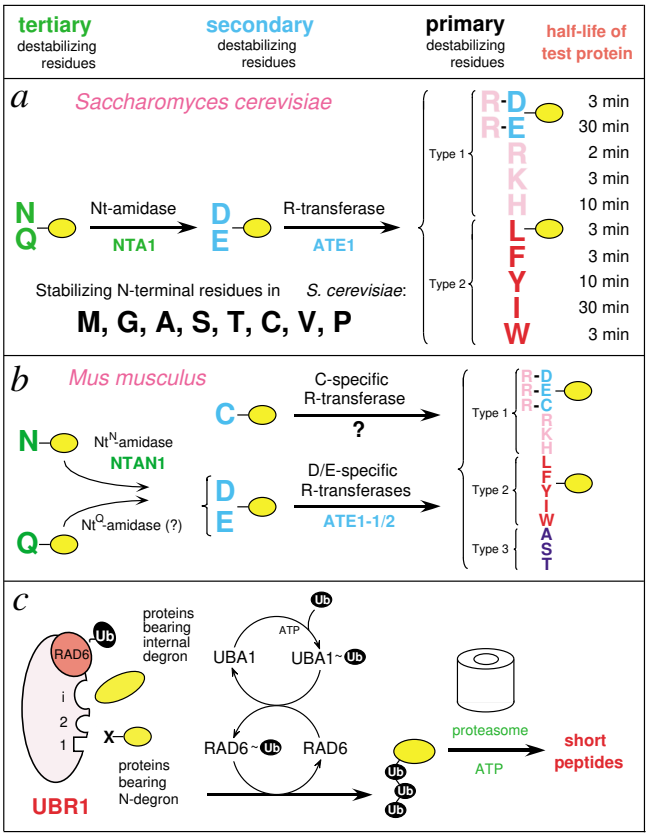

Fig. 6 The N-end rule pathway. Notations in the yeast (***a***) and mouse (***b***) pathways show type 1 (purple) and type 2 (red) primary, secondary (light blue) and tertiary (green) destabilizing N-terminal residues; yellow ovals indicate the rest of a protein substrate. ***a***, The *in vivo* half-lives of X-βgals, β-galactosidase-based test proteins in *S. cerevisiae*[48] (right). X-βgal proteins bearing stabilizing N-terminal residues (black) are metabolically stable ($t_{1/2}$, more than 20 h). The tertiary destabilizing residues N (Asn) and Q (Gln) are converted into secondary destabilizing residues D (Asp) and E (Glu) by N-terminal amidohydrolase (Nt-amidase), encoded by *NTA1*. D and E are conjugated to R (Arg), one of the primary destabilizing residues, by Arg–RNA protein transferase (R-transferase), encoded by *ATE1*. ***b***, In the mammalian N-end rule pathway, the deamidation step is mediated by two distinct enzymes, Nt^N-amidase and Nt^Q-amidase, specific for N-terminal Asn and Gln residues, respectively [54]. In vertebrates, the set of secondary destabilizing residues contains not only Asp and Glu but also Cys (C), which is a stabilizing residue in yeast[55]. In mammals but not in yeast, Ala (A), Ser (S) and Thr (T) are primary (type 3) destabilizing residues[48]. ***c***, *S. cerevisiae* UBR1 has two binding sites for the primary destabilizing N-terminal residues of either proteins or short peptides. The type 1 site is specific for basic N-terminal residues Arg, Lys and His. The type 2 site is specific for bulky hydrophobic N-terminal residues Phe, Leu, Trp, Tyr and Ile. UBR1 contains yet another substrate-binding site (i), which targets proteins bearing internal (non-N-terminal) degrons. In yeast, these proteins include the CUP9 repressor[57]. A complex of UBR1 and the ubiquitin-conjugating (E2) enzyme RAD6 produces a substrate-linked multi-ubiquitin chain[48].

to that of CDC34, an essential cell-cycle regulator defined genetically by Leland Hartwell. In 1988, a collaboration between Breck Byer's and my laboratories demonstrated that CDC34 was also a ubiquitin-conjugating enzyme[44] (Fig. 5). This result transformed a hint from our ts85 work into a definitive demonstration of the involvement of the ubiquitin system in cell-cycle control.

In 1989, Finley and Bonnie Bartel discovered that ubiquitin genes other than *UBI4* (the polyubiquitin gene) were also quite unusual: *UBI1–UBI3* encoded fusions of ubiquitin to one protein of the large ribosomal subunit and one protein of the small ribosomal subunit, an arrangement conserved from yeast to humans[45] (Fig. 5). Kenneth Redman and Martin Rechsteiner independently identified these non-ubiquitin extensions as ribosomal proteins. The transient presence of ubiquitin in front of a ribosomal protein moiety (ubiquitin was rapidly cleaved off by deubiquitinating enzymes) was found to be essential for efficient biogenesis of the ribosomes[45]. Ubiquitin acts, in these settings, not as a degradation signal but as a molecular chaperone. The fusion-imposed 1:1 molar ratio of free ubiquitin to a free ribosomal protein (Fig. 5) sets an upper limit for the number of newly produced ribosomes relative to the number of newly formed ubiquitin molecules. This tight link, through DNA-encoded fusions of ubiquitin and ribosomal proteins, is one of the few understood regulatory interactions between protein synthesis and protein degradation.

The enormous expansion of the ubiquitin field in the last decade stemmed mainly from these functional insights of the 1980s, which demonstrated both the involvement of ubiquitin conjugation in important biological processes and the striking diversity of these processes, from the cell cycle[28, 29, 44] to DNA repair[43], ribosome biogenesis[45] and stress responses[42]. Many more functions have been added to this list since 1990.

How are proteins recognized as substrates for ubiquitin conjugation? The first solution to this problem was produced in 1986, when Andreas Bachmair and Finley discovered the first degradation signals in short-lived proteins[46]. We constructed ubiquitin fusion proteins in which ubiquitin was followed by a reporter moiety such as *Escherichia coli* β-galactosidase, and expressed them in *S. cerevisiae*. The first advance took place when we learned that the ubiquitin moiety of these fusion proteins was rapidly removed by deubiquitinating enzymes regardless of the identity of the residue at the C-terminal side of the cleavage site, with Pro being the sole exception. Thus was born the ubiquitin fusion technique, which made it possible to place, *in vivo*, any desired residue (except Pro) at the N-terminus of a protein of interest[46]. The presence of Met at the N-termini of nascent proteins and the substrate specificity of cytosolic Met aminopeptidases did not allow this level of experimental freedom before the discovery of the ubiquitin fusion technique[47].

Using this method, Bachmair and Finley discovered that the *in vivo* half-life of a test protein was strongly dependent on the identity of its N-terminal residue, a simple relation called the N-end rule[46]. The underlying ubiquitin-dependent pathway, called the N-end-rule pathway (Fig. 6), was later found to be present in all eukaryotes, from fungi to plants and mammals, and even in prokaryotes, which lack ubiquitin[48]. Yet another degradation signal identified in 1986 was the N-terminal ubiquitin moiety of a fusion protein under conditions that precluded its removal by deubiquitinating enzymes[46]. This signal is targeted by a distinct pathway of the ubiquitin system[49].

A family of signals, called N-degrons, that give rise to the N-end rule is still the best-understood set of degradation signals. An N-degron consists of a substrate's destabilizing N-terminal residue and an internal Lys residue, the latter being the site of ubiquitin attachment[48,50]. The E2–E3 ubiquitin ligase (Fig. 6*c*) binds to the substrate's N-terminal residue and forms a multi-ubiquitin chain linked to a substrate's Lys residue, the selection of which is often the result of stochastic choice among several sterically suitable Lys residues[48]. This bi-partite organization is also characteristic of subsequently identified degradation signals

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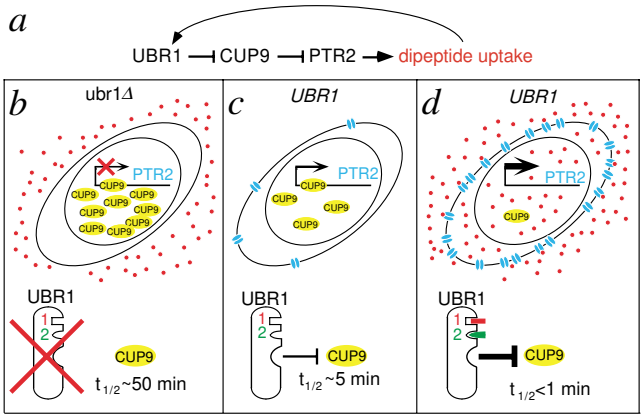

Fig. 7 Ubiquitin-dependent activation of peptide import in *S. cerevisiae*[20]. ***a***, Genetic diagram of the peptide transport circuit. ***b***, UBR1 is required for di-peptide uptake. In the absence of UBR1 (*ubr1Δ*), the transcriptional repressor CUP9 is long-lived, accumulates to high levels and extinguishes the expression of peptide transporter encoded by *PTR2*. The *ubr1Δ* cells cannot import di-peptides (red dots). ***c***, In a *UBR1* cell growing in the absence of extracellular di-peptides, UBR1 targets CUP9 for degradation ($t_{1/2}$, about 5 min), resulting in a lower steady-state concentration of CUP9 and weak but substantial expression of the PTR2 transporter (blue double ovals). ***d***, In *UBR1* cells growing in the presence of extracellular di-peptides, some of which bear destabilizing N-terminal residues, the imported di-peptides bind to the basic (type 1; red rectangle) or hydrophobic (type 2; green wedge) residue-binding sites of UBR1. Binding of either type of di-peptide to UBR1 allosterically increases the rate of UBR1-mediated degradation of CUP9. The resulting decrease of the half-life of CUP9 from about 5 min to less than 1 min leads to a further decrease in CUP9 levels, and consequently to a strong induction of the PTR2 transporter[57].

in cyclins, transcription factors and other short-lived proteins. One unique feature of N-degrons is that substrates bearing certain destabilizing N-terminal residues are chemically modified *in vivo*, through their enzymatic deamidation or arginylation, before a substrate can be bound by the ubiquitin ligase[48,51] (Fig. 6).

Having been the first ubiquitin-dependent pathway to be defined through molecular genetic methods, the N-end rule pathway (Fig. 6) was also the setting in which several essential insights relevant to the entire ubiquitin system were first made, including the discovery of specific multi-ubiquitin chains and their function in proteolysis[24]. In 1985, Hershko and Heller suggested, on the basis of chemical modification data, that some ubiquitin moieties in multi-ubiquitinated proteins might be linked together in a chain. In 1989, Vincent Chau and colleagues in my laboratory demonstrated the existence of protein-linked multi-ubiquitin chains, found them to have unique topology (ubiquitin–ubiquitin bonds through Lys48 of ubiquitin) and showed that these chains were required for degradation of test proteins[24]. We proposed that the main function of a substrate-linked multi-ubiquitin chain is to bind the substrate to the proteasome[24]. The complexity and multiplicity of ways in which a substrate is delivered to the proteasome is demonstrated by the recent discovery that ubiquitin ligases themselves physically interact with specific subunits of the 26S proteasome[52].

Subunit selectivity of protein degradation was yet another fundamental feature of the ubiquitin system that was first discovered in the N-end rule pathway. Erica Johnson and David Gonda demonstrated in 1990 that this pathway can eliminate one subunit of an oligomeric protein selectively, leaving intact the other subunits of the same protein molecule[53]. It is specifically the subunit conjugated to a multi-ubiquitin chain that gets destroyed. Subunit selectivity of proteolysis underlies large differences in the *in vivo* half-lives of subunits in oligomeric proteins. This essential feature of the ubiquitin system is both powerful and flexible, in that it allows protein degradation to be wielded as an instrument of either positive or negative control. Among many examples are activation of transcription factor NF-κB through degradation of its inhibitory ligand IF-κB, and inactivation of cyclin-dependent kinase activity through degradation of a regulatory cyclin subunit.

The emerging functions of the N-end rule pathway have been described[54,55]. Among these functions, the best understood is the essential role of this pathway in a positive feedback circuit that regulates the import of peptides in *S. cerevisiae*[56,57] (Fig. 7). Imported peptides bearing destabilizing N-terminal residues bind to the recognition sites for N-end rule substrates in UBR1, the pathway's E3 enzyme. This binding allosterically activates yet another substrate-binding site of UBR1, leading to accelerated degradation of the transcriptional repressor CUP9. The resulting derepression of expression of the peptide transporter PTR2 greatly increases the cell's capacity to import peptides[57]. This circuit (Fig. 7) is the first example of small compounds being natural allosteric regulators of the ubiquitin system.

A backward glance: It's Moscow, and the year is 1968. The author, a chemistry undergraduate both cocky and insecure, is listening to a leading Russian biochemist, a man in his forties whose education and entire life were warped by the combined cruelties of Stalinism and the Lysenko-led destruction of Russian genetics. The great man was telling me something he considered self-evident: "Ah, Alex, don't waste your time on genetics. It's all ancient Greece, beautiful in a strange way, but next to useless. They keep tormenting fruit flies, but it's us biochemists who will produce the understanding that really matters." Having spent a day reading genetic papers, I sensed that he could not be right, that genetics was essential too. Over the next three decades, the dynamic interaction of genetics and biochemistry kept yielding insights that could not be produced by biochemistry or genetics alone. These advances, many of them technical in nature, have transformed biology, and are beginning to be felt in medicine.

The early history of the ubiquitin field recapitulates, in a microcosm, the essential interaction between biochemistry and genetics that underlies the phenomenon of modern biology. Biochemical studies by Hershko, Ciechanover and their colleagues revealed a mechanistically unexpected, most curious but functionally obscure pathway of protein degradation. Molecular genetic (as well as biochemical) work proved necessary for discovering the first physiological functions of ubiquitin-dependent proteolysis and the first degradation signals in short-lived proteins. Methods and approaches developed in this work, including the ubiquitin fusion technique[47], continue to be of use in the ubiquitin field and beyond.

The vast expansion of ubiquitin studies over the last decade, with hundreds of laboratories around the world working on the ubiquitin system and its legion of biological functions, is a sight to behold. The fundamental understanding of this system, and recent insights into its roles in health and disease will have a profound influence on the realm of therapeutic drugs. The reason is not just the obvious one—promising drug targets among the ubiquitin system's components and substrates—but also the possibility of developing drugs that could direct this system to destroy (and thereby to inhibit functionally) any protein target.

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Avram Hershko
Unit of Biochemistry, Technion-Israel Institute of Technology
Faculty of Medicine, P.O. Box 9649, Haifa, 31096, Israel

Aaron Ciechanover
Unit of Biochemistry, Technion-Israel Institute of Technology
Faculty of Medicine, P.O. Box 9649, Haifa, 31096, Israel

Alexander Varshavsky
Smits Professor of Cell Biology, Division of Biology
California Institute of Technology
1200 East California Blvd., Pasadena, CA 91125

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