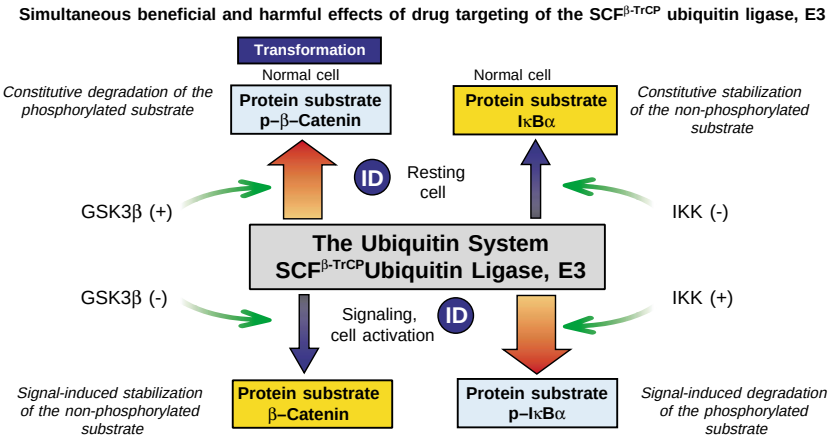

Fig. 4 Drug targeting of an E3 enzyme can become a 'double-edged sword'. In the resting cell (top), IκBα (blue arrow and beige box, upper right) is not phosphorylated and degraded slowly, whereas β-catenin is phosphorylated constitutively by GSK3β and is degraded rapidly following ubiquitination by the SCF$^{\beta\text{-TrCP}}$–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].

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

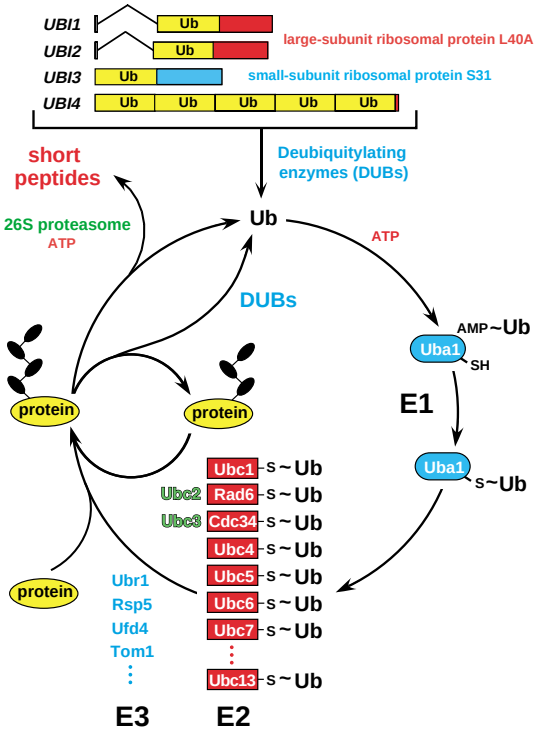

Fig. 5 The ubiquitin system of *S. cerevisiae*[51]. The yeast ubiquitin genes, two of which (*UBI1* and *UBI2*) contain introns, encode fusion proteins of ubiquitin (yellow rectangles) to itself (*UBI4*) or to one of the two specific ribosomal proteins (*UBI1–UBI3*) (red and blue rectangles). These fusion 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.

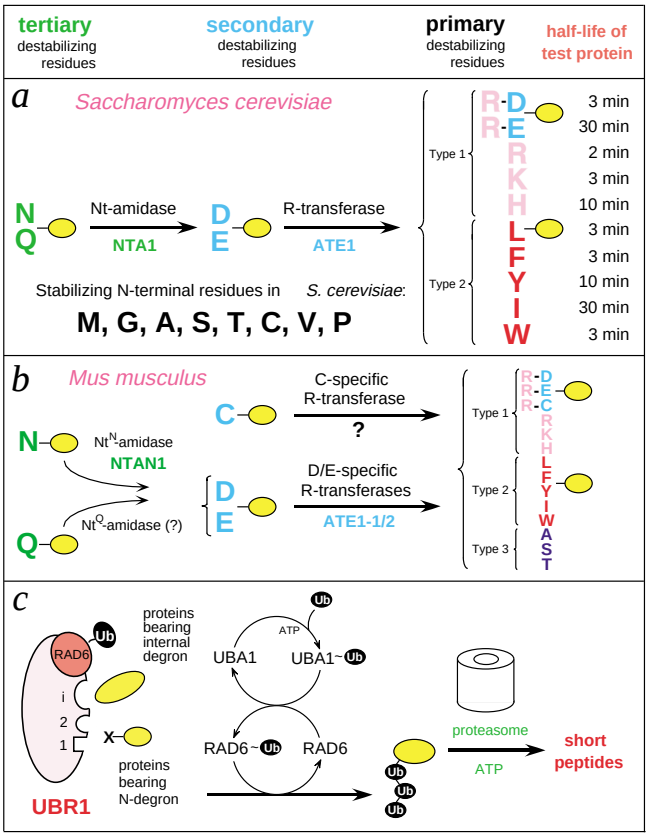

Fig. 6 The N-end rule pathway. Notations in the yeast (***a***) and mouse (***b***) pathways show type 1 (purple) and type 2 (red) primary, secondary (light blue) and tertiary (green) destabilizing N-terminal residues; yellow ovals indicate the rest of a protein substrate. ***a***, The *in vivo* half-lives 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

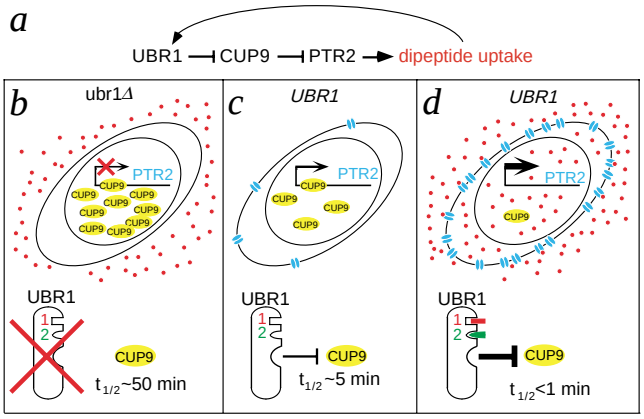

Fig. 7 Ubiquitin-dependent activation of peptide import in *S. cerevisiae*[20]. *a*, Genetic diagram of the peptide transport circuit. ***b***, UBR1 is required for di-peptide uptake. In the absence of UBR1 (*ubr1Δ*), the transcriptional repressor CUP9 is long-lived, accumulates to high levels and extinguishes 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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