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Mini Review

Tracing the history of the ubiquitin proteolytic system: The pioneering article

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ABSTRACT

A series of findings made by several researchers during a two-decade period between the mid-1950s and mid-1970s raised the suspicion that the lysosome might not be the organelle that degrades the bulk of cellular proteins under basal conditions. These findings predicted the existence of a nonlysosomal, adenosine triphosphate (ATP)-dependent proteolytic system. Yet, following the initial discovery of such activity in a crude cell extract, it was a single article published in this journal [A. Ciechanover, Y. Hod, A. Hershko, A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes, Biochem. Biophys. Res. Commun. 81 (1978) 1100–1105], my first study as a graduate student of Avram Hershko, that made it clear that the system that catalyzes the activity is novel and complex, and does not follow the paradigm in the field of proteolysis where a single protease typically cleaves its substrate; here at least two components were required to carry out this activity, and one of them was an unusual, small, and heat-stable protein later identified as ubiquitin.

Introduction

Between the 1950s and 1980s, scientists were fascinated mostly with the genetic code—how the genome is transcribed to transcriptome, which is then translated to the proteome. Although it was obvious that proteins that are synthesized must be degraded, many regarded intracellular proteolysis as an end process, and the putative mechanism that carries it out as nonspecific. As a result, the problem of intracellular proteolysis had remained a neglected research area studied by only a few. Progress in the field was minute, and new knowledge was mostly descriptive and phenomenological in nature, and not mechanistic. In retrospect, realizing the immeasurable scope of modification by ubiquitin and ubiquitin-like proteins, one can say that the development of the field shows in a most illuminating way that besides curiosity, creativity, and originality, fashionable trends play an important role in determining the timing of when scientific discoveries are made.

Breakthroughs in biomedicine are not made overnight, but rather via a series of small steps along the endless route of progress; real "eureka" moments are rare and are typically appreciated as such only in retrospect. Importantly, the definition of novelty in science is difficult at times, because the initial entry of researchers into an unexplored territory is based, in many cases, on preliminary findings of others, although many of these earlier findings are indirect and only suggestive. The unraveling of the ubiquitin proteolytic system is not an exception. Several important and pioneering findings made between the mid-1930s and mid-1970s laid the foundation of the field. Initially, it was demonstrated that the proteins of the organism are in a "dynamic state" of synthesis and degradation. Later findings demonstrated that proteolysis requires metabolic energy, which appeared paradoxical thermodynamically given that energy is invested in a process that is exergonic by nature. These findings, which were demonstrated in both prokaryotes and eukaryotes, suggested that the process is highly specific, and that different substrates are degraded distinctively under different pathophysiological conditions. The lysosome was discovered during the early 1950s, and for two decades (between the mid-1950s and mid-1970s) it was assumed that intracellular proteins are degraded within this organelle. However, several lines of experimental evidence, including the use of specific lysosomal inhibitors that did not affect degradation of intracellular proteins, had suggested that the degradation of intracellular proteins is nonlysosomal in nature, leading to the discovery of the ubiquitin system during the late 1970s and early 1980s. Could the ubiquitin system have been discovered earlier? Possibly yes. This could have happened by chance (see the cha–cha–cha [chance–challenge–charge] theory of scientific discoveries by Daniel Koshland [1]). As we now know, the system was not discovered by chance but rather by challenge—mostly as a natural response to developments in the field—to findings that could not be explained by assuming that the lysosome is the degrading organelle and to "requirements" from the putative system that could not be fulfilled by the 'lysosomal hypothesis'. A new system and concept(s) were needed to explain all of these new findings and assumptions, gathering them under a unifying umbrella.

When tracing the history of a major discovery, it is at times possible to identify one single article that describes the essentials of

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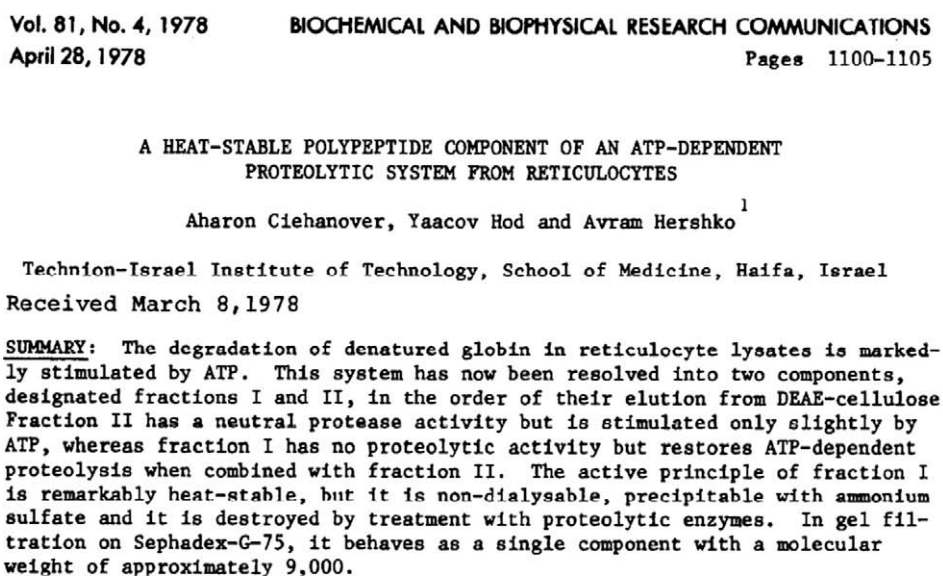
the entire discovery. A wonderful example is that of the structure of the double helix of DNA [2]. In most instances this is not the case, but it is nevertheless possible to point to a single article, or a handful of articles, that laid the foundation to the discovery. This is the case with the ubiquitin system, where the pioneering findings were described in a single article published in this journal [3] (Fig. 1). The core finding indicated that proteolysis is not carried out by a single protease, as had been the case for known proteases at the time, but rather by a complex multicomponent machinery. This paved the road for additional critical discoveries [4,5] that followed shortly thereafter, including that of the adenosine triphosphate (ATP)-dependent proteolysis factor 1 (APF-1)/ubiquitin tagging mechanism, which unraveled the basic principles and mode of action of the system, allowed prediction of its various components, and enabled portrayal of a model of nonlysosomal ATP-dependent proteolysis—a model that has withstood the test of time. The vast majority of further developments in the field were based on the findings and predictions described in these three articles [3–5]. As noted, in the first article, we demonstrated that proteolysis is not catalyzed by a single protease, as was the paradigm with most proteases that had been studied until that time; rather, degradation required two complementing fractions resolved from the crude extract: one containing the "protease" and one containing a small heat-stable protein—later identified as ubiquitin—that stimulated proteolysis [3]. Because the two fractions were crude, we assumed that they could have contained more complementing factors, as was indeed shown later. Studying the mechanism of action of the small protein subsequently led to the discovery of covalent conjugation of the substrate by ubiquitin [4,5] as a marking step that precedes proteolysis. The tagging, which has become the hallmark of the system, could explain two major and related requirements/assumptions. The first is the high specificity and selectivity of the system toward its numerous substrates. Proteolysis—the act of scission—appears to be non-specific and irreversible, yet the marking must be specific and reversible. The discovery of this preceding step made researchers turn their attention to the question of why certain proteins are tagged at a certain time and under certain pathophysiological conditions and how specific tagging occurs. The second major requirement/assumption is the ability of the protease(s) and its substrates to live "peacefully" in the same compartment given that the protease(s) should destroy only ubiquitin-tagged proteins but not unmarked substrates. In principle, substrates and proteases need to be "fenced" from one another to prevent untoward destruction. Ubiquitin conjugation serves, among other functions, to overcome a "fence" of recognition between the protease and its substrate. During the preceding period, when the lysosomal hypothesis prevailed, the separating/protecting fence between the two was the lysosomal membrane. Yet, this fence could not explain the specificity of the system given that substrates gained access to the lysosomal lumen via macro- or microautophagy of invaginated vesicles, and all proteins contained in these vesicles, which are basically all of the cytosolic proteins, should have been destroyed at the same rate. This was clearly not the case, as different proteins have different half lives, and even the stability of a single protein can very under different conditions. The ubiquitin tagging mechanism not only provided a new type of fence in the form of a posttranslational modification as a prerequirement for recognition by the protease, but also offered, at the same time, an explanation for the high specificity of the proteolytic process, namely that proteins are ubiquitinated in a regulated manner.

At that time, the only protein that was known to be modified by ubiquitin was histone H2A (uH2A, protein A24). Yet, unlike the modification for proteolysis that required the generation of a polyubiquitin chain where one moiety of ubiquitin is attached to the other, the histone molecule was modified only once [6] and the modification did not serve to destabilize it. In addition, the mechanism of modification and the enzymes that catalyze it were not known at the time. However, the structure of the histone adduct was known; it was a bifurcated protein where the C-terminal residue of ubiquitin, Gly76, was linked in an isopeptide bond to the ε-NH_2 group of internal Lys119 of the histone molecule. Thus, although conjugation of ubiquitin as a proteolysis signal presented a new paradigm and obviously a novel mechanism, the knowledge of the chemical structure of an already known ubiquitin conjugate paved the road to the unraveling of the mechanism of conjugation, isolation of the enzymes involved, and understanding of the base for energy requirement for the apparently exergonic process of intracellular proteolysis. For a detailed history of the field of proteolysis and the ubiquitin system, the reader is referred to numerous review articles written on the subject (see, e.g., Refs. [7–14]). Here, I mostly describe the early history of the field and the contribution of the 1978 *Biochemical and Biophysical Research Communications* (BBRC) article [3] to its development. According to my view, this was the first article in the history of the



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A HEAT-STABLE POLYPEPTIDE COMPONENT OF AN ATP-DEPENDENT PROTEOLYTIC SYSTEM FROM RETICULOCYTES

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SUMMARY: The degradation of denatured globin in reticulocyte lysates is markedly stimulated by ATP. This system has now been resolved into two components, designated fractions I and II, in the order of their elution from DEAE-cellulose. Fraction II has a neutral protease activity but is stimulated only slightly by ATP, whereas fraction I has no proteolytic activity but restores ATP-dependent proteolysis when combined with fraction II. The active principle of fraction I is remarkably heat-stable, but it is non-dialysable, precipitable with ammonium sulfate and it is destroyed by treatment with proteolytic enzymes. In gel filtration on Sephadex-G-75, it behaves as a single component with a molecular weight of approximately 9,000.

Fig. 1. Title, abstract, and publication details of the BBRC article [3] that is the subject of the current article.

ubiquitin system as we currently know it, and it clearly served as the starting point of the exciting road to its discovery.

The dynamic state of the body proteins

The idea that the organism's proteins are in a dynamic state of synthesis and degradation is hardly 70 years old. Beforehand, proteins were viewed as stable components that were subjected only to minor "wear and tear"; dietary proteins were believed to function primarily as energy-providing fuel, and there was no metabolic linkage between them and the structural and functional proteins of the body. This concept was challenged by Rudolf Schoenheimer at Columbia University. Schoenheimer had escaped racial Germany and joined the Department of Biochemistry at Columbia headed by Hans T. Clarke [15–17]. There Schoenheimer met Harold Urey, who discovered deuterium. David Rittenberg, who recently received his PhD in Urey's laboratory, and Sarah Ratner who graduated with Clarke and joined Schoenheimer, and together they entertained the idea of "employing a stable isotope as a label in organic compounds, destined for experiments in intermediary metabolism, which should be biochemically indistinguishable from their natural analogues" [15]. Urey succeeded in enriching nitrogen with ^{15}N, which provided Schoenheimer, Rittenberg, and Ratner with a tag for the amino acids that they used for their studies on protein dynamics. They discovered that following administration of ^{15}N-labeled tyrosine to rat, only approximately 50% was recovered in the urine, whereas "most of the remainder is deposited in tissue proteins. An equivalent of protein nitrogen is excreted" [18]. In contrast to the prevailing dogma at that time, this and other experiments demonstrated unequivocally that (i) the body structural and functional proteins are in a dynamic state of synthesis and degradation, and (ii) there is a cross-talk between ingested dietary proteins and the body's own proteins. Schoenheimer was invited to deliver the prestigious Edward K. Dunham Lecture at Harvard University in 1941, where he presented his revolutionary findings. After his untimely tragic death shortly after, his lecture notes were edited by Clarke and his associates Rittenberg and Ratner, and were published in a small book by the Harvard University Press. The editors called the book *The Dynamic State of Body Constituents* [19], adopting the title of Schoenheimer's presentation at the Harvard University lecture. In the book, the new hypothesis was clearly presented: "The simile of the combustion engine pictured the steady state flow of fuel into a fixed system, and the conversion of this fuel into waste products. The new results imply that not only the fuel, but the structural materials are in a steady state of flux. The classical picture must thus be replaced by one which takes account of the dynamic state of body structure" [19]. The idea that proteins are turning over was not accepted easily and was challenged as late as the mid-1950s. For example, Hogness and colleagues studied the kinetics of β-galactosidase in *Escherichia coli* [20] and summarized their findings: "To sum up: there seems to be no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover, our experiments have shown that the proteins of growing *E. coli* are static. Therefore it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a 'dynamic state'" [20]. The authors used the term "dynamic state" twice in a single paragraph, and this was not incidental given that they challenged Schoenheimer's hypothesis directly.

The lysosome and intracellular protein degradation

During the mid-1950s, Christian de Duve discovered the lysosome (see, e.g., Refs. [21,22]). The lysosome was recognized first in the rat liver as a membrane-surrounded vacuolar structure that contains various hydrolytic enzymes that function optimally at an acidic pH. The definition of the lysosome has been broadened ever since its discovery because it has been recognized that lysosomal digestion of proteins from within the cell (autophagy) or from the extracellular milieu (heterophagy) is a dynamic and complex process that involves numerous stages required for the organelle's formation and maturation occurring continuously before, during, and after digestion of the lysosomal targets.

The discovery of the lysosome, along with additional experiments that further strengthened the notion that cellular proteins are indeed in a constant state of synthesis and degradation, led scientists to assume that they had at hand an organelle that can mediate degradation of intracellular proteins. Although it was clear almost from its discovery that the lysosome is involved in degradation of extracellular proteins (targeted via receptor-mediated endocytosis, pinocytosis, and phagocytosis), its role in degradation of intracellular proteins had remained elusive for a long period afterward. The finding that the proteases were separated from their substrates by a membrane provided an explanation for controlled—but not specific—degradation. An important problem that had remained unsolved at the time was how the substrates are translocated into the lysosomal lumen, where they become exposed to the activity of the lysosomal proteases and degraded. The discovery of autophagy helped to partially resolve this problem (reviewed in Refs. [23–26]). In microautophagy, portions of the cytoplasm are transferred into the lysosome by direct invagination of the lysosomal membrane and subsequent budding of vesicles into the lysosomal lumen. These vesicles, which contain the entire repertoire of the cytosolic proteins, are then digested by the lysosomal hydrolases. Under more extreme conditions (e.g., starvation), mitochondria, endoplasmic reticulum membranes, and other cytoplasmic entities can be engulfed by a process called macroautophagy.

However, over a period of more than two decades, between the mid-1950s and the late 1970s, it became apparent that the lysosome cannot be involved in the degradation of at least certain classes of cellular proteins. First was the observation that proteins vary in their stabilities: measurement of half-lives of different proteins showed that they span three orders of magnitude, from a few minutes to many days. Thus, whereas the $t_{1/2}$ of ornithine decarboxylase (ODC) is 12 min, that of glucose-6-phosphate dehydrogenase (G6PD) is 15 h (for review articles, see, e.g., Refs. [27,28]). Also, rates of degradation of several proteins were shown to vary with changing physiological conditions such as availability of nutrients or hormones. It was difficult to reconcile the findings of distinct and changing half-lives of different proteins with the mechanism of action of the lysosome, where the microautophagic vesicle engulfs and contains the entire repertoire of cytosolic proteins that, therefore, are expected to be destroyed at the same rate.

Another source of concern about the lysosome as the organelle in which intracellular proteins are degraded was the finding that specific and general inhibitors of lysosomal proteases had different effects on the fate of different groups of proteins, suggesting that different proteolytic machineries act in the cell [29,30]. An elegant experiment in this respect was carried out by Brian Poole and his colleagues at Rockefeller University [31,32]. Poole was studying the effect of acid ionophores (that inactivate the lysosomal membrane H^+ pump), and of weak bases (lysosomotropic agents such as ammonium chloride and chloroquine that accumulate in the lysosomal lumen and titrate the H^+ ions), that independently dissipate the low intralysosomal acidic pH, thus inactivating its proteases. It was assumed that this mechanism also underlies the anti-malarial activity of chloroquine and similar drugs, where they inhibit the activity of the lysosome of the parasite, "paralyzing" its ability to digest the host's hemoglobin during the intraerythrocytic stage of its life cycle [31]. Poole and colleagues metabolically

labeled intracellular proteins in macrophages using ^{3}H[leucine] and "fed" the labeled macrophages with dead macrophages/debris of macrophages that had been previously labeled with [^{14}C]leucine. They assumed correctly that the debris and proteins of the dead macrophages will be phagocytosed/pinocytosed by the live cells and targeted to the lysosome for degradation, while at the same time, they will be able to study the question of whether the lysosome also degrades the metabolically labeled intracellular proteins. The researchers monitored the effect of the lysosomotropic agents on the degradation of the two protein populations and found that the drugs specifically inhibited the degradation of extracellular proteins, but not that of intracellular proteins. Poole and colleagues summarized these experiments and explicitly predicted—in an elegant and poetic way—the existence of an as yet unknown nonlysosomal proteolytic system that degrades intracellular proteins: "Some of the macrophages labeled with tritium were permitted to endocytize the dead macrophages labeled with ^{14}C. The cells were then washed and replaced in fresh medium. In this way we were able to measure in the same cells the digestion of macrophage proteins from two sources. The exogenous proteins will be broken down in the lysosomes, while the endogenous proteins will be broken down wherever it is that endogenous proteins are broken down during protein turnover."

Another concern, albeit a weaker one, about the lysosomal hypothesis was the thermodynamically paradoxical requirement for metabolic energy that was observed for the degradation of both eukaryotic [33,34] and prokaryotic [35] proteins. Proteolysis per se is an exergonic process, and the energy requirement for intracellular degradation made researchers believe that energy cannot be consumed directly by the proteases and is used indirectly. As Melvin Simpson summarized his findings, "The data can also be interpreted by postulating that the release of amino acids from protein is itself directly dependent on energy supply. A somewhat similar hypothesis, based on studies on autolysis in tissue minces, has been advanced recently, but the supporting data are very difficult to interpret. However, the fact that protein hydrolysis as catalyzed by the familiar proteases and peptidases occurs exergonically, together with the consideration that autolysis in excised organs or tissue minces continues for weeks long after phosphorylation or oxidation ceased, renders improbable the hypothesis of the direct energy dependence of the reactions leading to protein breakdown" [33]. Thus, the puzzling finding of energy requirement was left with vague explanations, for example, that there is a linkage between protein degradation and protein synthesis (where energy is required): "The fact that a supply of energy seems to be necessary for both the incorporation and the release of amino acids from protein might well mean that the two processes are interrelated. Additional data suggestive of such a view are available from other types of experiments. Early investigations on nitrogen balance by Benedict, Folin, Gamble, Smith, and others point to the fact that the rate of protein catabolism varies with the dietary protein level. Since the protein level of the diet would be expected to exert a direct influence on synthesis rather than breakdown, the altered catabolic rate could well be caused by a change in the rate of synthesis" [33]. The observation by Hershko and Tomkins—nearly 20 years after Simpson's discovery of the energy requirement for degradation of intracellular proteins—that the activity of tyrosine aminotransferase (TAT) was stabilized following depletion of ATP [36] corroborated these earlier findings and suggested that energy may be required at an early stage of the proteolytic process, probably before the proteolytic scission occurs. Yet it did not provide a clue as to the mechanism and classes of proteins involved. Energy could still be used for a general lysosomal mechanism, for example, one that involves transport of TAT into the lysosome. However, energy could also be required for degradation via a nonlysosomal mechanism, for example, for phosphorylation of TAT that would sensitize it to degradation by an as yet unknown protease, or for a modification that activates the putative protease. Later, and based on the known mechanisms of action of the lysosome, it was shown that energy is required for the transport of substrates into the lysosome [37] and for the maintenance of the low intralysosomal pH [38], thereby providing possible explanations for this enigma where energy is used indirectly. It should be noted that energy inhibitors almost completely abolished degradation of all cellular proteins, suggesting more than one role for ATP in protein catabolism that affects all mechanisms and classes of proteins involved—known as well as unknown. Therefore, the usefulness of these inhibitors as experimental tools for the discovery of the nonlysosomal system(s) was limited. In bacteria, that lack lysosomes, an argument involving an energy requirement for lysosomal degradation could not have been proposed, but other more direct effects of ATP hydrolysis could have affected proteolysis, including phosphorylation of the substrates and/or the proteolytic enzymes, and activation of the protease(s). In any event, the requirement of metabolic energy for protein degradation in both prokaryotes and eukaryotes, a process that is thermodynamically exergonic, strongly indicated that proteolysis is highly regulated and specific in cells, and that a similar principle/mechanism has been preserved throughout evolution. Inferring from the possible more direct requirement for ATP in the degradation of proteins in bacteria, it was not too unlikely to assume a similar mechanism in the degradation of cellular proteins in eukaryotes.

It should be noted, however, that the assumption that the degradation of intracellular proteins is mediated by the lysosome was nevertheless logical. Proteolysis results from direct interaction between target substrates and proteases; therefore, it was clear that active proteases cannot reside freely in the cytosol, which would have resulted in destruction of the cell. Thus, it was accepted that any proteolytic machinery that mediates degradation of intracellular proteins must also be endowed with a mechanism that separates—physically or chemically—between the proteases and their substrates, and enables them to interact only when needed: the lysosomal membrane provided the separating "fence". However, to explain the shortcomings of the lysosomal hypothesis (e.g., different stabilities of different substrates, differential effects of lysosomal inhibitors, energy requirement), researchers provided different explanations. According to one model, it was proposed that different proteins have different sensitivities to lysosomal proteases, and that their half-lives *in vivo* correlate with their sensitivities to the action of lysosomal proteases *in vitro* [39]. To explain an extremely long half-life for a protein that is nevertheless sensitive to lysosomal proteases, or alterations in the stability of a single protein under various physiological states, it was suggested that although all cellular proteins are engulfed in the lysosome, only the short-lived proteins are degraded, whereas the long-lived proteins can exit back into the cytosol: "To account for differences in half-life among cell components or of a single component in various physiological states, it was necessary to include in the model the possibility of an exit of native components back to the extralysosomal compartment" [40]. According to a different model, the selectivity is determined by the binding affinities of the different proteins to the lysosomal membrane that control their entry rates into the lysosome, and subsequently their degradation rates [41]. More recent studies have shown that, at least for stress-induced macroautophagy, a general sequence of amino acids, KFFERQ, directs via binding to a specific "receptor," and along with cytosolic and lysosomal chaperones, the regulated entry of many cytosolic proteins into the lysosomal lumen. This hypothesis can explain the mass entry into the lysosome of a large population of proteins that were found to contain a homologous sequence, but not the targeting for degradation of a specific protein under defined conditions [42,43]. It should be emphasized that neither of these

hypotheses had been substantiated to a point where it could explain, alone or in some combination, the weaknesses in the lysosomal hypothesis. In the absence of any alternative, and with lysosomal degradation as the most logical explanation for targeting all known classes of proteins at the time, de Duve summarized his view on the subject in a review article published during the mid-1960s: "Just as extracellular digestion is successfully carried out by the concerted action of (lysosomal; AC) enzymes with limited individual capacities, so we believe is intracellular digestion" [44]. The problem of different sensitivities of distinct protein groups to lysosomal inhibitors, as well as the accumulating data on different and changing stabilities of distinct proteins, had remained unsolved and may have triggered the search for a nonlysosomal proteolytic system.

Progress in identifying the elusive nonlysosomal proteolytic system(s) was hampered by the lack of cell-free preparations that could faithfully reproduce the cellular proteolytic events—degrading proteins in a specific and energy-requiring mode. An important breakthrough was made by Rabinovitz and Fisher who found that rabbit reticulocytes degrade abnormal amino acid analog-containing hemoglobin [45]. Their experiments modeled hemoglobinopathies, a group of diseases caused by mutated hemoglobins that, unlike the wild-type hemoglobin (their normal counterpart), are rapidly degraded in the reticulocyte. Thus, the abnormal mutated hemoglobin chains in sickle cell anemia, or the excess of unassembled normal hemoglobin chains in certain types of thalassemias where the pairing chain is not synthesized, are rapidly degraded in the reticulocyte [46,47]. Reticulocytes are terminally differentiating red blood cells that no longer contain lysosomes: these organelles were expelled during the maturation process. Therefore, it was postulated that the degradation of hemoglobin in these cells is mediated by a nonlysosomal machinery. Etlinger and Goldberg [48] were the first to isolate and characterize a cell-free proteolytic preparation from reticulocytes. The crude extract selectively degraded abnormal hemoglobin in an ATP hydrolysis-dependent manner and acted optimally at a neutral pH. A similar system was isolated and characterized later by our own group during the initial phase of my graduate studies with Avram Hershko [49] (Fig. 2). Additional efforts by our group subsequently led to the resolution, characterization, and purification of the major enzymatic components from this extract, and to the discovery of the ubiquitin tagging mechanism and the ubiquitin proteolytic system.

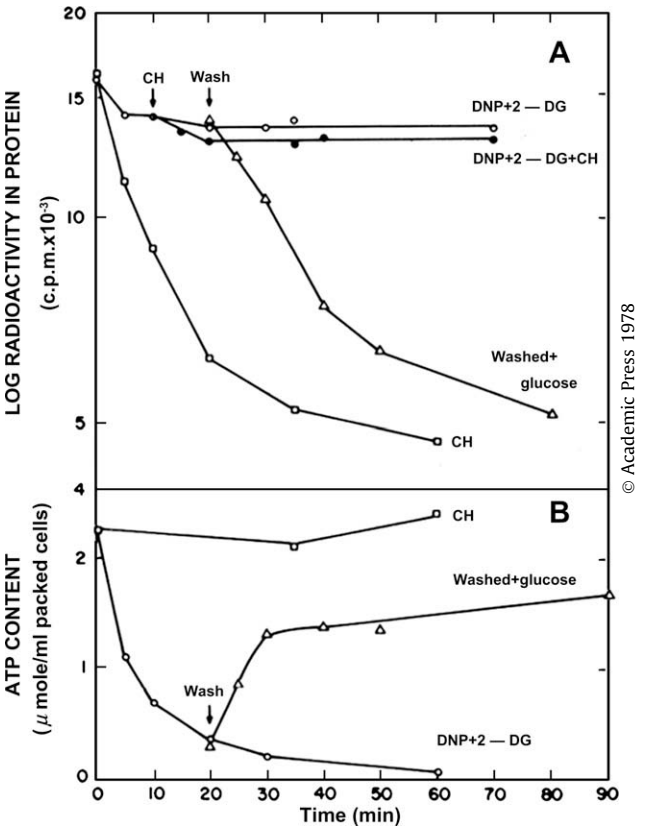

Fig. 2. Energy-dependent degradation of abnormal, amino acid analog-containing proteins in intact reticulocytes. Rabbit reticulocytes were preincubated with the amino acid analog 4-thialysine, followed by the addition of [^{3}H]leucine. At time 0, the labeled amino acid was washed. ○, ATP was trapped by the addition of 2-deoxyglucose (2-DG) and 2,4-dinitrophenol (DNP); ●, ATP was trapped as above and protein synthesis was inhibited by the addition of cycloheximide (indicated by an arrow marked CH); △, DNP and 2-DG were added initially, but were washed (indicated by an arrow marked Wash) and replaced with a complete medium containing glucose and CH; □, CH was added at time 0. (A) Radioactivity in cell proteins. (B) Cellular ATP content (source: Ref. [49]).

The ubiquitin–proteasome system

The reticulocyte extract [48,49] turned out to be a rich source for the initial identification and subsequent purification and characterization of the ubiquitin–proteasome system enzymes. With the initial hypothesis that a single ATP-dependent protease may catalyze the reaction, we thought that the first step toward its isolation should be removal of hemoglobin, the main (>80%) protein in the reticulocyte extract. This could be achieved via fractionation of the lysate on the anion exchange resin diethylaminoethyl cellulose (DEAE), where hemoglobin does not adsorb to the resin at neutral pH. Fractionation of the crude lysate on this resin brought with it the first surprising result [3] (Fig. 3); as expected, the breakthrough fraction (designated fraction I) that contained mostly hemoglobin, did not have any proteolytic activity. Yet, the high-salt eluate (eluted with 0.5 M KCl) that apparently contained all of the other remaining proteins (designated fraction II) had also only residual activity; the "lost" proteolytic activity could be reconstituted and recovered upon the addition of the two fractions (Fig. 3). This finding was important and served as a lesson for the future dissection of the system and isolation of its components. First, it suggested that the system is not composed of a single "classical" protease that had evolved evolutionarily to acquire energy

TABLE 1: Resolution of the ATP-Dependent Cell-Free Proteolytic System Into Complementing Activities

Enzyme fraction	Degradation of [^{3}H]globin percent/h	
	-ATP	+ATP
lysate	1.5	10.0
fraction I	0	0
fraction II	1.5	2.7
fraction I and fraction II	1.6	10.6

Fig. 3. Anion exchange chromatography of rabbit reticulocyte lysate and resolution of the ATP-dependent proteolytic activity into two complementing activities (the original table as appeared in Ref. [3]).

dependence (although later, energy-dependent proteases such as the mammalian 26S proteasome [see below] and the prokaryotic *Lon* gene product, have been described). Rather, it is made of at least two components—and probably more—because fractions I and II were crude extracts that together represented the entire protein population of the lysate. Second, learning from this reconstitution experiment and the essential dependence between the two active fractions, we reconstituted activity from resolved fractions

whenever it was lost during further purification steps. This biochemical "complementation" approach led to sequential discovery of additional enzymes/components of the system that were all required to be present in the reaction mixture to catalyze proteolysis of the tested protein substrate. At the end of an effort of several years, the essential components of the system were identified, enabling us to propose the two-step proteolytic cascade model—marking of the substrate by ubiquitin followed by its subsequent degradation by a downstream protease that recognizes only ubiquitin-marked substrates. Free and reusable ubiquitin is released during the process. It should be noted however, that the initial observation of the two complementing fractions (I and II) left us with no paradigm to follow because it did not fit the two-component paradigm—the requirement for only two components – a protease and a substrate—that are needed for the proteolytic "tango". In an attempt to explain the requirement for two complementing factors, we hypothesized that the two fractions could contain, for example, an inhibited protease and its activator, but other explanations were considered as well.

With the two fractions at hand, we initiated our efforts to identify, purify, and characterize the components of the system. We decided to isolate first the active component from fraction I. It was found to be a small, approximately 8.5-kDa heat-stable protein that we designated APF-1. The reason was that at the same time we started to isolate other complementing components and decided to number them sequentially. APF-1 was later identified as ubiquitin (the term APF-1 is used in this review up to the point where its identification as ubiquitin is described, where the terminology is changed accordingly). The decision to start the purification efforts with fraction I was judicious because we suspected that it contains mostly hemoglobin and only one component that was necessary to stimulate fraction II-mediated proteolysis of labeled globin, the substrate we studied. In contrast, we suspected that fraction II contains more than one component, and that turned out to be true. If we would have initiated our purification efforts with fraction II, the road would have been significantly bumpier and the journey would have been slower. Admittedly, we were also lucky using globin, the model substrate we studied at the time; later studies showed that fraction I contains additional components—beyond APF-1/ubiquitin—necessary for the degradation of other substrates. Thus, if we had used one of these substrates, it would have slowed us down significantly even with our efforts to characterize the active component contained in fraction I. The fact that for reconstitution of the degradation of globin (and later of lysozyme and bovine serum albumin) the only component required from fraction I was APF-1, and that APF-1 turned out to be small and heat-stable, made our purification efforts and the deciphering of its mechanism of action relatively easy. All in all, starting this voyage with APF-1 was critically important to our later success, and was the result of a combination of understanding the biochemistry of the anion exchange chromatography, but also of serendipity and luck. Thus, (i) the establishment of the cell-free, energy-requiring proteolytic system, (ii) the resolution of the crude extract into two complementing fractions (I and II), (iii) the decision to initiate the purification and characterization efforts with fraction I, and (iv) the initial characterization of APF-1 as a small heat-stable protein, all were described in a short BBRC article published in 1978 [3]. This publication, according to my view, was the first in the history of the ubiquitin system. More important, however, it laid the cornerstone to further fundamental discoveries because it unraveled one of the most important characteristics of the system—being multicomponent and, therefore, probably multistep. This initial study paved the road—methodologically and conceptually—for the future isolation and characterization of the other components of this cascade-structured, highly regulated, and critically important proteolytic system.

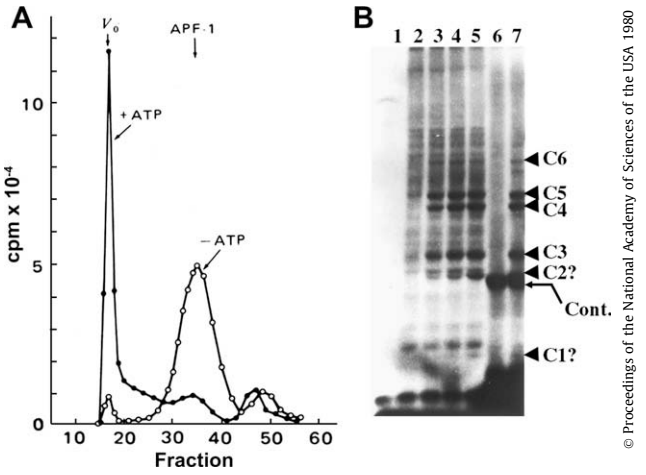

Fig. 4. ATP-dependent conjugation of APF-1 (later identified as ubiquitin) to target proteolytic substrates. (A) ATP-dependent shift of APF-1 to high-molecular-mass "compound" following incubation with fraction II. ^{125}I-labeled APF-1 was incubated in the absence or presence of ATP along with crude fraction II, and the reaction mixtures were resolved via gel filtration chromatography. Shown are the radioactive counts in the resolved fractions. (source: Ref. [4]). (B) ATP-dependent covalent conjugation of APF-1 to proteolytic substrates. Labeled APF-1 was incubated in the presence of crude fraction II in the absence (lane 1) or presence (lanes 2–5) of ATP, and in the absence (lane 2) or presence (lanes 3–5) of increasing concentrations of unlabeled lysozyme. In a similar experiment, unlabeled APF-1 was incubated in the presence of ^{125}I-labeled lysozyme and fraction II in the absence (lane 6) or presence (lane 7) of ATP. Reaction mixtures were resolved via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were visualized via autoradiography. Marked by C1–C6 are specific adducts between APF-1 and lysozyme (source: Ref. [5]).

The finding that APF-1 is heat stable allowed its rapid purification [50] and dissection of its mechanism of action. We found that multiple molecules of APF-1 are covalently linked to the proteolytic substrate in an ATP-dependent reaction catalyzed by enzymes contained in fraction II [4,5] (Fig. 4), and hypothesized that this modification renders the substrate susceptible to degradation by a downstream protease. We also found that free and reusable APF-1 is recycled either directly from the substrate before it is degraded, or at the end of the process, following degradation. The removal of APF-1 by recycling enzymes prior to degradation was suggested to serve a "correction" function where substrates were conjugated mistakenly, or to rescue substrates that were refolded or otherwise regained their normal structure and function [5].

Based on all of these findings, in 1980 we proposed a model (Fig. 5) according to which protein substrate modification by multiple moieties of APF-1 targets it for degradation by a downstream, as-yet-unidentified (at that time) protease that does not recognize the unmodified substrate; following degradation, reusable APF-1 is released [5]. The proposed model has withstood the test of time, and now—nearly three decades later—it still appears to correctly represent the basic steps involved in ubiquitin-mediated proteolysis.

Amino acid analysis of APF-1 along with its known molecular mass and other general characteristics raised the suspicion that APF-1 is ubiquitin [50], a known protein of previously unknown function. Indeed, Wilkinson and his colleagues Urban and Hass confirmed unequivocally that APF-1 is indeed ubiquitin [51]. Ubiquitin, a small 76-residue polypeptide, was first purified during the isolation of thymopoietin [52] and was subsequently described as ubiquitously expressed in all kingdoms of living cells—prokaryotes and eukaryotes alike [53]. Interestingly, it was initially described as a lymphocyte-differentiating factor that acted via stimulation of adenylate cyclase [53,54]. Accordingly, it was named UBIP (ubiquitous immunopoietic polypeptide) [53]. However, later studies showed that ubiquitin is not involved in the immune response

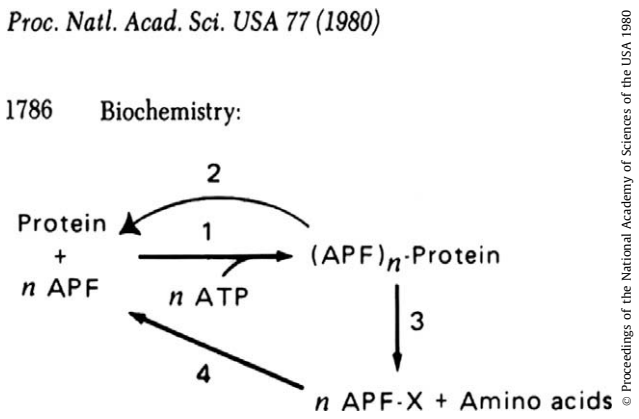

Fig. 5. The APF-1 (ubiquitin) system proteolytic cycle as envisioned in 1980 [5]. (1) ATP-dependent conjugation of multiple moieties of APF-1 to the target substrate. This step was suggested to be catalyzed by APF-1–protein amide synthetase, and subsequent studies demonstrated that it is catalyzed by three enzymes that act in concert: E1, the ubiquitin-activating enzyme; E2, a ubiquitin–carrier protein (designated later ubiquitin-conjugating enzyme); and E3, a substrate-specific ubiquitin–protein ligase. (2) Removal of ubiquitin from the intact substrate. This step was originally suggested to be catalyzed by an amidase or a "correction enzyme," and it was later discovered to be catalyzed by ubiquitin-specific proteases (USPs) or deubiquitinating enzymes (DUBs). (3) Degradation of the APF-1-conjugated substrate to amino acids with release of APF-1 bound to short peptides/degradation products. This step was originally suggested to be catalyzed by peptidases, and it was later discovered to be catalyzed by the 26S proteasome complex. It is now known that the proteasome releases short peptides that are further degraded to amino acids by downstream amino- and carboxypeptidases. It is not clear whether the most proximal ubiquitin moiety (ies) in the chain is released bound to short peptides, because such products have not been isolated. These moieties can also be degraded along with their conjugated substrate. It is clear, however, that the more distal moieties are released as free and reusable molecules (see step 4). (4) Recycling of APF-1 from the short peptides/degradation products. It is now known that ubiquitin is recycled via the activity of USPs/DUBs, some of which are 26S proteasome-associated and some of which are free (see step 3). (source: Ref. [5]).

[55] and that it was a contaminating endotoxin in the preparation that was responsible for the adenylate cyclase and T-cell differentiating activities. Furthermore, the sequence of several eubacteria and archaebacteria genomes, as well as biochemical analyses in these organisms (unpublished), showed that ubiquitin is restricted only to eukaryotes. The finding of ubiquitin in bacteria [53] was probably due to contamination of the bacterial extract with yeast ubiquitin derived from the yeast extract in which the bacteria were grown. Although in retrospect the name ubiquitin is a misnomer given that it is not ubiquitous and is restricted to eukaryotes, it has remained the accepted name of the protein. It might be a good idea that the names of other novel enzymes and components of the ubiquitin system, as well as of other systems, should remain as initially coined by their discoverers.

But how was the idea that APF-1 and ubiquitin are at all related conceived? It is the convergence of two independent fields—histone modification and intracellular proteolysis—that led to it. During the mid-1970s, it was found that a single ubiquitin moiety is covalently conjugated to a small fraction of nucleosomal histone H2A and H2B. Although the function of these adducts has remained elusive until the early 2000s, their structure was already unraveled at that time. The structure of the ubiquitin conjugate of H2A (uH2A, also designated protein A24) was deciphered by Goldknopf and Busch [56,57] and by Hunt and Dayhoff [58], who found that the two proteins are linked through a fork-like, branched isopeptide bond between the carboxy-terminal glycine of ubiquitin (Gly76) and the ε-NH_2 group of an internal lysine (Lys119) of the histone molecule. Based on size similarities between APF-1 and ubiquitin, and on the covalent bond generated between APF-1 and the substrate that had chemical characteristics of a peptide bond (and, therefore, was similar to the already known peptide bond between ubiquitin and H2A), researchers at the Fox Chase Cancer Center in Philadelphia, (where Hershko and I worked at the time and collaborated with Irwin A. Rose; these researchers included Drs. Leonard Cohen, Martin Nemer, and Alfred Zweidler), suggested that APF-1 may be similar or identical to ubiquitin. It should be noted that there were important differences between the cases of proteolysis and histone modification; for degradation, multiple moieties of APF-1 were required, whereas the histone molecules were modified only once. Indeed, the modification did not appear to affect the histone's stability. Our initial characterization suggested that APF-1 is probably ubiquitin [50], which was subsequently shown unequivocally by Wilkinson and his colleagues [51]. Interestingly, the elucidation of the role of monoubiquitination of histones needed to wait additional two decades; only recently was it shown to be part of the epigenetic control mechanisms where transcription is regulated via different modifications of nucleosomal histones (for a review article, see Ref. [59]).

The identification of APF-1 as ubiquitin, and the discovery that a high-energy isopeptide bond, similar to the one that links ubiquitin to histone H2A, also links it to the target proteolytic substrate, resolved the enigma of energy requirement for intracellular proteolysis and paved the road to the untangling of the complex mechanism of ubiquitin activation and later isopeptide bond formation [60–62]. Using this newly discovered mechanism along with immobilized ubiquitin as covalent affinity bait, the three enzymes that are involved in the cascade reaction of ubiquitin conjugation were purified: (i) E1, the ubiquitin-activating enzyme; (ii) E2, the ubiquitin–carrier protein; and (iii) E3, the ubiquitin–protein ligase [61,62]. Interestingly, the process turned out to be similar to that of amino acid activation for the "canonical" ribosomal (catalysed by aminocyl-tRNA synthetase and mediated by tRNA) or nonribosomal peptide bond formation [63]. The discovery of an E3 that appeared to be a specific substrate-binding component, suggested a possible solution to the enigma of the high selectivity of the system and the varying stabilities of different protein substrates; it was hypothesized that they are recognized and targeted by distinct ligases.

During a short period, the ubiquitin tagging hypothesis received substantial support, most importantly from experiments conducted in intact nucleated cells, as the initial studies were carried out in a cell-free reconstituted system isolated from reticulocytes [64–68]. One shortcoming of these experiments was that, cell-free systems do not always faithfully reproduce processes occurring in the intact cell. Also, because the reticulocyte is a terminally differentiating cell that is involved extensively in degradation of most of its systems prior to maturation, the hypothesis that the newly discovered mechanism is unique to this cell and not universal was rather attractive.

At this point, the only missing link was the identification of the downstream protease, which according to the model was assumed to recognize specifically ubiquitinated substrates. Tanaka and colleagues found that ATP has two distinct roles in the degradation of proteins in the reticulocyte extract [69], whereas Hershko and colleagues showed that ATP is required not only for conjugation of ubiquitin but also for conjugate degradation [70]. Hough and colleagues partially purified and characterized a high molecular mass alkaline protease that degraded—in an ATP-dependent mode—ubiquitin adducts of lysozyme, but not untagged lysozyme [71]. This protease, which was later called the 26S proteasome, fulfilled all of the criteria for being the specific proteolytic arm of the ubiquitin system. The protease was further characterized by Waxman and colleagues, who found that it is an unusually large enzyme (~2.5 mDa) [72]. An important advance in the field was the discov-

ery that a smaller neutral multisubunit 20S protease complex, which was purified along with the larger 26S complex and was suggested to be part of it [73], is similar to a "multicatalytic proteinase complex" (MCP) that was described earlier in the bovine pituitary gland by Wilk and Orlowski [74]. This 20S protease is ATP-independent, and has several distinct catalytic activities, cleaving on the carboxy-terminal side of hydrophobic, basic, and acidic residues. Later direct studies suggested that the 20S complex is the core catalytic particle of the larger 26S complex [75,76]. However, strong and direct evidence that the active mushroom-shaped 26S protease is generated through ATP-dependent assembly of two distinct subcomplexes—the catalytic 20S cylinder-like MCP and an additional 19S ball-shaped subcomplex (which was predicted to have a regulatory role)—was provided only during the early 1990s by Hoffman and colleagues [77], who mixed the two purified particles and generated the active 26S enzyme.

Concluding remarks

The evolvement of proteolysis as a centrally important regulatory mechanism is a remarkable example for the evolution of a novel biological concept and the accompanying battles to change trends, fashions, and paradigms. The six-decade journey between the mid-1930s and mid-1990s began with fierce discussions on whether cellular proteins are static, as has been thought for a long time, or are turning over. The discovery of the dynamic state of proteins was followed by the discovery of the lysosome, which was believed—between the mid-1950s and mid-1970s—to be the organelle within which intracellular proteins are destroyed. This occurred at the time when the double helix was discovered, and many in the scientific community were mesmerized by the secrets of the genetic code—how the genome is transcribed to the transcriptome, which is then translated to the proteome. Only a handful of researchers were interested in protein degradation not only because the process was regarded as an end and nonspecific disposal, but also because it was assumed that it is mediated by the lysosome and, therefore, that there is no novel mechanism(s) still waiting to be discovered. Slowly crawling, for more than two decades, were independent lines of experimental evidence that gradually eroded the 'lysosomal hypothesis' and led to the evolvement of a new concept according to which the bulk of intracellular proteins are degraded—under basal metabolic conditions—via a nonlysosomal machinery. This resulted in the discovery of the ubiquitin system during the late 1970s and early 1980s.

With the identification of the reactions and enzymes that are involved in the ubiquitin–proteasome cascade, a new era in the protein degradation research field began during the mid 1980s and early 1990s. Studies showing that the system is involved in targeting of key regulatory proteins and in regulating basic cellular processes started to emerge. They were followed by numerous additional studies on the underlying mechanisms involved in the recognition of these specific proteins, each with its own unique mode of targeting. The unraveling of the human genome exposed the existence of hundreds of distinct E3s, attesting to the complexity and the high specificity and selectivity of the system. Two important advances in the field were the discovery of the nonproteolytic functions of ubiquitin, such as activation of transcription and routing of proteins to the vacuole, and the discovery of modification by ubiquitin-like proteins, which are also involved in numerous nonproteolytic functions, such as directing proteins to their subcellular destinations, protecting proteins from ubiquitination, and controlling entire processes such as autophagy (see, e.g., Ref. [78]). All of these studies have led to the emerging realization that this novel mode of covalent conjugation by ubiquitin and its family members plays a key role in regulating—via proteolytic and nonproteolytic mechanisms—a broad array of cellular process, including cell cycle and division, growth and differentiation, activation and silencing of transcription, apoptosis, the immune and inflammatory response, signal transduction, receptor-mediated endocytosis, various metabolic pathways, and maintenance of the cellular quality control. The discovery that ubiquitin modification plays a role in routing proteins to the lysosome/vacuole, and that modification by unique ubiquitin-like proteins controls autophagy (see, e.g., Refs. [24–26,78]) closed an historical cycle because it demonstrated that the two apparently distinct systems—the ubiquitin system and the lysosome/vacuole—communicate with one another, operate via common mechanisms, and are in many ways inseparable. The simple assumptions that they are distinct—which guided us during the early days and initiated the search after a nonlysosomal system—turned out to be important during those days because the clear boundaries allowed defined characterization of the nonlysosomal system. Yet, nature is more complex than any of our assumptions, and the pendulum now appears to come to rest at the middle, where autophagy/targeting to the lysosome and ubiquitination not only are tightly related, but also serve important cellular functions under both basal and stressed conditions in health and disease. 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. Although 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, may result in a broad array of phenotypes. Likewise, acquired changes in the activity of the system can also evolve into certain pathologies. The disease states associated with the ubiquitin system can be classified into two groups: (i) those that result from loss of function, that is, mutation in a ubiquitin system enzyme or in a recognition motif in the target substrate that results in stabilization of certain proteins; and (ii) those that result from gain of function, that is, abnormal or accelerated degradation of the protein target. Better understanding of the processes and identification of the components involved in the degradation of key regulatory proteins will lead to the development of mechanism-based drugs that will specifically target only the involved proteins. Although the first drug, a specific proteasome inhibitor, is already on the market [79,80] and is used successfully to combat multiple myeloma, it appears that we are entering a new era of development of novel drugs that target specific ligases, such as aberrant Mdm2 or E6-AP that mediate accelerated degradation of the tumor suppressor p53, or SCF complexes that are involved in driving the cell cycle and activation of nuclear factor–kappa B (NF-κB) (see, e.g., Ref. [81]).

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Lecture of the author delivered in Stockholm, Sweden, on December 8, 2004 (see Ref. [9]).

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