TIMELINE

Proteolysis: from the lysosome to ubiquitin and the proteasome

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Abstract | How the genetic code is translated into proteins was a key focus of biological research before the 1980s, but how these proteins are degraded remained a neglected area. With the discovery of the lysosome, it was suggested that cellular proteins are degraded in this organelle. However, several independent lines of experimental evidence strongly indicated that non-lysosomal pathways have an important role in intracellular proteolysis, although their identity and mechanisms of action remained obscure. The discovery of the ubiquitin–proteasome system resolved this enigma.

The concept of protein turnover is barely 60 years old. Previously, the protein components of the body were viewed as essentially stable constituents that were subject to only minor 'wear and tear', whereas dietary proteins, which were believed to function primarily as energy-providing fuel, acted as separate entities, independent from the structural and functional proteins of the body. This concept was challenged by Rudolf Scheonheimer who used ^{15}N-labelled amino acids to show that bodily protein components are turning over extensively — that is, they are continuously synthesized and degraded. In his book *The Dynamic State of Body Constituents*[1], he summarized his experiments in the following way: "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." However, the idea that proteins are turning over was not widely accepted, and was challenged as late as the mid-1950s. For example, Hogness and colleagues[2] studied the kinetics of β-galactosidase in *Escherichia coli* and summarized their findings by stating, "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'."

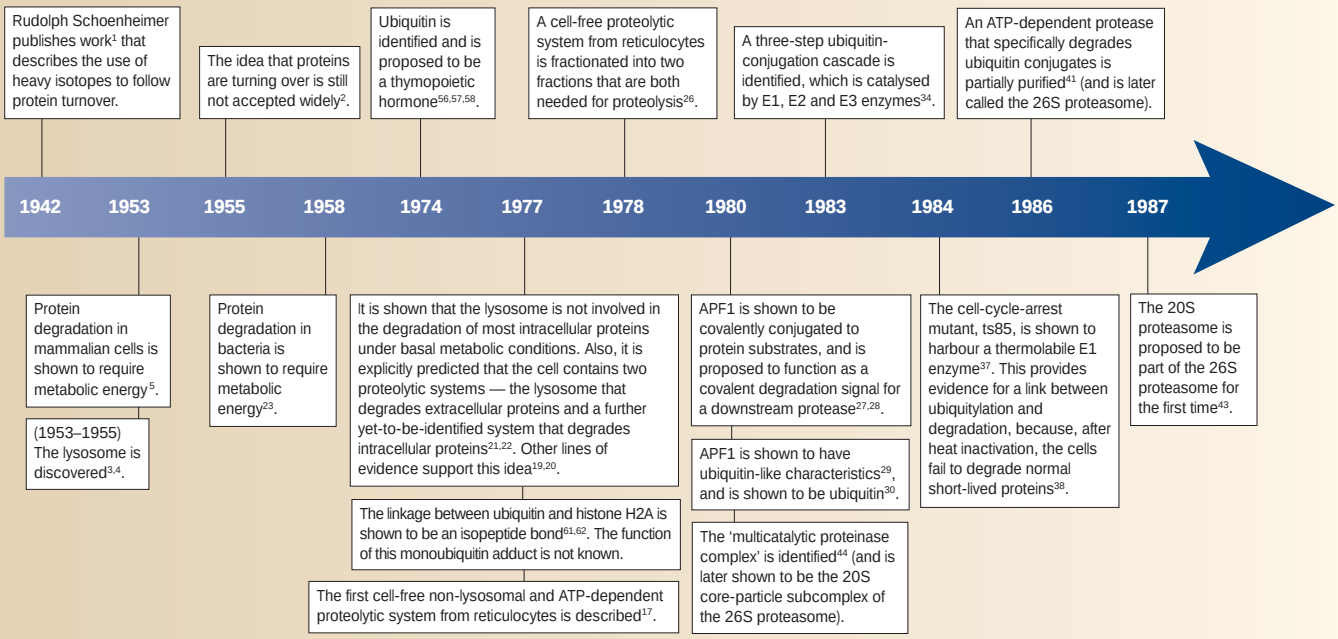
This article reviews the revolution that occurred in the field of intracellular proteolysis. This includes the realization that proteins are, indeed, turning over extensively, that this process is specific, and that the stability of many proteins is regulated individually and can vary under different conditions. It also describes the search for the underlying mechanism (or mechanisms), the discovery of the lysosome, and the simple logical assumptions that led to the hypothesis that intracellular proteolysis probably occurs in this organelle. Finally, the emerging experimental data that strongly indicated that the degradation of most cellular proteins under basal metabolic conditions must be mediated by a non-lysosomal machinery — which led to the discovery of the ubiquitin signalling system and the proteasome — will also be dicussed. The discovery of the ubiquitin–proteasome system resulted in another important development — the realization that regulated proteolysis is involved in controlling a broad array of cellular processes such as the cell cycle and cell division, apoptosis, transcription, antigen presentation, signal transduction, receptor-mediated endocytosis, protein quality control and the modulation of diverse metabolic pathways. Intracellular proteolysis was therefore transformed from a neglected process and research area into an important field in modern biology (see the TIMELINE for the history and key discoveries of the field of intracellular proteolysis).

Mechanisms of intracellular proteolysis

The discovery of the lysosome (see, for example, REFS 3,4; see also FIG. 1 and BOX 1) was a turning point in the studies on protein degradation. Several independent experiments had substantiated the idea that cellular proteins are in a constant state of synthesis and degradation (see, for example, REF. 5), so the parallel discovery of an organelle that contains a broad array of secluded proteases with different specificities provided, for the first time, a machinery that could potentially mediate intracellular proteolysis. However, over a period of more than two decades, between the mid-1950s and the late 1970s, accumulating lines of independent experimental evidence indicated that the degradation of at least certain classes of cellular protein under particular physiological conditions must be non-lysosomal.

First, an important discovery in this respect was the unravelling of the basic functional mechanism of the lysosome — microautophagy. During this process, which occurs under basal metabolic conditions, portions of the cytoplasm that contain the entire cohort of cellular proteins are segregated within a membrane-bound compartment. This compartment then fuses with a primary nascent lysosome, which results in the digestion of its protein contents. Under

Timeline | Important discoveries and milestones in the five-decade history of intracellular protein degradation*



*The timeline shows, in my opinion, the most important discoveries in the five-decade history of intracellular protein degradation. It stops after describing the two main, basic discoveries in the ubiquitin field — ubiquitin conjugation as a degradation signal, and the proteasome. Later important developments in the field have been described in numerous review articles (see, for example, REF. 69). Furthermore, for an interesting historical perspective of the ubiquitin system that stresses the importance of this system in health and disease, see REF. 70. APF1, ATP-dependent proteolysis factor-1; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein (ubiquitin-conjugating enzyme); E3, ubiquitin-protein ligase.

more extreme conditions — for example, starvation — mitochondria, endoplasmic-reticulum membranes, glycogen bodies and other cytoplasmic entities can be engulfed in a process that is known as macroautophagy (see, for example, REF. 6). It was conceptually difficult to reconcile this mode of non-selective degradation with the emerging concept that different proteins are degraded with distinct half-lives — particularly as protein half-lives can vary from minutes to days and can be markedly affected by changing pathophysiological conditions, such as nutrient or hormone availability (for reviews, see REFS 7,8). Interestingly, later evidence[9] indicated that lysosomal degradation might actually be specific and be mediated by the recognition of a defined motif in the target protein (KFERQ), although the existence of a similar sequence in ~30% of cellular proteins made it unlikely that such a mechanism could be substrate specific. However, it could function as part of a general mechanism that mediates substrate transport across the lysosomal membrane, although this would not be the only mechanism, as substrate entry into lysosomes is also mediated by other mechanisms, such as vesicle-membrane fusion and the formation of multivesicular bodies (MVBs; BOX 1).

Second, the discovery that specific and general inhibitors of lysosomal proteases have different effects on different populations of proteins made it clear that different proteolytic machineries function in the cell: the discovery that the degradation of endocytosed/extracellular proteins was significantly inhibited, whereas only a limited effect was observed on the degradation of long-lived proteins and almost no effect could be detected on the degradation of short-lived and abnormal/mutated proteins made it clear that different proteins are targeted by different proteolytic machineries.

Finally, the thermodynamically paradoxical observation that the degradation of cellular proteins requires metabolic energy and, more importantly, the emerging evidence that the proteolytic machinery might require energy in a direct manner were in contrast with the known mode of action of lysosomal proteases — that is, that under the appropriate acidic conditions and similar to all known proteases, they degrade proteins exergonically.

The hypothesis that the degradation of intracellular proteins is mediated by the lysosome was nevertheless logical. In general, proteolysis seemed to result from the direct interaction of substrates with proteases, and because it was clear that active proteases cannot be free in the cytosol, the most sound assumption was that intracellular protein degradation is lysosomal. Nobody could have predicted that a new mode of post-translational modification — polyubiquitylation — is needed for substrates to be specifically recognized by a giant protease that is about half the size of a ribosome. At the time, the lysosomal membrane — rather than the requirement for such a modification — seemed to provide the essential barrier between the protease (or proteases) and its substrates. It was just necessary to explain how proteins enter the lysosome and are degraded in a selective manner. 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 sensitivity to the action of lysosomal proteases *in vitro*[10]. 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 proposed that, although all cellular proteins are engulfed by the lysosome, only the short-lived proteins are degraded, whereas the long-lived proteins exit back into the cytosol[11]. According to a different model, selectivity is determined by the binding affinity of the different proteins for the lysosomal membrane and their subsequent entry into the lysosome, a process that controls their degradation rate[12]. The requirement for energy was described as

indirect, and necessary, for example, for protein transport across the lysosomal membrane[13] or for the activity of the H^+ pump, which is required for the maintenance of the acidic intralysosomal pH that is necessary for the optimal activity of the proteases[14]. "Just as extracellular digestion is successfully carried out by the concerted action of enzymes with limited individual capacities, so, we believe, is intracellular digestion", summarized Christian de Duve[15], the discoverer of the lysosome.

Progress in identifying the putative non-lysosomal proteolytic system (or systems) was hampered by the lack of a cell-free preparation that could faithfully replicate the cellular proteolytic events — that is, a preparation that could degrade proteins in a specific, energy-requiring, yet non-lysosomal manner. An important breakthrough came from Rabinovitz and Fisher, who found that rabbit reticulocytes efficiently degrade abnormal haemoglobin that contains amino-acid analogues[16]. As reticulocytes are immature, terminally differentiating red blood cells and do not contain lysosomes, it was postulated that the degradation of haemoglobin is mediated by a non-lysosomal machinery. Etlinger and Goldberg[17] were the first to isolate a cell-free proteolytic preparation from reticulocytes. The crude extract selectively degraded abnormal haemoglobin, required ATP hydrolysis and functioned optimally at a neutral pH, which strongly indicated that the proteolytic activity was non-lysosomal. A similar system was isolated and characterized shortly afterwards by Hershko, Ciechanover and colleagues[18], who later resolved, characterized and purified its components — an acheivement that resulted in the discovery of the ubiquitin signalling system (see below).

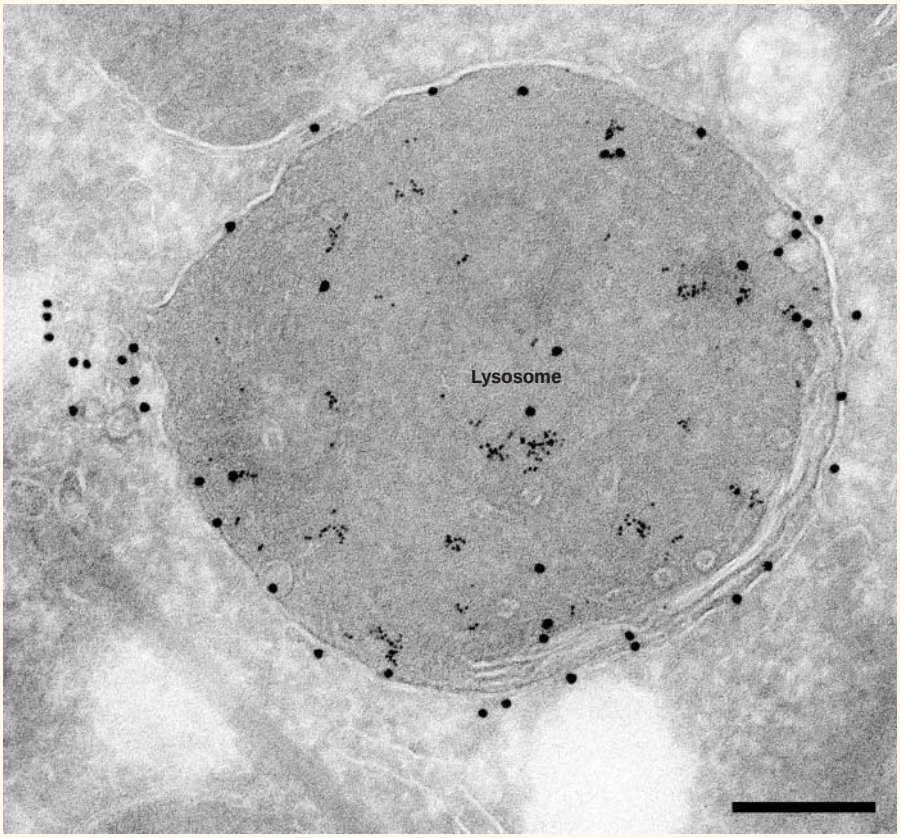


Figure 1 | **The lysosome.** Ultrathin cryosections of a human hepatoma G2 cell that had been loaded for three hours with bovine serum albumin (BSA)–gold (5-nm particles) and immunolabelled for the late endosomal/lysosomal marker CD63 (10-nm particles). The marker mostly decorates the lysosomal membrane, whereas the BSA resides in the lumen of the organelle. Bar, 200 nm. This figure was provided courtesy of Viola Oorschot and Judith Klumperman, Department of Cell Biology, Utrecht University Medical Center, Utrecht, The Netherlands.

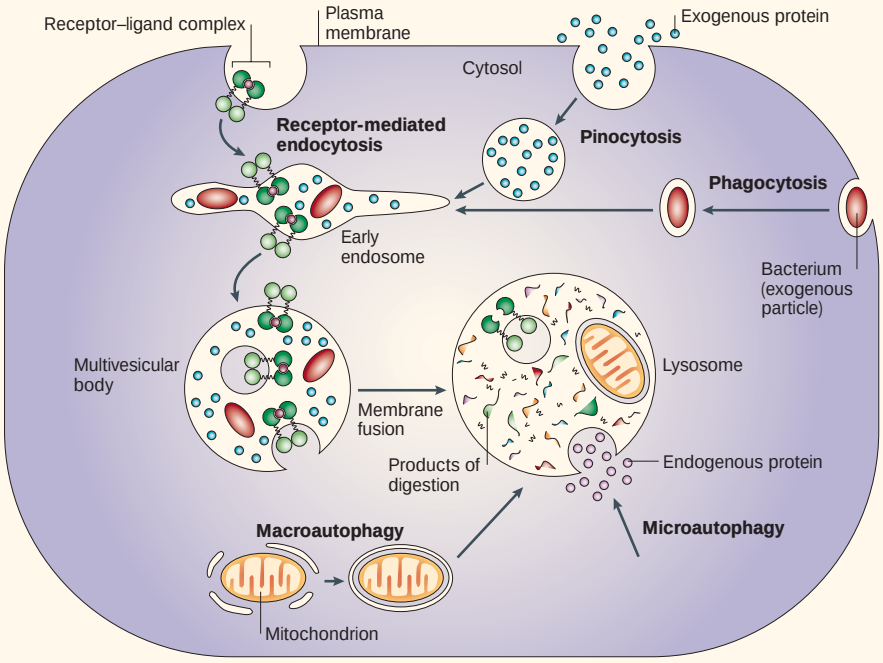
The lysosome and cellular proteolysis

As mentioned above, the functional mechanism (or mechanisms) of the lysosome could not be reconciled with several key emerging characteristics of intracellular protein degradation, such as the heterogeneous stability of individual proteins, the effect of nutrients and hormones on their degradation, the differential effect of selective inhibitors on the degradation of different classes of protein, and the dependence of intracellular proteolysis on metabolic energy.

The evolvement of methods to monitor protein kinetics in cells together with the development of specific and general lysosomal inhibitors resulted in the identification of different classes of cellular proteins (long- and short-lived), and the discovery of the differential effects of the inhibitors on these classes of proteins (see, for example, REFS 19,20). For example, Poole and his colleagues metabolically labelled endogenous proteins in living macrophages with ^{3}H-leucine and then 'fed' them with dead macrophages that had been previously labelled with ^{14}C-leucine. In this way, they were able to monitor, within one cell, the digestion of the same macrophage proteins that were presented to the cell from two different sources — from within the cell (^{3}H-labelled proteins) and from the extracellular milieu (^{14}C-labelled proteins). They followed the effect of lysosomotropic agents on the degradation of these two protein populations — specifically, they studied the effect of the weak bases chloroquine and ammonium chloride, which enter the lysosome and neutralize the H^+ ions, and the acid ionophore X537A, which dissipates the H^+ gradient across the lysosomal membrane. Treatment with these agents increases the intralysosomal pH, which results in the inhibition of the lysosomal proteases that function optimally at an acidic pH. They found that these drugs specifically inhibited the degradation of extracellular proteins, but not that of intracellular proteins[21]. Poole summarized these experiments by explicitly predicting the existence of a non-lysosomal proteolytic system that degrades intracellular proteins: "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."[22]

The metabolic energy requirement for the degradation of both eukaryotic[5] and prokaryotic[23] proteins was difficult to understand. Proteolysis is an exergonic process and the thermodynamically paradoxical energy requirement for intracellular proteolysis made researchers believe that the energy could not be consumed directly by proteases or the proteolytic process *per se*, and therefore must be used indirectly. As Simpson summarized his findings[5]: "…the fact that protein hydrolysis as catalyzed by the familiar proteases and peptidases occurs exergonically,

Box 1 | The lysosome



The lysosome (FIG. 1) was first recognized biochemically in rat liver as a vacuolar structure that contains various hydrolytic enzymes, which function optimally at an acidic pH. It is surrounded by a membrane that endows the enclosed enzymes with a latency that is required to protect the cellular contents from their action[3,4,11,14]. The definition of the lysosome has been broadened over the years (see figure). This is because it has been recognized that the digestive process is dynamic and involves numerous stages of lysosomal maturation, together with the digestion of both exogenous proteins and particles, as well as the digestion of endogenous proteins and cellular organelles. Exogenous proteins are targeted to the lysosome through receptor-mediated endocytosis and pinocytosis, and exogenous particles are targeted by phagocytosis; these three processes are known as heterophagy. Endogenous proteins and cellular organelles are targeted by microautophagy and macroautophagy, respectively. The lysosomal/vacuolar system, as we now recognize it, is a heterogeneous, discontinuous digestive system that also includes structures that are mostly devoid of hydrolases — for example, early endosomes that contain endocytosed receptor–ligand complexes and pinocytosed/phagocytosed extracellular contents. At the other extreme, it includes the residual bodies — the end products of the completed digestive processes of heterophagy and autophagy. In between these extremes, there are: primary/nascent lysosomes that have not yet been engaged in any proteolytic process; early autophagic vacuoles that might contain intracellular organelles; intermediate/late endosomes and pinocytic/phagocytic vacuoles (heterophagic vacuoles) that contain extracellular contents/particles; and multivesicular bodies, which are the transition vacuoles between endosomes/heterophagic vacuoles and the digestive lysosomes.

The figure shows the digestive processes that are mediated by the lysosome: specific receptor-mediated endocytosis; pinocytosis (the nonspecific engulfment of extracellular fluid); phagocytosis (the engulfment of extracellular particles); and autophagy (the engulfment of intracellular proteins (microautophagy) and organelles (macroautophagy)).

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." With the discovery of lysosomes in eukaryotic cells, it could be argued, for example, that energy is required for the transport of substrates into the lysosome or for the maintenance of the low intralysosomal pH (see above). The observation by Hershko and Tomkins that the activity of tyrosine aminotransferase was stabilized following the depletion of ATP[24] indicated that energy might be required at an early stage of the proteolytic process, possibly before proteolysis occurs. However, it did not rule out, for example, a possible role for the lysosome in the process, or a role for energy in another, non-proteolytic process that might lead to the inactivation of the enzyme. In bacteria, which lack lysosomes, the former argument could not have been proposed, but other indirect effects of ATP hydrolysis could have affected proteolysis in *E. coli*, such as the maintenance of the 'energized membrane state'. According to this model, proteins can become susceptible to proteolysis by changing their conformation, for example, following their association with cellular membranes that maintain a local, energy-dependent gradient of a certain ion. However, such an effect was ruled out[25], and it seemed that, at least in bacteria, energy is required directly for the proteolytic process, although the proteolytic machinery in prokaryotes had not been identified at that time. The metabolic energy requirement for protein degradation in both prokaryotes and eukaryotes indicated that energy is required directly for the proteolytic process, most probably for the regulation of it, and that a similar principle/mechanism must have been preserved during the evolution of the two kingdoms. The description of the cell-free proteolytic system in reticulocytes[17,18], which also lack lysosomes, further strengthened the idea that energy is probably directly required for the proteolytic process in eukaryotes as well, although, here too, the underlying mechanisms remained enigmatic at the time. However, the description of the cell-free system enabled the underlying mechanism (or mechanisms) to be unravelled.

The ubiquitin–proteasome system

The cell-free proteolytic system from reticulocytes[17,18] turned out to be an extremely important source for the purification and characterization of the enzymes that are involved in the ubiquitin–proteasome system (FIG. 2). Ciechanover and Hershko first found that fractionation of the crude reticulocyte cell extract on an anion-exchange resin yielded two fractions, I and II, which were both required to reconstitute the energy-dependent proteolytic activity that is found in the crude extract[26]. This was an important observation and a valuable lesson for the future dissection of the system, as it indicated that the system is not composed of a single 'classic' protease that evolved to acquire energy dependence, but that it has at least two components (although single proteases that require energy — the mammalian 26S proteasome (see below) and the prokaryotic *Lon* gene product — were discovered later). Learning from this discovery, the researchers reconstituted the activity using the resolved

fractions whenever they encountered a loss of activity during further purification steps. This biochemical 'complementation' approach resulted in the discovery of further enzymes of the system, which are all required in the reaction mixture to catalyse the multistep proteolysis of a target substrate. The active component from fraction I was characterized and found to be a small, ~8.5-kDa heat-stable protein[26]. A plausible hypothesis was, for example, that the active component in fraction I could be an activator for a protease in fraction II. Extremely important findings that paved the way for future developments in the field were that several moieties of this heat-stable protein — which had been designated ATP-dependent proteolysis factor-1 (APF1) — are covalently conjugated to the target substrate when it is incubated in the presence of fraction II, and that this modification requires ATP[27,28].

The discovery that APF1 is covalently conjugated to protein substrates and stimulates their proteolysis in the presence of ATP and crude fraction II led to the proposal, in 1980, of a model in which protein-substrate modification by several moieties of APF1 targets it for degradation by a downstream, as-yet-unidentified protease that cannot recognize the unmodified substrate. In this model, reusable APF1 is released following protein-substrate degradation[28]. Amino-acid analysis of APF1, along with its known molecular mass and other general characteristics raised the suspicion that APF1 is ubiquitin[29], a known protein of previously unknown function (BOX 2). Indeed, Wilkinson and colleagues showed unequivocally that APF1 is indeed ubiquitin[30]. This discovery, and the discovery that the mode of APF1 attachment to the substrate[31] is similar to the one that links ubiquitin to histone H2A (BOX 2), resolved the enigma of the energy requirement for intracellular proteolysis (see, however, below), and paved the way to understanding the complex mechanism of isopeptide-bond formation. This process turned out to be similar, in principle, to the mechanism of peptide-bond formation that is catalysed by tRNA synthetase following amino-acid activation during protein synthesis or during the non-ribosomal synthesis of short peptides[32]. Using the unravelled mechanism of ubiquitin activation and immobilized ubiquitin as a 'covalent' affinity bait, the three enzymes that are involved in the cascade reaction of ubiquitin conjugation were purified by Hershko, Ciechanover and colleagues. These enzymes are: enzyme-1 (E1), the ubiquitin-activating enzyme; E2, the ubiquitin carrier protein (ubiquitin-conjugating enzyme); and E3, the ubiquitin-protein ligase[33,34] (FIG. 2a). The discovery of an E3

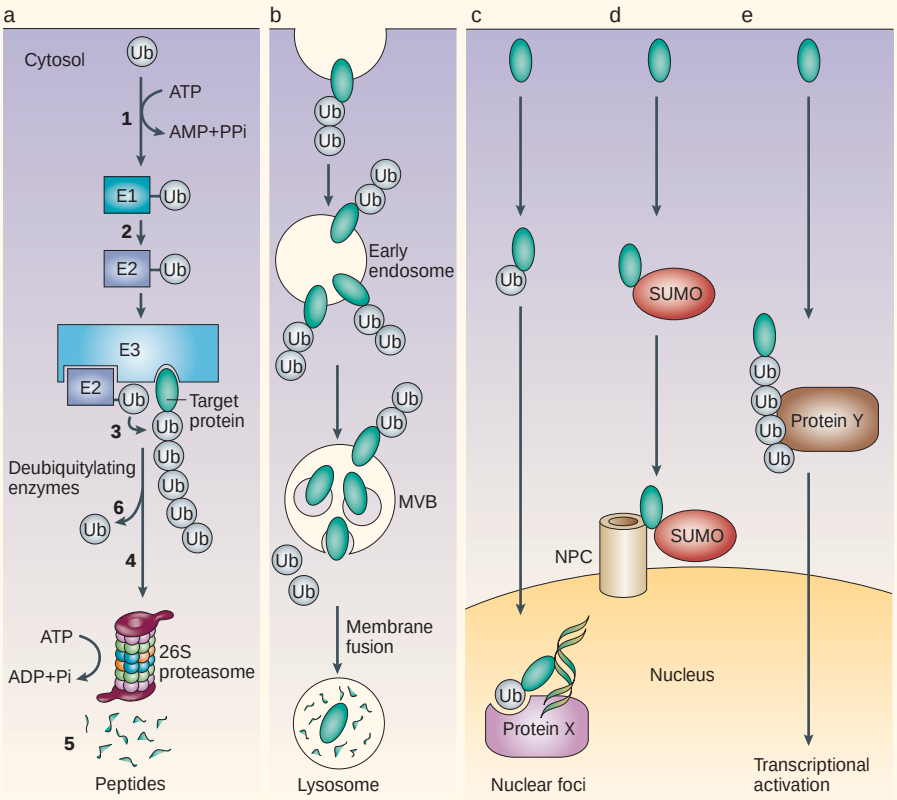


Figure 2 | **Some of the various functions of modification by ubiquitin and ubiquitin-like proteins.** Proteasome-dependent degradation of cellular proteins (**a**). Ubiquitin is activated by the ubiquitin-activating enzyme (E1; step 1), and its transfer to a ubiquitin carrier protein (ubiquitin-conjugating enzyme; E2; step 2) then follows. The E2 enzyme and the protein substrate both bind specifically to a particular ubiquitin-protein ligase (E3), and the activated ubiquitin moiety is then transferred to the protein substrate (step 3). The successive conjugation of ubiquitin moieties generates a polyubiquitin chain that functions as a signal to target the protein substrate to the 26S proteasome for degradation (step 4). The substrate is degraded to short peptides (step 5), and reusable ubiquitin is released by deubiquitylating enzymes (step 6). Mono- or oligoubiquitylation (**b**) targets membrane proteins for degradation in the lysosome. Monoubiquitylation (**c**) or a single modification by a ubiquitin-like protein — for example, SUMO (**d**) — can target proteins to different subcellular destinations such as nuclear foci (which contain Protein X in this figure) or the nuclear pore complex (NPC). Modification by ubiquitin-like proteins can have further non-proteolytic functions, such as protecting proteins from ubiquitylation or activating E3 enzymes (not shown). The generation of a polyubiquitin chain (**e**) can activate transcriptional regulators either directly or indirectly, the latter occurring through the recruitment of other proteins (Protein Y is shown) or the activation of upstream components such as kinases. In the case of non-proteolytic functions, the polymerization of ubiquitin is mediated using an internal lysine residue in the ubiquitin moieties (for example, Lys63) that is different to the Lys48 residue that is involved in signalling proteolysis (see also BOX 2). MVB, multivesicular body; Pi, inorganic phosphate; Ub, ubiquitin. This figure was modified with permission from REF. 68 © (2004) Taylor and Francis Ltd.

enzyme, which is the specific substrate-binding component of the system, indicated a possible solution to the problem of specificity and the varying stabilities of different proteins — they might be specifically recognized and targeted by different ligases.

The ubiquitin-tagging hypothesis quickly received substantial support. For example, Chin and colleagues injected labelled ubiquitin and haemoglobin into HeLa cells and then denatured the injected haemoglobin by oxidizing it with phenylhydrazine. They found that ubiquitin conjugation to globin is markedly enhanced by the denaturation of haemoglobin, and that the concentration of globin–ubiquitin conjugates was proportional to the rate of haemoglobin degradation[35]. Hershko and colleagues observed a similar correlation for abnormal, short-lived proteins that contained amino-acid analogues[36]. A previously isolated mammalian cell-cycle-arrest mutant, which loses the ubiquitin–histone-H2A conjugate at the permissive temperature (BOX 2), was found by Finley, Ciechanover and Varshavsky to harbour a thermolabile E1 (REF. 37). Following

Box 2 | Ubiquitin

Ubiquitin is a small (76 residue), heat-stable and highly evolutionarily conserved protein. It was first purified during the isolation of thymopoietin[56] and was subsequently found to be ubiquitously expressed in cells from all kingdoms of life, including prokaryotes[57]. Interestingly, it was initially found to have lymphocyte-differentiating properties, a characteristic that was attributed to the stimulation of adenylate cyclase[58]. It was therefore named UBIP (ubiquitous 'immunopietic' polypeptide[57]). However, later studies showed that ubiquitin is not involved in the immune response[59], and that it was a contaminating endotoxin in the preparation that probably stimulated the adenylate cyclase and the T-cell-differentiating activity. Furthermore, the sequencing of several eubacterial and archaebacterial genomes, together with functional studies in these organisms, showed that ubiquitin is actually restricted only to eukaryotes. The identification of ubiquitin in bacteria[57] was probably due to contamination of the bacterial extract with yeast ubiquitin, which was derived from the yeast extract in which the bacteria were grown. Importantly, although the name ubiquitin is a misnomer because this protein is not as ubiquitous as was previously thought, it has remained the name of the protein for historical reasons.

An important breakthrough in the field of ubiquitin research was the discovery that a single ubiquitin moiety can be covalently conjugated to histones, particularly to histones H2A and H2B. The ubiquitin conjugate of H2A (which was designated protein A24) was characterized by Goldknopf and Busch[60,61] and by Hunt and Dayhoff[62], who found that the two proteins are linked through an isopeptide bond between the C-terminal glycine of ubiquitin (Gly76) and the ε-NH_2 group of an internal lysine (Lys119) of the histone. It should be noted that a bifurcated isopeptide bond between two different proteins that is generated post-translationally and is probably dynamic (that is, synthesized and hydrolysed) had not been described before. The isopeptide bond in this histone–ubiquitin conjugate is identical to the bond that was proposed for the linkage between ubiquitin and its target proteolytic substrates[31], and between the ubiquitin moieties in the polyubiquitin chain[63,64]. The polyubiquitin chain is synthesized on the substrate and functions as a recognition signal for proteolysis by the 26S proteasome (BOX 3). In this polyubiquitin chain, the linkage is between Gly76 of one ubiquitin moiety and the internal Lys48 of the previously conjugated ubiquitin moiety. The role of the monoubiquitin modification of histones in the regulation of transcription (for a recent review, see, for example, REF. 65) is unlike that of polyubiquitylation in proteolysis (FIG. 2; BOX 3; please also refer to the main text for further details).

heat inactivation, the cells fail to degrade normal short-lived proteins[38]. Although the cells did not provide direct evidence for substrate ubiquitylation as a destruction signal, this work nevertheless provided the strongest direct linkage between ubiquitin conjugation and protein degradation. In addition, because the work was carried out using a cell-cycle-arrest mutant, these observations enabled the researchers to predict the possible involvement of the ubiquitin system in controlling cell division — a hypothesis that later turned out to be correct.

At this point, the only missing link was the identification of the downstream protease that would specifically recognize ubiquitylated substrates. Tanaka and colleagues identified a second ATP-requiring step in the reticulocyte proteolytic system, which occurred after ubiquitin conjugation[39], and Hershko and colleagues showed that the energy is required for conjugate degradation[40]. An important advance in the field was a discovery by Hough and colleagues, who partially purified and characterized a high-molecular-mass alkaline protease that degraded ubiquitin conjugates of lysozyme, but not untagged lysozyme, in an ATP-dependent manner[41]. This protease, which later became known as the 26S proteasome (BOX 3), fitted all the necessary criteria for being the specific proteolytic arm of the ubiquitin system. This idea was confirmed, and the protease was further characterized by Waxman and colleagues, who found that it is an unusually large, ~1.5-MDa enzyme that is unlike any other known protease[42]. A further advance in the field was the finding[43] that a smaller, neutral, multisubunit 20S protease complex, which was discovered together with the larger 26S complex, is similar to a 'multicatalytic proteinase complex' (MCP) that was described earlier by Wilk and Orlowski in bovine pituitary gland[44]. This 20S protease is ATP-independent and has several distinct catalytic activities, for example, it cleaves on the C-terminal side of hydrophobic, basic and acidic residues. Hough and colleagues raised the possibility — although they did not show it experimentally — that this 20S protease can be a part of the larger 26S protease that degrades the ubiquitin conjugates[43]. Later studies showed that, indeed, the 20S complex is the core catalytic particle of the larger 26S complex[45,46]. However, direct evidence that the active, double-'mushroom'-shaped 26S protease is generated through the assembly of two distinct subcomplexes — the catalytic 20S cylinder-like MCP and a further 19S ball-shaped subcomplex (that was predicted to have a regulatory role) — was provided by Hoffman and colleagues[47], who mixed the two purified particles to generate the active 26S enzyme (BOX 3).

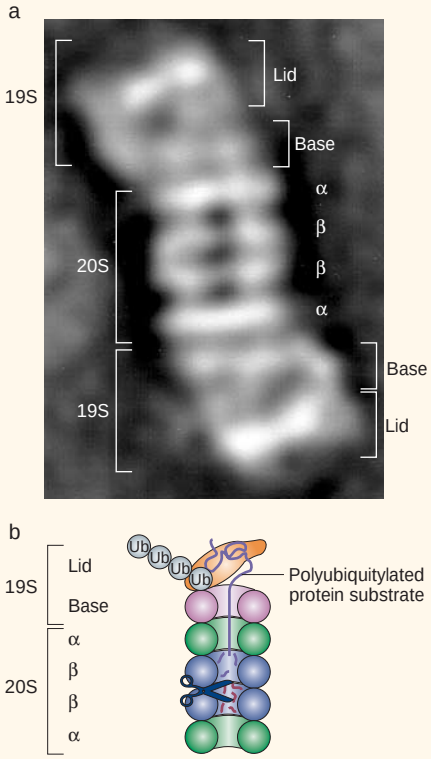
Concluding remarks

The emergence of proteolysis as a centrally important regulatory mechanism is a remarkable example of the evolution of a new biological concept and the accompanying battle to change paradigms. The journey between the early 1940s and early 1990s began with fierce discussions regarding whether proteins are stable, as had been thought for a long time, or were 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 in which intracellular proteins are degraded. Independent lines of experimental evidence gradually eroded the 'lysosomal hypothesis' and led to it being substituted by a new hypothesis in which most intracellular proteins are degraded — under basal metabolic conditions — by a non-lysosomal machinery. This resulted in the discovery of the ubiquitin system in the late 1970s and early 1980s.

With the identification of the reactions and enzymes that are involved in the ubiquitin–proteasome cascade (FIG. 2a), a new era in the protein-degradation field began in the late 1980s and early 1990s. Studies began to show that the system is involved in targeting key regulatory proteins — such as light-regulated proteins in plants, transcription factors, cell-cycle regulators, tumour suppressors and promoters (see, for example, REFS 48–52). These studies were followed by numerous investigations into the mechanisms that underlie the degradation of specific proteins, with each having its own unique mode of recognition and regulation. The recent unravelling of the human genome highlighted the existence of hundreds of distinct E3 enzymes, which confirms the complexity, high specificity and selectivity of the system.

Two further important advances in the field were the discovery of non-proteolytic functions for ubiquitin, such as in the activation of transcription, and the discovery of ubiquitin-like proteins (FIG. 2). Some of the latter proteins function through the covalent modification of their targets, and are also involved in numerous non-proteolytic functions such as directing proteins to their subcellular destination (FIG. 2d) and protecting other proteins from ubiquitylation.

Box 3 | The 26 proteasome



The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitylated proteins to produce small peptides (see figure). It is composed of two subcomplexes — a 20S core particle (CP) that carries the catalytic activity, and a 19S regulatory particle (RP). The 20S CP is a barrel-shaped structure that is composed of four stacked rings, two identical outer α-rings and two identical inner β-rings. The eukaryotic α- and β-rings are each composed of seven distinct subunits, which gives the 20S complex the general structure of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The catalytic sites are localized to some of the β-subunits. One or both ends of the 20S barrel can be capped by a 19S RP that is composed of 17 distinct subunits — 9 in a 'base' subcomplex, and 8 in a 'lid' subcomplex. One important function of the 19S RP is to recognize polyubiquitylated proteins. Several ubiquitin-binding subunits of the 19S RP have been identified, but their biological roles and mode of action have not been discerned. A second function of the 19S RP is to open an orifice in the α-ring, which allows the substrate to enter the proteolytic chamber. In addition, as a folded protein cannot fit through the narrow proteasomal channel, it is thought that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel-opening and the substrate-unfolding functions require metabolic energy and, indeed, the base of the 19S RP contains six different ATPase subunits. Following substrate degradation, short peptides that have been derived from the substrate are released, as is reusable ubiquitin. For a recent review on the proteasome, see REF. 66.

Part **a** of the figure shows an electron-microscopy image of a 26S proteasome from *Saccharomyces cerevisiae*, and part **b** shows a schematic representation of the structure and function of the 26S proteasome. Ub, ubiquitin. Part **a** was reproduced with permission from REF. 67 © (1998) Elsevier. Part **b** was modified with permission from *Nature Reviews Molecular Cell Biology* REF. 66 © (2004) Macmillan Magazines Ltd.

Others have different functions in the ubiquitin signalling system that do not involve the covalent modification of target proteins. Further interesting discoveries include the finding that the ubiquitin-like protein Apg12 is essential for the function of the vacuole in autophagy in *Saccharomyces cerevisiae*[53] (later studies produced similar findings in mammals), and that ubiquitylation functions as a signal for sorting into endosomes[54] and the MVB pathway[55] (BOX 1). The latter sorting pathways are complex and involve the modification of both the cargo substrates and the components of the vesicular system. These discoveries closed an exciting historical cycle, linking together what were thought to be two distinct systems — lysosomal digestion and modification by ubiquitin and ubiquitin-like proteins. All these studies have led to the emerging realization that this mode of covalent conjugation has a key role in regulating — through both proteolytic and non-proteolytic mechanisms — a broad array of cellular processes. These include: the cell cycle and cell division; cell growth and differentiation; the activation and silencing of transcription; apoptosis; immune and inflammatory responses; signal transduction; receptor-mediated endocytosis and the sorting of proteins in the cell; various metabolic pathways; and protein quality control. As there are numerous substrates that need to be targeted and processes that have to be regulated, it has not been surprising to discover that aberrations in the system are implicated in the pathogenesis of many diseases, including several malignancies and neurodegenerative disorders. It seems that one important goal of the era that we are now entering will be to discover new drugs that target specific processes — for example, drugs that inhibit the aberrant E3(MDM2)-mediated targeting of the p53 tumour suppressor, which is observed in many malignancies.

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Competing interests statement

The author declares no competing financial interests.

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