ESSAY

The unravelling of the ubiquitin system

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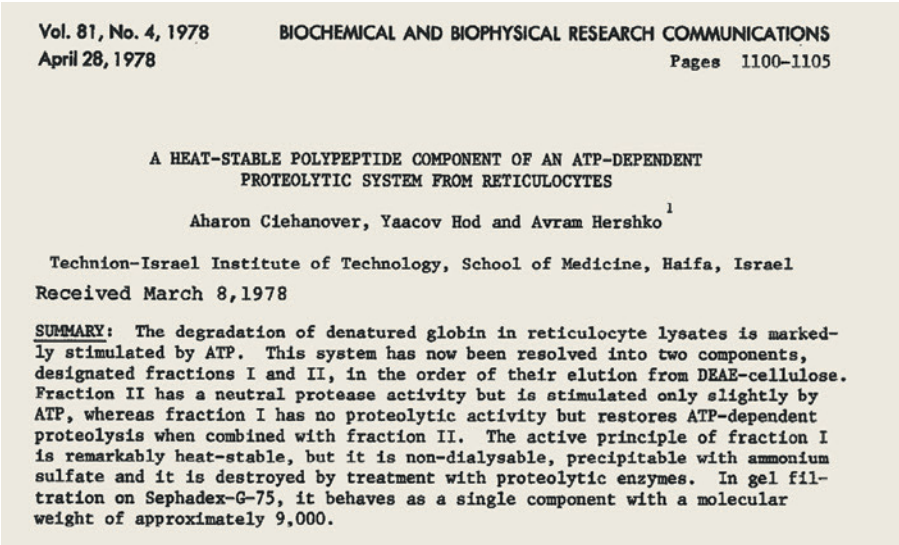
Abstract | Today, many scientific discoveries are made using a top-down experimental approach. The ubiquitin system was discovered using a 'classic' bottom-up approach to tackle the question: 'how are cellular proteins selectively degraded?' A simple proteolytic assay, which used a crude cell-extract, was all that was required to address this question; it was followed by fractionation and reconstitution experiments to decipher the role of the components in this multi-step process. This 'biochemistry at its best' approach, which was published in a periodical that today would not be regarded as highly visible, provided magnificent findings.

Both the ubiquitin–proteasome proteolytic system and post-translational modifications by ubiquitin and ubiquitin-like proteins are involved in nearly all cellular processes. Aberrations in this system give rise to numerous diseases, including different malignancies and neurodegeneration. Interestingly, the modification of proteins by ubiquitin and the important part that these modifications play in targeting proteins for degradation were not discovered by screening, data mining or a systems biology approach. Rather, they came to life through the old 'classical' scientific approach of asking a biological question, which in this case was 'how are intracellular proteins degraded?' and, in particular, 'what is the identity of the mechanism (or mechanisms) that endows the degradation process with its high selectivity and specificity?'

The pioneering studies of Rudolph Schoenheimer taught us that cellular proteins are turning over[1], yet the mechanism (or mechanisms) underlying this had remained elusive. With the discovery of the lysosome by Christian de Duve[2], the proteolytic apparatus was thought to have been identified. However, the mechanism of degradation by the lysosome that was known at the time — involving small portions of the cytosol that contain an aliquot of the entire cytosolic proteome undergoing what is now known as microautophagy — could not explain the substrate specificity and selectivity of the proteolytic process. For example, one could not explain how misfolded, mutated or otherwise damaged proteins are recognized and removed, while the vast majority of functional proteins are spared, or how regulatory proteins such as cell cycle regulators or transcription factors are destroyed in a timed and programmed manner when they are not needed but are kept intact and active when they are. Another unsolved mystery was the role of metabolic energy in the process[3] — why is an investment of energy required for the hydrolysis of peptide bonds, which is an exergonic process? This finding could not be explained in simple thermodynamic terms. Although it was known that the acidification of the lysosome required ATP[4], the fact that a role for lysosome-mediated microautophagy in selective protein degradation had been ruled out, along with the finding that energy is also required for protein degradation in prokaryotes that do not have lysosomes[5], raised the hypothesis that ATP is required for a regulatory step (or steps) in the proteolytic process. The most direct evidence that the lysosome does not mediate selective intracellular proteolysis came from an experiment by Brian Poole[6], showing that lysosomotropic agents (that is, agents that preferentially accumulate in lysosomes) such as chloroquine — which neutralize lysosomal pH and thus inhibit the activity of lysosomal proteases — abolished the degradation of extracellular proteins that reach the lysosome via different endocytic routes but had no effect on the degradation of intracellular proteins. Brian Poole summarized his findings by predicting the existence of a non-lysosomal 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" (REF. 6).

These open questions and experimental findings drove the search for the elusive non-lysosomal and ATP-dependent protease or proteolytic system that degrades intracellular proteins in a specific manner. Admittedly, only a handful of researchers were interested in the problem, as the attention of the scientific community was at the time (the 1970s) focused on deciphering the factors and regulatory mechanisms involved in the central dogma of biology — unlocking the genetic code of protein synthesis. Along with



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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.

Figure 1 | **The title and abstract of the first manuscript that prompted additional studies and resulted in the discovery of the ubiquitin proteolytic system.** This study describes the fractionation of a crude cell-extract into two complementary fractions (see also TABLE 1). The active component in one fraction was identified as a small 8.5 kDa protein (later identified as ubiquitin). The finding that the activity in the crude extract is made of two complementing activities, rather than a single energy-requiring protease, prompted further fractionation, whch later resulted in the discovery of the cascade of conugating enzymes and the proteolytic machinery. Reprinted with permission from REF. 9, Elsevier.

Table 1 | **ATP and two enzymatic components are required for protein degradation**

Enzyme fraction	Degradation of ^{3}H-globin (percent per hour)	
	–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

ATP and the enzymatic activity of two complementing fractions of the cell-free proteolytic system are required for degradation of the model substrate ^{3}H-globin. The energy-requiring proteolytic activity in crude reticulocyte lysate was resolved into two essential components using anion exchange chromatography: fraction I, which contains the proteins that did not adsorb to the resin, and fraction II, which contains the proteins that were adsorbed and eluted with high salt. Reprinted with permission from REF. 9, Elsevier.

Avram Hershko, my graduate studies mentor, we selected the reticulocyte, the terminally differentiating red blood cell, as our model system, because it was known that it expels its lysosomes during differentiation in the bone marrow but continues to degrade its proteins and protein-based machineries until its final maturation and ejection into the peripheral circulation[7]. Indeed, Etlinger and Goldberg[8] demonstrated that intact reticulocytes and, importantly, high-speed centrifugation supernatant prepared from them, degraded amino acid analogue-containing abnormal haemoglobin in an ATP-dependent manner by an unknown mechanism.

The first, and arguably the most important mechanistic clue came from experiments that were described in a short study published in 1978 in *Biochemical and Biophysical Research Communications* (BBRC)[9] (FIG. 1). The idea behind the crucial experiment was, obviously, to purify and then characterize the elusive ATP-dependent protease. Surprisingly, in the first attempt to purify it, we were already left without a paradigm: typically, the 'tango' of proteolysis is danced by two — a protease and a substrate. Here, fractionation of the crude lysate on an anion exchange resin revealed that the proteolytic activity required for the degradation of our model substrate was made of two necessary components and required ATP (TABLE 1). This finding raised the hypothesis that more than two components may be needed because the two fractions were crude and reflected the division of the entire cellular proteome according to protein behaviour on the resin. This indeed proved to be the case and, shortly after, using different chromatographic approaches and reconstituted cell-free assays, we began isolating additional factors. The first component was a small (molecular weight of ~9.0 kDa) heat-stable protein that we called ATP-dependent proteolysis factor 1 (APF1)[9], which we later found to be covalently attached to the target substrates by an ATP-requiring reaction; we hypothesized that APF1 probably signalled them for degradation by a downstream protease that had not yet been identified[10,11] (FIG. 2). Along with our collaborator, Irwin A. Rose, we proposed a model for the entire proteolytic cycle that has withstood the test of time and is accepted to be largely correct[11] (FIG. 3).

According to the model, *n* molecules of APF1 are covalently attached to the substrate, which marks it for recognition by a downstream protease that degrades the substrate and recycles APF1 for reuse. A regulatory function was also proposed, which involves the removal of APF1 from the substrate before its degradation, in case the substrate refolds to its native form or the modification (or modifications) that rendered it susceptible for destruction is removed or corrected.

Shortly after, APF1 was identified as ubiquitin, a previously-known protein that was, at that point, of unknown function[12,13]. Ubiquitin had previously been found to be covalently attached to fractions of both histone H2A and histone H2B. In this case, the link was identified as an isopeptide bond between the carboxy-terminal Gly76 residue of ubiquitin and an ε-NH_2 group of

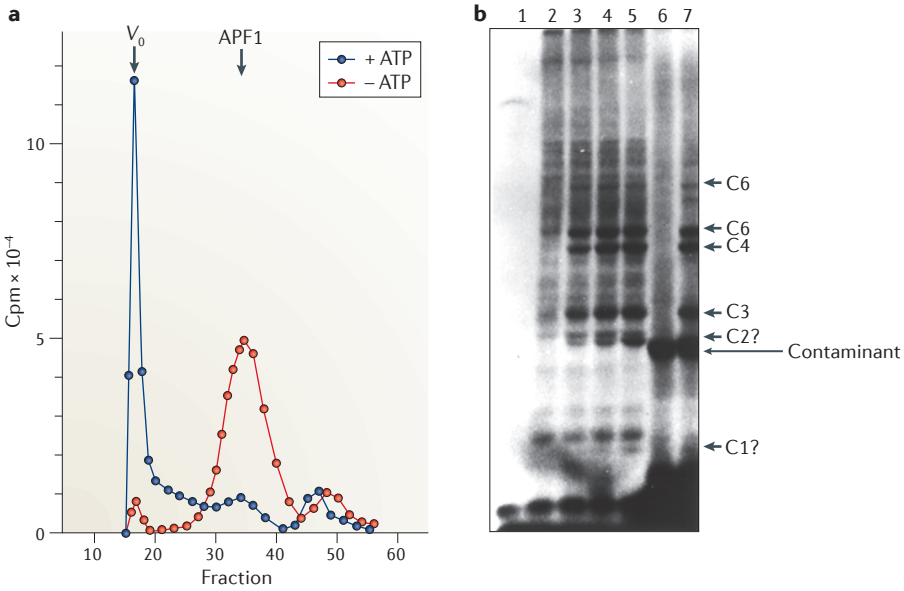

Figure 2 | **APF1 is covalently conjugated to proteolytic substrates, presumably marking them for degradation by a downstream protease. a** | ^{125}I-labelled ATP-dependent proteolytic factor 1 (APF1; later identified as ubiquitin) purified from fraction I (see TABLE 1) was incubated with crude fraction II in the absence or presence of ATP, and the mixtures were resolved by gel filtration chromatography. Shown are the radioactivity levels of the different fractions. The addition of ATP resulted in a shift of almost all the radioactivity to the high molecular-mass zone of the chromatographic separation. The arrow underneath APF1 points to the peak of radioactivity of the APF1 resolved from a system incubated without ATP (red), or to the remaining free APF1 left in a reaction mixture that was incubated with ATP (blue). **b** | ^{125}I-labelled APF1 purified from fraction I was incubated with crude fraction II in the absence (lane 1) or presence (lanes 2–5) of ATP. When increasing amounts of unlabelled lysozyme were added (lanes 3–5), new bands (conjugates, denoted by C1–C6) containing labelled APF1 appeared. To demonstrate that these newly formed bands also contained lysozyme, unlabelled APF1 was incubated with fraction II and ^{125}I-labelled lysozyme in the absence (lane 6) or presence (lane 7) of ATP. Bands of molecular mass similar to those in lanes 3–5 appeared. Presented is the autoradiogram of the SDS–PAGE-resolved reaction mixtures. The difference in molecular mass between adjacent conjugates is similar to the molecular mass of APF1, suggesting that multiple molecules of APF1 are conjugated to each protein target molecule. As we now know, this can be the result of either the generation of a polyubiquitin chain that is anchored to a single lysine residue or of the conjugation of several single ubiquitin moieties to multiple lysine anchors in the protein target (that is, multiple monoubiquitylations). Part **a** reprinted with permission from REF. 10, US National Academy of Sciences. Part **b** reprinted with permission from REF. 11, US National Academy of Sciences. Cpm, counts per million.

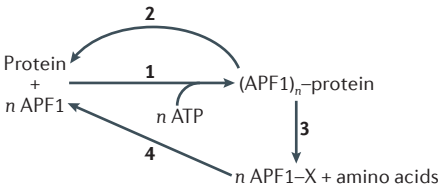

Figure 3 | **Model of the APF1 (ubiquitin)-mediated proteolytic pathway as proposed in 1980.** Step 1: a substrate protein is covalently conjugated by *n* molecules of ATP-dependent proteolytic factor 1 (APF1; later identified as ubiquitin) in an ATP-dependent reaction. It was later found that this step is catalysed by three types of enzyme that act in concert: a ubiquitin-activating enzyme (E1), ubiquitin-carrier proteins (E2 enzymes; also known as ubiquitin-conjugating enzymes (UBCs)) and ubiquitin-protein ligases (E3 enzymes). An E1 enzyme consumes two ATP molecules for each APF1 it activates. Step 2: APF1 can be removed (de-conjugated) in case the protein substrate was conjugated mistakenly, or in case it re-folds to its native form. This reaction was later found to be catalysed by deubiquitylating enzymes (DUBs). Step 3: the APF1-tagged substrate is degraded into free amino acids by a putative downstream protease, thereby releasing partially conjugated APF1 molecules (APF1–X; 'X' denotes a lysine residue or a short peptide derived from the substrate that is still bound to APF1). This reaction was later found to be catalysed by the 26S proteasome. We now know that the proteasome releases short peptides — not amino acids — that are later degraded to amino acids by cytosolic amino- and carboxy-peptidases. Step 4: the release of X by an amidase (now known as one of many DUBs) and the recycling of free APF1 for reuse by an E3 enzyme. Reprinted with permission from REF. 11, US National Academy of Sciences.

an internal lysine in the histone molecule[14]. Much later, this modification, which is mostly involved in gene silencing, was found to be a physiologically significant part of the 'epigenetic code' (REF. 15).

The convergence of identities of APF1 and ubiquitin, and the realization that ubiquitin can modify a protein by forming a peptide bond, helped us to understand the nature of the linkage between ubiquitin and the proteolytic target substrate, understand why ATP is required for this modification and to predict the existence of conjugation enzymes and machinery. From the terminally differentiating reticulocyte, the road took us to showing that the system is 'universal', and that ubiquitin mediates the degradation of proteins in nucleated cells as well[16]. The last two missing links in the chain of events leading to protein degradation were found shortly after. First, the conjugation machinery was shown to consist of three types of enzyme that act in concert: a ubiquitin-activating enzyme (E1), ubiquitin-carrier proteins (E2 enzymes; also known as ubiquitin-conjugating enzymes) and ubiquitin ligases (E3 enzymes)[17]. The second and last link was the identification of the 26S proteasome — a previously discovered protease complex with a then unknown function — as the proteolytic arm of the system[18,19]. Earlier, we had predicted this protease would specifically degrade ubiquitin-tagged proteins and recycle ubiquitin[11], a function that is now known to be carried out by deubiquitinases (DUBs), which are either integral to or associated with the proteasome, or by proteasome-independent DUBs.

The sequencing of the human genome in 2000 revealed the entire landscape of the ubiquitin system. It is made of ~1,500 components, many of which (~800) are E3 enzymes that recognize the myriad substrates of the system and endow it with its high specificity and selectivity. We now know that the modification of proteins by ubiquitin and ubiquitin-like proteins has important roles in almost all cellular processes, some of which are carried out by targeting proteins for degradation and others, such as the regulation of signalling, that are performed by non-proteolytic functions. Aberrations in the ubiquitin–proteasome system cause many diseases such as malignancies and neurodegenerative disorders. This has driven the development of drugs that modulate the activity of different components of the system.

The discovery of the ubiquitin system was the result of an attempt to solve a curiosity-driven question, of which many people either were unaware or did not consider important or biologically relevant. Technically, the experimental approach was based on embarrassingly simple biochemistry, involving setting the right output assay and purifying the basic components of the system. The core of success was the first experiment (TABLE 1), which taught us that it is not a 'classic' duo of protease–substrate that carries out the proteolysis, but rather a novel system that is potentially comprised of multiple components. We regard the BBRC article[9] as the most critical publication from which all other publications on the ubiquitin system emanated. Thinking of today's publication culture, one cannot help but conclude that it does not matter in which journal one publishes but rather what one publishes.

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Competing interests statement
The author declares no competing interests.