

Preface

The finding that cellular proteins are turning over—synthesized and degraded constantly—has traversed a torturous road from its discovery in the 1940s until it has reached its current central position as a major regulatory pathway. The dynamic state of the proteome was discovered by Rudolph Schoenheimer who used radiolabeled compounds to demonstrate that proteins are in a constant state of generation and destruction (1). Yet, the extent, the mechanisms and the physiological significance of roles of protein degradation have remained elusive for many years. Simpson reported that degradation of labeled proteins in liver slices requires metabolic energy (2). This—thermodynamically paradoxical finding—where investment of energy is still required for the degradation of energy-rich macromolecules—proteins—to low energy small molecules—amino acids—has been corroborated in many studies ever since in both eukaryotes and prokaryotes. Since proteolysis is an exergonic process, the requirement for energy had remained an enigma. Simpson tried to explain that “*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*”. He concluded however by saying that “*...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*” (2). The basic principle that cleavage of a peptide bond is exergonic, has not and could not have been challenged. Yet, the simple notion that proteases cannot exist in one compartment with their substrates without an energy barrier separating them, along with the high specificity of the process as we currently know it, makes energy investment in an

apparently exergonic process something we all accept now. Yet, the road to this acceptance has been long.

The discovery of the lysosome by Dr. Christian de Duve (reviewed in Ref. 3) has resolved some of the enigmas. Since the lysosome contains many acidic proteases, it was suggested that it must play a role in the degradation of intracellular proteins. Mortimore demonstrated a direct correlation between accelerated protein degradation that follows deprivation of nutrients to perfused liver, and increased lysosomal autophagy that is accompanied by a variety of structural alterations in the lysosomal system. Both the accelerated degradation and the structural changes could be reversed by re-supplementation of amino acids and hormones or serum (see for example Ref. 4). It was found that energy is required for activity of the lysosomal membrane proton pump that maintains the low intralysosomal pH necessary for optimal activity of the proteases (5). Different lines of experimental evidence along with the development of specific inhibitors strongly suggested that multiple pathways are involved in intracellular protein degradation, and the lysosome plays a role only in certain aspects of this process. Proteins were classified into short- and long-lived (reviewed in Refs. 6,7), but their different stability could not be explained based on the known mechanism of action of the lysosome that involves micro- and macroautophagy. During this process, entire droplets of cytosol and even subcellular organelles are engulfed with all the contained proteins digested at similar rates. While it was clear that lysosomal proteases are neither selective nor specific, certain studies still attempted to attribute specificity to lysosomal degradation. According to one model for example, all cellular proteins are engulfed into the lysosome, but only short-lived proteins that are sensitive to lysosomal proteases are degraded, whereas the more resistant, long-lived proteins escape back into the cytosol (8). The development of specific inhibitors of lysosomal proteases and of lysosomotropic agents—weak bases such as chloroquine or ammonium chloride—that are entrapped within the lysosome and increase the pH, thus inactivating lysosomal proteases—enabled researchers to examine in more detail the existence of distinct proteolytic pathways. Knowles and Ballard (9) and Neff and colleagues (10) demonstrated that leupeptin, antipain and chymostatin—specific lysosomal protease inhibitors—inhibit selectively degradation of long-lived but not of short-lived and abnormal proteins.

Poole and colleagues demonstrated that the lysosomotropic agent chloroquine selectively inhibits enhanced protein breakdown induced in cultured cells by depletion of serum, but has no effect on the degradation of cellular proteins under basal metabolic conditions (11). In an extremely elegant experiment, yet ingenious in its simplicity, he showed that chloroquine does not inhibit the degradation of endogenous cellular proteins that were metabolically labeled with ^3H -leucine, but at the same time and in the same ^3H -leucine-labeled cells, strongly inhibits the degradation of either endocytosed BSA, or endocytosed soluble cellular proteins that were prepared from identical cells metabolically labeled with ^{14}C -leucine (11). He concluded that intracellular proteins degraded under stress, or endocytosed/pinocytosed extracellular proteins are degraded within lysosomes following their engulfment from the cytosol or transfer from the cell membrane to the lysosome along the vacuolar system, respectively. In contrast, under basal metabolic conditions, intracellular proteins, and in particular short-lived ones, are degraded by a yet unidentified non-lysosomal system(s) (reviewed in Refs. 7 and 12).

To identify and characterize this non-lysosomal system, Etlinger and Goldberg chose the reticulocyte as a model system. This cell lacks lysosomes and is involved in extensive degradation of its organelles and enzymatic systems prior to maturation in the bone marrow and conversion to a circulating erythrocyte. They found that the reticulocyte contains an ATP-dependent proteolytic system that degrades abnormal, amino acid analog-containing, short-lived proteins (13). Working in parallel, Hershko and Ciechanover fractionated the reticulocyte extract and found that a small—~8.0 kDa—heat stable protein is necessary to reconstitute proteolysis of a model substrate in a crude lysate from which it was first removed during fractionation (14). The protein was designated ATP-dependent Proteolysis Factor-I (APF-1), as it became clear that the system contains several additional factors that may act in concert. Mechanistic studies revealed that multiple moieties of APF-1 are covalently conjugated—in an ATP-dependent mode—to the substrate (15). This surprising finding led the two researchers, along with Rose, to propose a model according to which degradation of a protein via the system involves two steps (i) conjugation of multiple molecules of APF-1 to the substrate, and (ii) degradation of the tagged substrate with release of reusable APF-1 (16). Parallel studies

identified APF-1 as ubiquitin, a known protein of hitherto unknown function (17, 18). Ubiquitin was discovered by Goldstein and colleagues as a protein that induces differentiation of B and T cells, and is ubiquitously distributed in prokaryotes and eukaryotes, hence its name (19). Later analyses revealed that it is not involved in regulating lymphocytes development, and that prokaryotes do not express it. Yet, the descriptive name was retained. An interesting finding related to ubiquitin was the identification by Busch and colleagues of the nucleolar protein A24 (see for example Ref. 20). Structural analyses (21,22) revealed that A24 has a unique bifurcated structure in which ubiquitin is conjugated—in an isopeptide bond—via its C-terminal Gly⁷⁶ to the ϵ -NH₂ group of Lys¹¹⁹ of histone 2A. The function of protein A24 has remained an unsolved mystery to our days. Yet, the finding that its level is decreased following hydrolysis to its two components, histone H2A and ubiquitin, during liver regeneration (23) or erythropoiesis (24), as well as a later finding that it is associated preferentially to nucleosomes that are localized at the 5' end of actively transcribed genes (25), led to the hypothesis that it plays a role in transcriptional regulation. As noted, all the changes observed in the level of the protein involve its hydrolysis and re-assembly and not degradation and resynthesis. In light of our current understanding of the ubiquitin system, this is because proteins that are modified by a single moiety of ubiquitin cannot be recognized by the 26S proteasome, the protease of the system (see below) that degrades only multiply ubiquitinated substrates. Identification of APF-1 as ubiquitin and the known structure of A24 led to the hypothesis that the C-terminal Gly residue of ubiquitin must be activated prior to its conjugation in a mechanism that is enzymatically similar to the activation of amino acids by aminoacyl tRNA-synthetase during ribosome-based polypeptide synthesis, or to the activation of amino acids during ribosome-free oligopeptide biosynthesis (see for example Ref. 26). Indeed, experiments in fractionated extracts showed that intermediates similar to those generated during amino acid activation, are generated also during activation of ubiquitin (27). Using the deciphered activation of ubiquitin, Ciechnaover and Hershko used immobilized ubiquitin to purify, via mechanism-based “covalent” affinity chromatography and reversal of the activation reaction, the first enzyme in the ubiquitin pathway cascade, the ubiquitin-activating enzyme, E1 (28). Purification of the two other enzymes in the ubiquitin relay reaction, the

ubiquitin-carrier protein, E2 (later designated also the ubiquitin-conjugating enzyme, UBC), and the ubiquitin-protein ligase, E3 (29) followed shortly after. The many members of the E3 family bind the target substrates via defined motifs and endow the system with its high specificity. Generation of antibodies to ubiquitin allowed to demonstrate, for the first time, that the system is active also in nucleated cells *in vivo* and not only in the terminally differentiating reticulocyte—the model cell studied initially: a direct correlation was observed between levels of ubiquitin adducts and rates of abnormal protein degradation induced by incubation of the cells in the presence of amino acid analogues (30). Stronger and more direct evidence was later obtained by Varshavsky, Finley and Ciechanover, who characterized a known cell cycle arrest mutant that loses A24 at the non-permissive temperature. They identified the mutation as a thermolabile E1 that, when inactive, cannot re-conjugate ubiquitin to histone 2A (31). Heat inactivation of the enzyme leads to severe impairment in the degradation of short-lived abnormal proteins generated during incubation of the cells in the presence of amino acids analogs (32). The original observation by Yamada and colleagues that loss of the thermolabile E1 leads to arrest at the S/G2 phase, enabled the three researchers to predict that the ubiquitin system is required for cell cycle progression. This hypothesis was later corroborated by numerous studies demonstrating ubiquitin intermediacy in the degradation of many cell cycle regulators. Later studies led to the discovery that it is a polyubiquitin chain—in which the ubiquitin moieties are linked to one another—that generates the high molecular mass adducts and the proteolytic signal (33). Beforehand, a formal possibility still existed that the high molecular mass adducts represent multiple single moieties attached to distinct lysine residues. The chain is composed of ubiquitin moieties that are linked to one another via an isopeptide bond between the C-terminal Gly76 of one ubiquitin moiety and an internal Lys48 of the previously conjugated moiety (34). In parallel, the downstream protease—the 26S proteasome complex—was discovered (35-37), and the entire pathway could be studied in different experimental systems.

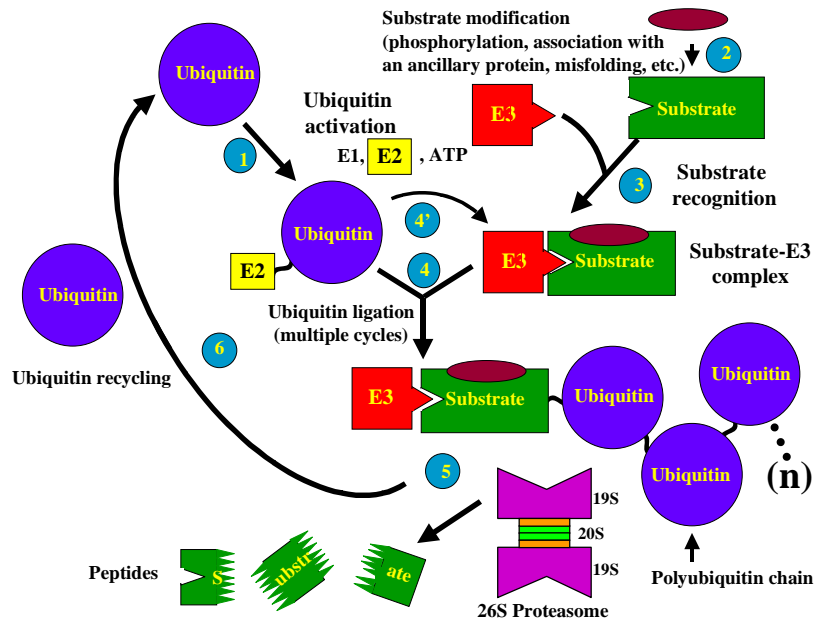
The first insights on the problem of specific substrate recognition began to emerge at that time. Using a biochemical approach, Hershko demonstrated that the N-terminal residue may play a role in recognition of certain model substrates (38), and that recognition is mediated via binding

of this residue to the ubiquitin ligase (39). Ciechanover showed that tRNA-Arg is required for the degradation of certain proteins (40) that have acidic N-termini. Arg-tRNA-protein transferase catalyzes a reaction that adds an Arg residue to the acidic N-terminal residue, and the conversion of the charge allows recognition of the substrate by the ubiquitin ligase E3 α (41). Varshavsky used a genetic approach in yeast to generate 20 distinct species of a derivative of β -galactosidase that differed solely in the identity of the N-terminal residue. Yet, the stability of the proteins varied significantly, which led to the formulation of a rule, the N-end rule, according to which the identity of the N-terminal residue determines the stability of the protein (42). Later studies revealed that the N-terminal recognition signal contains adjacent lysine residues that serve as ubiquitination anchors (43). Recognition of the N-terminal residue cannot provide a general targeting mechanism since the N-terminal residue of most substrates is not accessible for recognition by the ligase as it is acetylated (44). We now know that recognition of proteins by the ubiquitin system is far more complex than originally thought. Proteins are targeted by multiple ligases following recognition of different primary and secondary motifs, post-translational modifications and recognition in *trans* mediated by association with ancillary proteins. Yet, the discovery of N-terminal recognition, the first targeting motif, was of great importance, as it drew attention to a centrally important problem—the requirement for specific recognition of the substrates.

These early studies did not change the prevailing view that the main role of the system is to rid the cell from mutated/misfolded/abnormal proteins and the research focus remained on the puzzle of how the system selectively recognizes and eliminates abnormal proteins, leaving intact their normal counterparts, when the differences can be minute and sometime indiscernible. It was not until the early 1990s—when researchers started to discover that specific key cellular proteins, such as transcriptional and cell cycle regulators, are targeted by the system in a regulated manner (see for example Refs. 45-49)—that we have begun to see an exponential growth in the number of published studies and a general recognition of the role of the system in basic cellular processes such as regulation of transcription, cell cycle progression, growth and differentiation, the immune and inflammatory responses, and quality control. Not too long after that researchers started to

realize that aberrations in such a complex pathway underlie the pathogenesis of many diseases, both inherited and acquired. The discoveries of ubiquitin-like proteins and their role in non-proteolytic functions such as routing of certain proteins to their subcellular destinations or protecting others from ubiquitination and destruction, of mono-ubiquitination and its role in regulating the endocytic pathway, and of polyubiquitin chains that involve residues other than Lys48 in transcriptional regulation, have expanded the scope of ubiquitin conjugation beyond degradation and set it in a centrally important position among other regulatory mechanisms. The evolution of two distinct regulatory mechanisms, phosphorylation that is reversible and proteolysis that is irreversible, has been inevitable evolutionarily. For certain processes such as cell cycle progression, the unidirectional movement along a “one way” road must be controlled in a tight manner. Like the wife of Lot that on her way from Gomorrah “*looked back from behind him and she became a pillar of salt*” (Bible, Torah, Genesis, 19, 26), the cell cycle cannot look or go back.

Where is research on the ubiquitin system heading now? Important knowledge is still missing on the specific function of the E3s and their substrates. It is likely that, based on the recognition of common structural motifs such as the HECT domains, RING fingers and U-boxes, the human genome will unravel hundreds of novel ligases and lead to the discovery of their substrates, the processes involved, and the aberrations caused by selective malfunction of these enzymes. Some of these enzymes will have auto-ubiquitinating activity that may serve as a regulatory, “self-destructive” mechanism, others will have both *cis* and *trans* activities. Does BRCA1 have specific substrates? Which processes are derailed by its mutation? These and many other questions still await answers. Resolving the 3D structure of the ligases with their substrate may aid in developing mechanism-based specific drugs that will interfere with specific processes. Protease inhibitors are already making their way as potential drugs against many diseases such as malignancies and immune and inflammatory disorders (see for example Refs. 50, 51), yet they clearly cannot be specific and rely for their activity on a narrow toxicity window. Drugs that target specific ligases will affect a narrower subset of substrates. Better and even more specific drugs may be those that will interfere specifically with the



The ubiquitin proteasome pathway. (1) ATP-dependent activation of ubiquitin by the ubiquitin activating enzyme, E1, and by a ubiquitin-carrier protein (ubiquitin-conjugating enzyme, UBC), E2, to generate a high-energy E2-ubiquitin intermediate. (2) Modification of the substrate (phosphorylation or oxidation, for example), its association with an ancillary protein (chaperone or a viral protein, for example) or its misfolding are required for its recognition and specific binding to the ubiquitin ligase, E3 (3). (4) Generation of a substrate-anchored polyubiquitin chain catalyzed by direct transfer of the ubiquitin moiety from the E2-ubiquitin complex to the E3-bound substrate (RING finger E3s). (4') Generation of a substrate-anchored polyubiquitin chain catalyzed by transfer of the ubiquitin moiety from the E2-ubiquitin complex to the E3 to generate an additional E3-ubiquitin high energy intermediate from which the activated ubiquitin moiety is transferred to E3-bound substrate [catalyzed by HECT (*H*omologous to *E*6-*A*P *C*-*T*erminus) domain E3s]. U-box-containing E3s have also been described, but their mechanism of function has not been discerned. (5) Degradation of the polyubiquitinated substrate by the 26S proteasome complex with release of free and reusable ubiquitin (6) catalyzed by ubiquitin recycling enzymes (ubiquitin C-terminal hydrolases; deubiquitinating enzymes, DUBs; isopeptidases).

interaction of substrates with ancillary exogenous proteins, such as p53-E6 interaction (52). Interference with endogenous ancillary proteins such as molecular chaperones may prove to be extremely toxic. An additional line of research will involve dissection of non-proteolytic functions of ubiquitin and ubiquitin-like proteins, the requirements for specific substrate recognition and the role of ligases in these processes. All this new knowledge will not only broaden our basic knowledge on the ubiquitin system, but will drive the system from the test tube to the patient bed.

This conference has told us the story of the ubiquitin system, as we currently know it, glowing and shiny. From regulation of basic cellular processes such as cell cycle progression and transcription, through quality control and the pathogenetic mechanisms of disease, from X-ray crystallography of the 26S proteasome, to the interaction between substrates and their ligases, to the development of mechanism-based drugs to target specific aberrant processes. But this is the epilogue. It started differently. The history of intracellular protein degradation is an illuminating example of a modern “*Cinderella*”. She started her life in the garbage, literally, helping the cell cleaning it. Carving her way up the mountain, she taught us several important lessons. One is that cleaning garbage is a respected trade. Accumulation of mutated/misfolded/aggregated proteins underlies the pathogenesis of many diseases, including several neurodegenerative disorders such as Huntington’s disease. Maintaining the steady state level of growth stimulators, such as β -catenin and HIF-1 α , or tumor suppressors such as p53, is also essential. Accumulation of the first and accelerated degradation of the latter has been implicated in the pathogenesis of several malignancies. Finally, she taught us that normal proteins have to be destroyed as well: programmed destruction of cyclins allows cell cycle progression, whereas removal of transcriptional activators and their inhibitors, regulates specific gene expression. As King Solomon taught us thousands years ago “*To everything there is a season and a time to every purpose: A time to be born, and a time to die; a time to plant, and a time to pluck up that which is planted; a time to kill, and a time to heal; a time to break down, and a time to build up*” (Bible, Hagiograph, Ecclesiastes, 3, 1–3).

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