

Proteases: Multifunctional Enzymes in Life and Disease*^[5]

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Carlos López-Otín[‡] and Judith S. Bond^{§1}

From the [‡]Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, 33006 Oviedo, Spain and the [§]Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Our view of proteases has come a long way since P. A. Levene reported his studies on “The Cleavage Products of Proteoses” in the first issue of *The Journal of Biological Chemistry* published October 1, 1905 (1). Today, after more than 100 years and 350,000 articles on these enzymes in the scientific literature, proteases remain at the cutting edge of biological research.

Proteases likely arose at the earliest stages of protein evolution as simple destructive enzymes necessary for protein catabolism and the generation of amino acids in primitive organisms. For many years, studies on proteases focused on their original roles as blunt aggressors associated with protein demolition. However, the realization that, beyond these nonspecific degradative functions, proteases act as sharp scissors and catalyze highly specific reactions of proteolytic processing, producing new protein products, inaugurated a new era in protease research (2). The current success of research in this group of ancient enzymes derives mainly from the large collection of findings demonstrating their relevance in the control of multiple biological processes in all living organisms (3–11). Thus, proteases regulate the fate, localization, and activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, transduce, and amplify molecular signals. As a direct result of these multiple actions, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, hemostasis, blood coagulation, inflammation, immunity, autophagy, senescence, necrosis, and apoptosis. Consistent with these essential roles of proteases in cell behavior and survival and death of all organisms, alterations in proteolytic systems underlie multiple pathological conditions such as cancer, neurodegenerative disorders, and inflammatory and cardiovascular diseases. Accordingly, many proteases are a major focus of attention for the pharmaceu-

tical industry as potential drug targets or as diagnostic and prognostic biomarkers (12). Proteases also play key roles in plants and contribute to the processing, maturation, or destruction of specific sets of proteins in response to developmental cues or to variations in environmental conditions (13). Likewise, many infectious microorganisms require proteases for replication or use proteases as virulence factors, which has facilitated the development of protease-targeted therapies for diseases of great relevance to human life such as AIDS (12). Finally, proteases are also important tools of the biotechnological industry because of their usefulness as biochemical reagents or in the manufacture of numerous products (e.g. Ref. 14).

This outstanding diversity in protease functions directly results from the evolutionary invention of a multiplicity of enzymes that exhibit a variety of sizes and shapes. Thus, the architectural design of proteases ranges from small enzymes made up of simple catalytic units (~20 kDa) to sophisticated protein-processing and degradation machines, like the proteasome and meprin metalloproteinase isoforms (0.7–6 MDa) (15). In terms of specificity, diversity is also a common rule. Thus, some proteases exhibit an exquisite specificity toward a unique peptide bond of a single protein (e.g. angiotensin-converting enzyme); however, most proteases are relatively nonspecific for substrates, and some are overtly promiscuous and target multiple substrates in an indiscriminate manner (e.g. proteinase K). Proteases also follow different strategies to establish their appropriate location in the cellular geography and, in most cases, operate in the context of complex networks comprising distinct proteases, substrates, cofactors, inhibitors, adaptors, receptors, and binding proteins, which provide an additional level of interest but also complexity to the study of proteolytic enzymes.

This work aims at serving as a primer to a minireview series on proteases to be published in forthcoming issues of this Journal. This introductory article will focus on the discussion of the large and growing complexity of proteolytic enzymes present in all organisms, from bacteria to man. We will first show the results of comparative genomic analysis that have shed light on the real dimensions of the proteolytic space. The levels of protease complexity and mechanisms of protease regulation will then be addressed. Finally, we will discuss current frontiers and future perspectives in protease research.

The Vast Proteolytic Landscape

Proteases are the efficient executioners of a common chemical reaction: the hydrolysis of peptide bonds (16). Most proteolytic enzymes cleave α -peptide bonds between naturally occurring amino acids, but there are some proteases that perform slightly different reactions. Thus, a large group of enzymes known as DUBs (deubiquitylating enzymes) can hydrolyze isopeptide bonds in ubiquitin and ubiquitin-like protein conjugates; γ -glutamyl hydrolase and glutamate carboxypeptidase target γ -glutamyl bonds; γ -glutamyltransferases both transfer and cleave peptide bonds; and intramolecular autoproteses (such as nucleoporin and polycystin-1) hydrolyze only a single bond on their own polypeptide chain but then lose their proteolytic activity. Notably and under some conditions, proteases can also synthesize peptide bonds.

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

¹ To whom correspondence should be addressed. E-mail: jbond@psu.edu.

Proteases were initially classified into endopeptidases, which target internal peptide bonds, and exopeptidases (aminopeptidases and carboxypeptidases), the action of which is directed by the NH₂ and COOH termini of their corresponding substrates. However, the availability of structural and mechanistic information on these enzymes facilitated new classification schemes. Based on the mechanism of catalysis, proteases are classified into six distinct classes, aspartic, glutamic, and metalloproteases, cysteine, serine, and threonine proteases, although glutamic proteases have not been found in mammals so far. The first three classes utilize an activated water molecule as a nucleophile to attack the peptide bond of the substrate, whereas in the remaining enzymes, the nucleophile is an amino acid residue (Cys, Ser, or Thr, respectively) located in the active site from which the class names derive (supplemental Fig. 1). Proteases of the different classes can be further grouped into families on the basis of amino acid sequence comparison, and families can be assembled into clans based on similarities in their three-dimensional structures. Bioinformatic analysis of genome sequences has been decisive for establishing the dimensions of the complexity of proteolytic systems operating in different organisms (Fig. 1). The last release of *MEROPS* (merops.sanger.ac.uk), a comprehensive data base of proteases and inhibitors, annotates 1008 entries for human proteases and homologs, although it includes a large number of pseudogenes and protease-related sequences derived from endogenous retroviral elements embedded in our genome. A highly curated data base, the Degradome Database, which does not incorporate protease pseudogenes or these retrovirus-derived sequences, lists 569 human proteases and homologs classified into 68 families (17). Metalloproteases and serine proteases are the most densely populated classes, with 194 and 176 members, respectively, followed by 150 cysteine proteases, whereas threonine and aspartic proteases contain only 28 and 21 members, respectively.

The recent availability of the genome sequence of different mammals has allowed the identification of their entire protease complement (termed degradome) and their detailed comparison with humans (Fig. 1). The chimpanzee degradome is very similar to the human degradome, although it exhibits some remarkable differences in immune defense proteases like caspase-12 (18). Interestingly, mice and rats contain more protease genes (644 and 629, respectively) compared with humans despite the fact that their genomes are smaller (19, 20). These differences derive mainly from the expansion in rodents or the inactivation in humans of members of protease families (such as kallikreins and placental cathepsins) involved in immunological and reproductive functions (21, 22). The recent analysis of the degradome of other mammals such as the duck-billed platypus (*Ornithorhynchus anatinus*) has revealed some interesting findings on protease evolution. This fascinating monotreme also has more than 500 protease genes but lacks all genes encoding gastric pepsins, which are the archetypal digestive proteases widely conserved in all mammals (23). Birds, amphibians, and fish also contain large numbers of protease genes (382 in *Gallus gallus*, 278 in *Xenopus tropicalis*, and 503 in *Danio rerio*), although the protease annotation work in these species has not been as detailed as in mammals. Surprisingly, analysis of the protease content of invertebrates such as *Drosophila melanogaster* (a model organism with a gene content considerably lower than that in vertebrates) has shown the presence of more than 600 pro-

tease genes (24). The model plant *Arabidopsis thaliana* contains at least 723 protease-encoding genes, whereas a total of 955 protease genes have been annotated in the tree *Populus trichocarpa*. These marked differences are linked to the expansion of some protease families in *Populus*, especially the copia transposon endopeptidase family of aspartic proteases, which has 20 components in *Arabidopsis* and 123 in *Populus* (13). Genomic analyses have also shown that plants share with prokaryotes a set of serine proteases absent in other eukaryotes, which may be an indication of ancient endosymbiotic events leading to evolution of chloroplasts (25). Finally, there is a growing interest in analyzing the degradome of bacteria, viruses, fungi, and parasites as part of strategies aimed to define novel targets for therapeutic intervention (26–28). In this regard, the *MEROPS* Database annotates more than 100 protease genes in the genome of bacteria such as *Yersinia pestis* and *Legionella pneumophila* or in the malaria parasite *Plasmodium falciparum*, which cause devastating human diseases.

In summary, the emerging pattern derived from the global analysis of proteolytic systems is one of diversity and multiplicity. These comparative genomic studies have also provided valuable insights into the conservation, evolution, and functional relevance of this group of enzymes. Thus, it has become evident that, in addition to proteolytic routines conserved in all organisms, there are also specific roles played by unique proteases in different species. Nevertheless, further studies will be necessary to clarify the genetic and molecular basis underlying the evolutionary differences in the complex protease repertoire of all living forms.

Levels of Protease Complexity

Proteolytic enzymes are not mere catalytic devices working in isolation in their search for substrates to be hydrolyzed. Thus, many proteases link their catalytic domains to a variety of specialized functional modules or domains that provide substrate specificity, guide their cellular localization, modify their kinetic properties, and change their sensitivity to endogenous inhibitors. These non-catalytic domains include archetypal sorting signals that direct these enzymes to their proper location, autoinhibitory prodomains that prevent premature activation, and ancillary domains that facilitate homotypic interactions or heterotypic contacts with other proteins, substrates, receptors, or inhibitors. Some of these ancillary domains (like the epidermal growth factor domains) have been very successful in their incorporation into proteases and are present in a variety of enzymes from different families, whereas other domains (such as the thrombospondin repeats of ADAMTSs) have expanded within the same enzyme, forming long tandem repeats (29). Other proteases, including diverse members of the type II transmembrane serine protease family, exhibit a complex mosaic structure with up to six distinct ancillary domains located within a single polypeptide chain (30). This exuberant strategy of domain accretion and shuffling has also led to the creation of very peculiar structures, including protease-inhibitor chimeras or proteases with different catalytic units embedded in the same polypeptide chain (31). It is very likely that the substantial combinatorial activity observed in protease genes has been a driving force in the protease transition from nonspecific primitive enzymes to highly selective catalysts responsible for subtle proteolytic events that are at the heart of multiple biological processes.

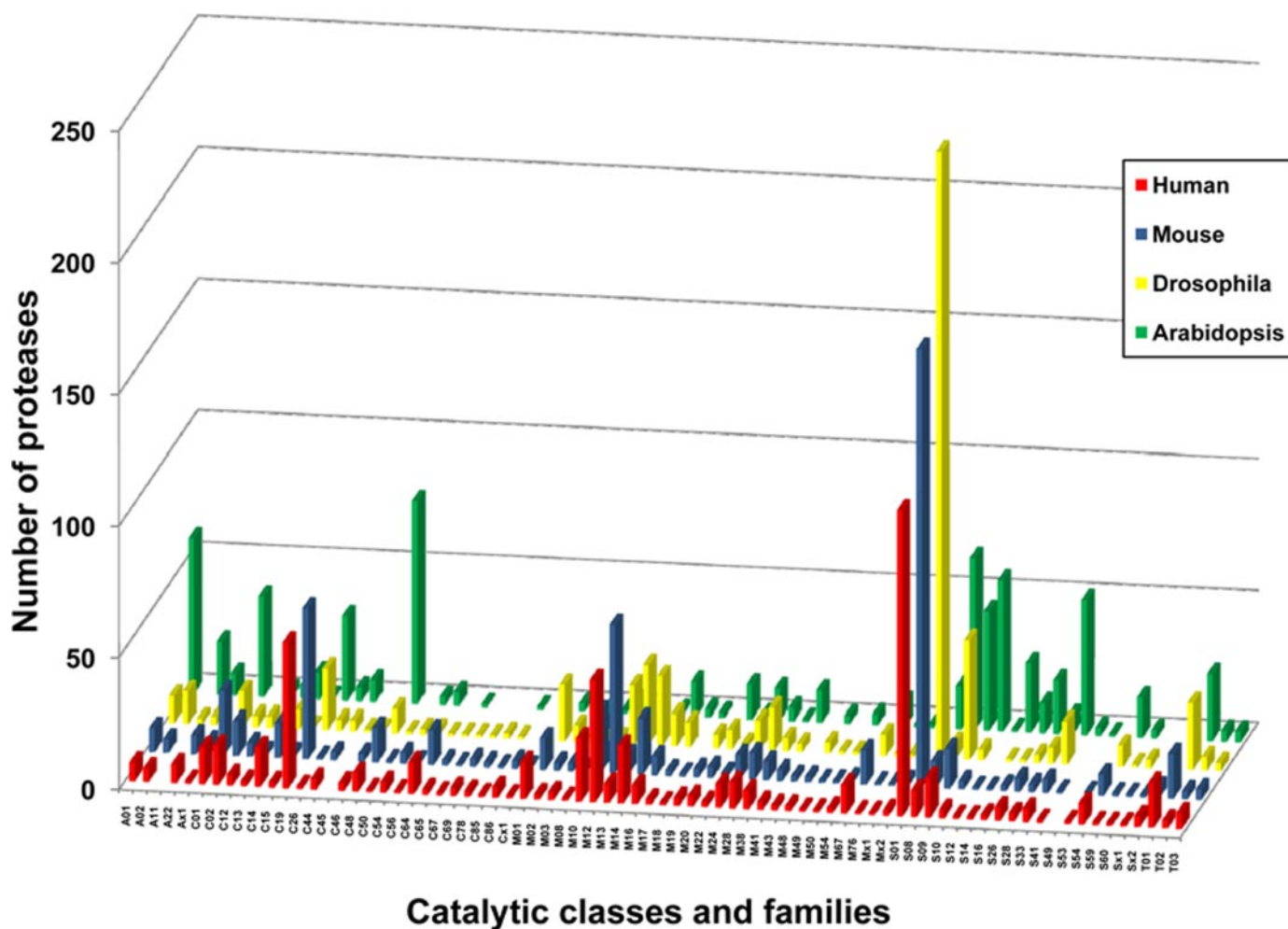


FIGURE 1. **Global view of the proteolytic landscape in representative eukaryotic genomes.** Proteases from human, mouse, *Drosophila*, and *Arabidopsis* are shown distributed in catalytic classes and families of related members. Catalytic classes are indicated as aspartic (A), cysteine (C), threonine (T), and serine (S) proteases and metalloprotease (M), and the associated numbers correspond to the different families of proteases belonging to each catalytic class. For example, there are only five distinct evolutionary families within the aspartic protease class, whereas there are 27 families in the metalloprotease class. The y axis shows the number of individual proteases identified thus far within a family for each species. For example, for the human aspartic protease family A01, there are five proteases, whereas in *Arabidopsis*, there are 55 members in this family. Data for human and mouse proteases are from Ref. 17 and the Degradome Database; data for *Arabidopsis* are from Ref. 13; and data for *Drosophila* are from the MEROPS Database (merops.sanger.ac.uk).

The complexity of proteases is further increased through post-transcriptional events such as alternative splicing and differential polyadenylation of genes encoding proteases (32, 33), by the occurrence of gene copy number variations or polymorphic variants that may contribute to the modification of protease functions or alter their regulatory mechanisms (34, 35), or by post-translational modifications such as glycosylation and phosphorylation. Finally, we must emphasize that, in many cases, proteases act in the context of complex cascades, pathways, circuits, and networks, comprising many protein partners that dynamically interact to form the so-called protease web (36). Accordingly, to understand the role of a certain protease in a given biological or pathological process, we must identify the mechanisms that regulate the expression and activity of the different enzymes and try to place them in the context of the multiple components that can influence its activity.

Mechanisms of Protease Regulation

Proteolytic processing represents an excellent strategy for increasing the diversity of the limited protein repertoire encoded

in the genome of any living system. However, in contrast to enzymes involved in other post-translational modifications, proteases catalyze essentially irreversible hydrolytic reactions, and consequently, they must be strictly regulated. The action of proteases can be controlled *in vivo* by several mechanisms: regulation of gene expression; activation of their inactive zymogens; blockade by endogenous inhibitors; targeting to specific compartments such as lysosomes, mitochondria, and specific apical membranes; and post-translational modifications such as glycosylation, metal binding, S–S bridging, proteolysis, and degradation.

To date, transcriptional mechanisms regulating gene expression are largely unknown for most proteases, although in some specific protease families of great relevance for human disease, such as matrix metalloproteinases, detailed information is already available about the variety of hormones, growth factors, cytokines, and chemokines controlling their expression in both normal and pathological conditions (37). The promoter regions of some of these genes have also been characterized, which has facilitated the identification of transcription factors such as Fos, Jun, NF- κ B, and

Cbfa1 and epigenetic mechanisms that mediate changes in the expression levels of these enzymes (38).

The activation of inactive protease precursors can be either autocatalytic or catalyzed by other proteases, although in some cases, protease activation requires additional factors or platforms such as the apoptosome, which mediates the activation of proapoptotic caspases (39). Protease activation may also be modulated by protein cofactors such as the tissue factor glycoprotein that binds to serine protease factor VIIa and initiates the coagulation cascade (40). Substrate-driven allosteric mechanisms of protease activation without prodomain cleavage have also been proposed for some metalloproteases (41).

All known endogenous protease inhibitors are proteins, although some microorganisms produce small non-protein inhibitors that block the proteolytic activity of host proteases. To date, the number of identified endogenous inhibitors is considerably lower than that of proteases. As an illustrative example, a total of 183 genes encoding protease inhibitors have been annotated in the rat genome, which markedly contrasts with the more than 600 protease genes present in this species (20). This unbalanced situation derives in part from the relaxed specificity of several inhibitors toward their target proteases, although there are also many proteases that are not blocked by any endogenous inhibitor, as their proteolytic activities are regulated at other levels. Protease inhibitors have been classified into families of structurally related members or according to the catalytic class of proteases targeted by them. Nevertheless, this classification is hampered by the occurrence of both compound inhibitors that contain inhibitor units of different protease classes and pan-inhibitors (such as α_2 -macroglobulin) that target enzymes of different classes through a trapping reaction induced after inhibitor cleavage by the targeted protease (42). Protease inhibitors can also be classified into four groups according to their mechanism of inhibition (12, 43). The canonical inhibitors, including serpins (serine protease inhibitors), block the active site of their target proteases through binding in a virtually substrate-like manner. By contrast, exosite-binding inhibitors like cystatins and some thrombin inhibitors bind a region adjacent to the active site, thereby preventing substrate access to this center but without directly blocking the catalytic residues. A third group of protease inhibitors, including TIMPs (tissue inhibitors of metalloproteinases), uses an intermediate mechanism based on a combination of the canonical and exosite-binding mechanisms. Finally, allosteric inhibitors (such as X-linked inhibitor of apoptosis protein, a caspase inhibitor) bind a region that is distantly located from the active site, but this binding prevents dimerization of the target protease and blocks its activity.

In addition to these main regulatory mechanisms, proteolysis may also be regulated or fine-tuned by epigenetic changes in the promoter regions of protease genes, control of mRNA stability, translation and degradation by transacting factors such as RNA-binding proteins and microRNAs, spatial and temporal protease compartmentalization, substrate interaction with inactive protease homologs that act as protease antagonists, shedding of substrate-binding domains, oligomerization, cellular internalization, and finally, autolysis reactions that lead to the termination of proteolytic activities. All these mechanisms must operate in a coordinate manner to assure that the correct substrates are processed at the right moment and in the appropriate environment, thereby

preventing the potentially harmful actions of uncontrolled proteases on living systems. Over the past years, our understanding of regulatory mechanisms acting at the level of individual proteases has considerably improved, but limited information is available on the global regulation of proteolytic systems. The emergence of high-throughput methodologies for profiling proteases in different organisms will contribute to define the regulatory mechanisms operating in the precise and dynamic control of the protease web.

Frontiers and Perspectives

At the beginning of the post-genome era, a large body of information is available about the composition and organization of proteolytic systems in many living organisms. These global genomic views have revealed that the protease landscape is vast and quite unexplored. Therefore, it is very likely that the size of the different degradomes will grow in the near future, as new enzymes with unusual structural designs and catalytic mechanisms are identified and characterized. The recent finding of two novel and evolutionarily conserved cysteine proteases called UfSP1 and UfSP2 represents an example of experimental work that has led to the unmasking of "hidden proteases" that had remained invisible to homology-based screening methods (44). Many newly identified proteases remain as *in silico* predictions without experimental evidence for enzymatic activity. A major challenge for the future will be to demonstrate enzymatic properties for these predicted proteases. The comparative genomic studies have also provided interesting information about conservation, neofunctionalization, and subfunctionalization events in the protease field. Thus, the lineage-specific expansion of reproductive proteases in rodents may help to explain some of the pronounced reproductive differences between mammalian species, whereas changes in immune-related proteases may reflect evolutionary diversification of host defense mechanisms in response to new environmental conditions (45).

In relation to the relevance of proteases for human disease, genomic studies will contribute to the elucidation of genetic diseases caused by mutations in protease loci as well as to the identification of protease gene polymorphisms associated with an increased susceptibility to certain diseases. These will provide excellent opportunities to design new generations of therapeutic inhibitors, such as those recently developed for targeting the proteasome in multiple myeloma (46) or dipeptidyl peptidase IV in type II diabetes (47).

Recent advances in different fields have also converged in the development of innovative strategies to profile the expression and activity levels of the multiple proteases present in complex cellular samples. Oligonucleotide microarrays, activity-based probes, and different fluorescence-based assays, including quantum dot-peptide conjugates, have been recently used for profiling and monitoring protease levels and activity. Novel methods are also being introduced to identify the *in vivo* substrates targeted by individual enzymes, a crucial step toward the functional characterization of those orphan proteases for which a biological role is yet unknown. The currently available strategies for de-orphaning proteases and identifying their *in vivo* functions are as diverse as the proteases themselves, although a first step toward this goal is frequently based on the determination of consensus cleavage sites for a protease by using phage-displayed peptide libraries, combinatorial fluorogenic substrate libraries, positional scanning synthetic

libraries, or mRNA-displayed protein libraries (48). Nevertheless, these methods only provide information about peptide sequences that can be cleaved but do not demonstrate that they are actually cleaved in their natural context, thus making necessary the utilization of additional approaches for linking a protease to its specific substrates. These approaches can be classified into two general categories: *ex vivo* proteomics-based methods and *in vivo* genetics-based methods (48). The genetic studies to identify *in vivo* protease substrates are usually based on the detection of non-processed substrates accumulated in tissues of knock-out mice deficient in specific proteases. Similar studies in other model organisms such as *C. elegans*, *D. melanogaster*, and *A. thaliana* have also allowed the identification of *in vivo* substrates of proteases (48), although these genetic strategies are hampered by the occurrence in most proteolytic systems of redundant and compensatory activities. A powerful alternative to loss-of-function animal models for substrate identification derives from the application of RNA interference techniques to the protease field (49). Nevertheless, it is unlikely that a single methodology will be sufficient to identify the substrates targeted by specific proteases under *in vivo* conditions. A system-wide approach termed degradomics (3) and involving the combination of biochemical studies, genetic tactics, cell-based assays, and proteomic methods will be necessary in the quest for the natural substrates of the multiple orphan proteases still present in all organisms. Degradomic studies will also be essential to define the regulatory and functional connections between all different components of proteolytic systems that form the protease web.

Finally, the detailed analyses of complex protease-mediated processes such as proteolytic regulation of transcription factor activity, protein ectodomain shedding, and regulated intramembrane proteolysis are challenges to be addressed in the near future. Hopefully, through a series of articles focused on structural and functional aspects of proteolytic systems as well as on the analysis of relevant biological processes regulated by them, this mini-review series will provide a current view of this complex group of protein sculptors that decisively influence the rhythms of cell life and death in all living forms.

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