Table I. Protein family World Wide Web sites

Protein family	Uniform resource locator
AAA	http://yeamob.pci.chemie.uni-tuebingen.de/AAA/Description.html
Chaperonins	http://bioc09.uthscsa.edu/~seale/Chap/chap.html
Cytokines and receptors	http://www.ocms.ox.ac.uk/~smb/cyt_web/
Esterases	http://ensam.inra.fr/cholinesterase/
G protein-coupled receptors	http://receptor.mgh.harvard.edu/GCRDBHOME.html
Glucoamylases	http://www.public.iastate.edu/~pedro/glase/glase.html
Glucocorticoid receptors	http://biochem1.basic-sci.georgetown.edu/GRR/GRR.HTML
Glycotransferases	http://bellatrix.pcl.ox.ac.uk/people/iain/glycosyltransferase.html
Histocompatibility proteins	http://histo.cryst.bbk.ac.uk/
Homeobox proteins	http://copan.bioz.unibas.ch/homeo.html
Kinesins	http://www.blocks.fhcrc.org/~kinesin/
Mutator transposons	http://www-leland.stanford.edu/~jeisen/Mutator/Mutator.html
Olfactory receptors	http://paella.med.yale.edu/cgi-bin/receptor_top/DB_CGI.p/query?FORM%3bHOME
P450-containing systems	http://base.icgeb.trieste.it/p450/
Protein kinases	http://www.sdsc.edu/projects/kinases/kinase_intro.html
RecA proteins	http://www-leland.stanford.edu/~jeisen/RecA/RecA.html
SNF2	http://www-leland.stanford.edu/~jeisen/SNF2/snf2.html
Thyroid hormone receptors	http://xanadu.mgh.harvard.edu/receptor/trrfront.html
Topoisomerases	http://ellington.pharm.arizona.edu/~bear/top/topo.html

At this time, we are aware of about 20 WWW sites dedicated to specific protein families and that provide up-to-date documentation and useful links (Table I). Protein family databases, such as the Blocks database, now have links to these sites to provide access for interpretation of database search results. Any biologist interested in setting up a site for other protein families can contact us (kinesin@sparky.fhcrc.org) for further information on the proWeb network.

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Lessons from the discovery of the ubiquitin system

It is now well known and widely accepted that, in eukaryotic cells, many proteins are targeted for degradation by covalent ligation to ubiquitin. It is also recognized that the ubiquitin system carries out the selective degradation of many important regulatory proteins. Notable examples are cell-cycle regulators, such as mitotic cyclins[1,2], G1 cyclins[3,4], some inhibitors of cyclin-dependent kinases[5,6] and proteins whose degradation is required for the onset of anaphase[7,8]. Other important regulatory proteins whose levels are controlled by ubiquitin-mediated degradation include the p53 tumor suppressor[9], the transcriptional regulator NF-κB and its inhibitor IκBα (Refs 10, 11), the *mos* proto-oncogene[12] and many transcription factors (reviewed in Refs 13, 14).

It has become increasingly evident that the specific and programmed degradation of short-lived regulatory proteins is a recurrent theme in temporally controlled processes. This seemingly wasteful way of destroying protein regulators might be essential to ensure irreversibility. Thus, ubiquitin-mediated protein degradation is of considerable current interest. Yet, not many are aware of how obscure this field was less than 20 years ago. Some imaginative models have been proposed to account for the selectivity of intracellular protein breakdown, such as one suggesting that all cellular proteins are rapidly engulfed by the lysosome, but only short-lived proteins are degraded in the lysosome, while long-lived proteins escape back to the cytosol[15]. The story of the discovery of the ubiquitin system might be instructive.

I became interested in the problem of how proteins are degraded in cells when I was a postdoctoral fellow in the laboratory of Gordon Tomkins at the University of California, San Francisco, between 1969 and 1971. At that time, Gordon's main pre-occupation was the mechanism by which steroid hormones cause the increased synthesis of specific enzymes. This problem was studied in cultured hepatoma cells, in which the enzyme tyrosine aminotransferase (TAT) is markedly induced by corticosteroids. It was a large laboratory, with many post-docs working on different aspects of TAT induction. So, I chose to study a different process that also regulates TAT levels: the degradation of the enzyme. I found that the degradation of TAT in hepatoma cells was completely inhibited by inhibitors of cellular ATP production[16]. These results confirmed and extended previous observations of Simpson[17] on the energy-dependence of protein degradation in liver slices. Similar energy requirement for the degradation of other proteins was subsequently observed in a variety of organisms (reviewed in Ref. 18).

Following my return to Israel, I continued to work on the mechanisms of intracellular protein degradation. I was very much intrigued by its energy-dependence, because proteolysis *per*

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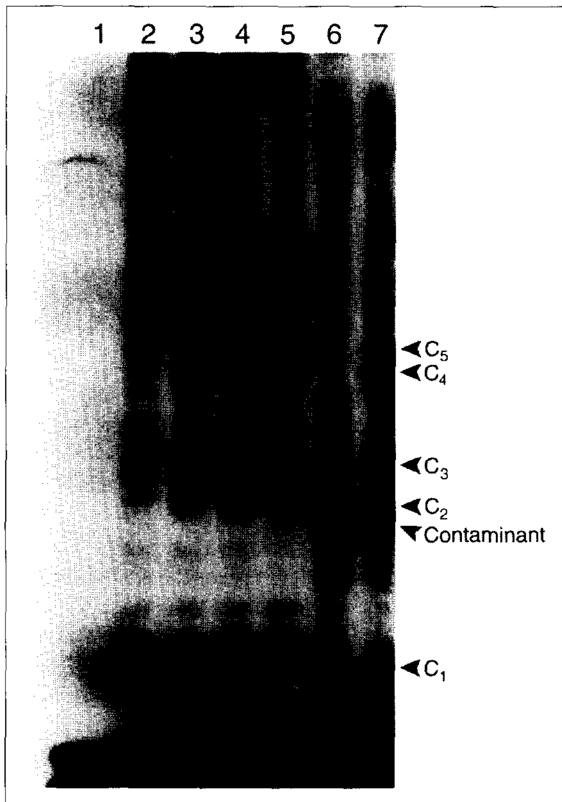

Figure 1
Covalent ligation of ubiquitin to lysozyme. This experiment showed us that ubiquitin is ligated to lysozyme, a good substrate of the ATP-dependent proteolytic system, and that several molecules of ubiquitin are linked to one molecule of lysozyme. All incubations contained Fraction 2 from reticulocytes. ^{125}I-labeled ubiquitin was added in lanes 1–5, and ^{125}I-labeled lysozyme in lanes 6–7. Lane 1, ^{125}I-ubiquitin incubated without ATP; lane 2, with ATP; lanes 3–5, with ATP and increasing concentrations of unlabeled lysozyme. Lane 6, ^{125}I-lysozyme incubated without ATP; lane 7, ^{125}I-lysozyme incubated with ATP and unlabeled ubiquitin. Following incubation, reaction products were separated by SDS-polyacrylamide gel electrophoresis. It can be seen that, in the presence of ATP, ^{125}I-ubiquitin is ligated to many endogenous proteins in Fraction 2 (lane 2). Upon the addition of unlabeled lysozyme, several new bands appeared (labeled C1 to C5), which increased with increasing concentrations of the lysozyme substrate. These are conjugates between lysozyme and ubiquitin, as shown by the observation that when the pattern of labeling was reversed, the same bands contained the label of ^{125}I-lysozyme (lane 7). The different bands contain increasing numbers of ubiquitin molecules linked to one molecule of lysozyme. From Ref. 23, with permission.

se is an exergonic process, which does not require energy. It seemed reasonable to assume that there was an unknown proteolytic system that uses energy to attain the high selectivity of intracellular protein degradation. I also thought that the best way to identify such a system is that of classical biochemistry: to reproduce ATP-dependent protein degradation in a cell-free system, and then to fractionate such a system in order to isolate and characterize its enzyme components.

From the very beginning, I thought that there might be multiple components (although I did not anticipate as many components as turned out to be the case!). My simplest working hypothesis at that time was that ATP might be required either to phosphorylate, and thus tag the protein substrate, or to phosphorylate and activate an unknown protease. In either case, at least two enzyme components were expected: a protein kinase that carries out the phosphorylation and a protease that eventually degrades the protein substrate.

An ATP-dependent, cell-free proteolytic system from reticulocytes was first established by Etlinger and Goldberg[19] and was then analysed by our fractionation–reconstitution studies. In this work, I was greatly helped by Aaron Ciechanover, who was then my graduate student. Our initial work was published in 1978 as a short paper[20] of which I am still very proud. Initially, reticulocyte lysates were fractionated on DEAE–cellulose into two crude fractions: Fraction 1, which is not adsorbed, and Fraction 2, which contains all proteins that are adsorbed to the resin and then eluted with high salt. Neither fraction was active by itself, but ATP-dependent proteolysis could be restored by the combination of the two fractions. This indicated the involvement of at least two components.

We decided to purify the component from Fraction 1 first, because it seemed a simpler task (Fraction 2 contains most non-hemoglobin reticulocyte proteins). The active component in Fraction 1 showed rather unusual features: it was stable to heating at 90°C, but appeared to be a protein by its sensitivity to treatment with proteolytic enzymes. By gel filtration on Sephadex, a molecular weight of about 9 kDa was determined[20]. We purified it to homogeneity by drastic heat treatment, followed by two steps of column chromatography. We called this small protein APF-1 (ATP-dependent proteolysis factor 1). The identity of APF-1 with ubiquitin was found by Wilkinson *et al.*[21] following our discovery of its conjugation to proteins, as described below.

The purification of ubiquitin from Fraction 1 allowed the elucidation of the mode of its action in the proteolytic system. This work was done in part in collaboration with Irwin Rose, who hosted me in his laboratory at Fox Chase Cancer Center in Philadelphia for a sabbatical year in 1978 and many times afterwards. At first, we thought that the small protein might be an activator or a regulatory subunit of some protease or other enzyme component of the system present in Fraction 2. To examine such possibility, purified ubiquitin was radio-iodinated and incubated with crude Fraction 2 in the presence or absence of ATP. The possible association of ^{125}I-labeled ubiquitin with an enzyme in Fraction 2 was examined by gel filtration chromatography. A dramatic ATP-dependent binding of labeled ubiquitin to high molecular weight material was observed. We were astonished to find, however, that ubiquitin was bound by a covalent amide linkage, as indicated by the stability of the 'complex' when treated with acid, alkali, hydroxylamine and boiled with SDS[22]. Analysis of the reaction products by SDS–polyacrylamide gel electrophoresis showed that ubiquitin is ligated to numerous high molecular weight proteins. As crude Fraction 2 from reticulocytes contains not only enzymes, but also endogenous substrates of the proteolytic system, we began to suspect that ubiquitin might be linked to protein substrates, rather than to an enzyme of the system. In support of this interpretation, we found that proteins that are good substrates for ATP-dependent proteolysis (such as lysozyme, lactalbumin or globin) form multiple conjugates with ubiquitin[23]. Figure 1 shows the experiment that convinced us that ubiquitin is ligated to the protein substrate. It might be seen that similar high molecular weight derivatives were formed when ^{125}I-ubiquitin was incubated with unlabeled lysozyme (Fig. 1, lanes 3–5), and when ^{125}I-lysozyme was incubated with unlabeled ubiquitin (Fig. 1, lane 7). Analysis of the ratio of ubiquitin to lysozyme radioactivities in the

various derivatives indicated that they consist of increasing numbers of ubiquitin molecules bound to one molecule of lysozyme. Based on these findings, in 1980 we proposed that the ligation of ubiquitin to proteins targets them for degradation by a protease that specifically acts on proteins with several ubiquitin molecules attached[23].

At the time of the above-described work, we did not know that APF-1 is similar to ubiquitin (I used the term ubiquitin for ease of description). This identification was made shortly afterwards by Wilkinson and co-workers[21]. Keith Wilkinson was then a postdoctoral fellow in Rose's laboratory, working on a different subject. I remember discussing our results with him and telling him that, with the exception of the transglutaminase reaction, I did not know of any other example of amide linkage between proteins. He replied that he knew one. It so happened that he had a friend, Michael Urban, who was a postdoctoral fellow in the laboratory of Alfred Zweidler, next to Rose's lab at Fox Chase, working on histone variants in development. Urban told him about a peculiar histone derivative, called protein A24. This protein was discovered and its structure elucidated by Busch and co-workers[24,25]. It is a branched protein, in which the carboxyl terminus of ubiquitin is linked by isopeptide linkage to the ε-amino group of Lys119 of histone 2A. Ubiquitin was first isolated by Goldstein and co-workers from the thymus and was mistakenly thought to be a thymic hormone; subsequently, it was found in all tissues and eukaryotic organisms and hence its name[26]. The identity of APF-1 with ubiquitin was suggested by the similarity of their conjugated forms; additional similarities indicated that this was indeed the case[21]. The role of the mono-ubiquitin conjugate of histone is still not understood; it might be involved in histone modification, rather than in its degradation. This story shows that it is good for postdocs and students from different laboratories to talk to each other.

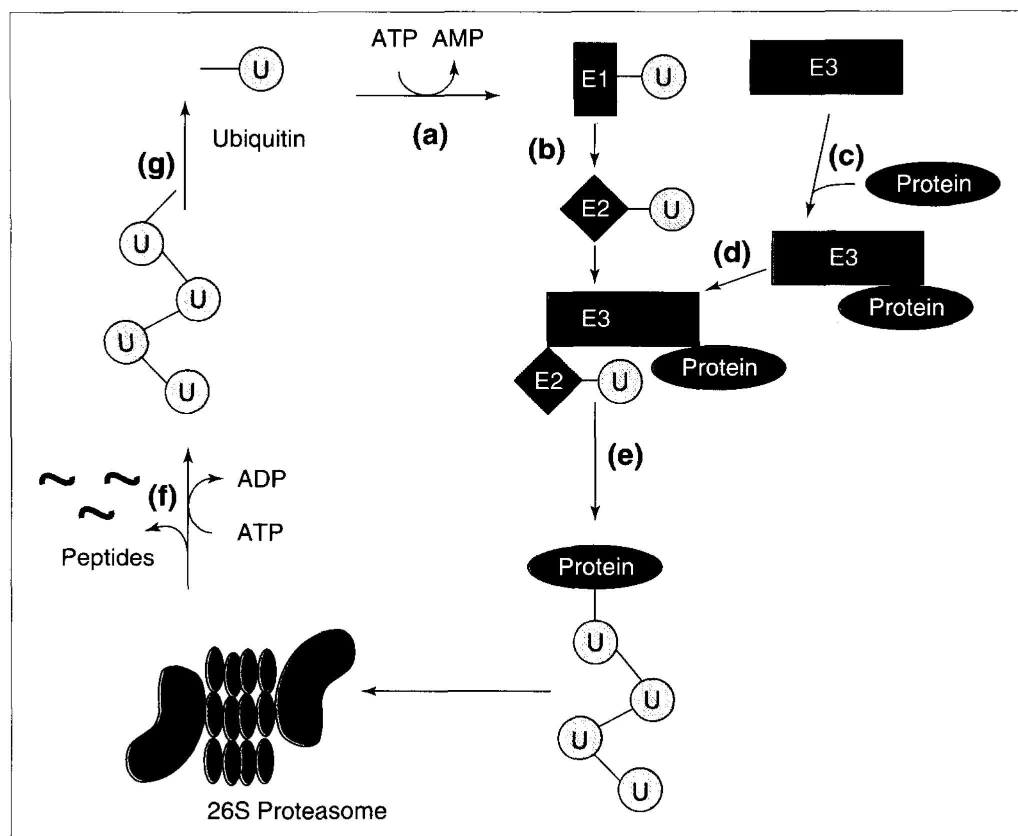

Figure 2
Main enzymatic steps in the ubiquitin proteolytic pathway. **(a)** ATP-dependent activation of ubiquitin with the formation of a high-energy thiolester bond with E1. **(b)** Transfer of activated ubiquitin to thiolester with E2. **(c)** Binding of the protein substrate to a specific ubiquitin ligase, E3. **(d)** Formation of an intermediary complex between E3, the protein substrate and E2–ubiquitin. **(e)** Ligation of ubiquitin to a lysine residue of the protein substrate, followed by the formation of a multi-ubiquitin chain. **(f)** ATP-dependent degradation of ubiquitinylated protein to small peptides by the 26S proteasome complex. **(g)** Recycling of free ubiquitin by the action of isopeptidases.

Following the discovery of ubiquitin ligation to protein substrates, we tried to identify and characterize the different enzymes of the ubiquitin pathway. We did this, again, by biochemical methods of fractionation, purification and reconstitution. An outline of the main enzymatic steps of this pathway, based on such studies, is shown in Fig. 2. This scheme summarizes about ten years of our work (between 1980 and 1990), as well as some of others' work.

We first described the ubiquitin-activating enzyme, E1, that carries out the ATP-dependent activation of the carboxy-terminal glycine residue of ubiquitin before ligation. E1 catalyses a two-step reaction sequence, in which ubiquitin adenylate is first formed, followed by the transfer of activated ubiquitin to a thiol site of E1 (Ref. 27).

Next, we found that two more enzymes are required for ubiquitin–protein ligation: a ubiquitin-carrier protein, E2, which accepts activated ubiquitin from E1 in a thiolester linkage and then transfers it to the protein substrate in a reaction requiring a ubiquitin–protein ligase, E3 (Ref. 28). E3 enzymes apparently have an important function in the selection of proteins suitable for degradation. We have studied in detail one species of ubiquitin–protein ligase, E3α. The enzyme binds specific protein substrates[29] and E2 (Ref. 30) and allows the transfer of ubiquitin from E2 to amino groups of the protein substrate. One signal in protein substrates recognized by E3α is the identity of the amino-terminal amino acid residue. We found that E3α contains distinct binding sites for basic and bulky-hydrophobic amino-terminal amino acid residues[31], providing an explanation for the 'N-end rule' discovered by Varshavsky and co-workers[32]. Other E3 enzymes, with other E2 partners, recognize other signals in specific proteins (reviewed in Refs 13, 14, 33).

By the action of such ubiquitinylating enzyme systems, ubiquitin is linked to proteins by isopeptide linkages between the carboxy-terminal

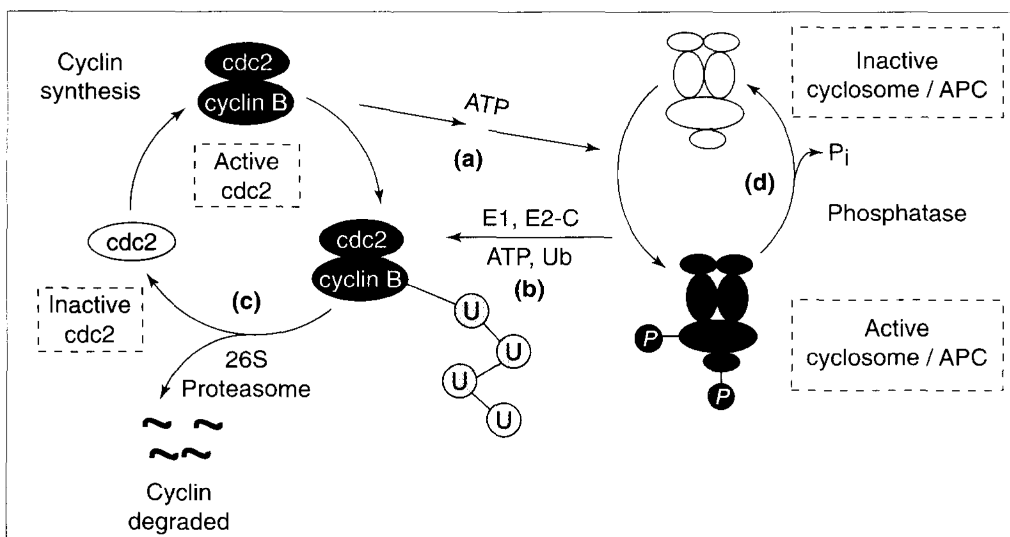

Figure 3
Proposed sequence of events in cell cycle-regulated degradation of cyclin B. **(a)** The active cdc2–cyclin B protein kinase converts the cyclosome/anaphase-promoting complex (APC) to the active, phosphorylated form at the end of mitosis. This process includes a time lag and might involve a cascade of phosphorylation reactions. **(b)** The active cyclosome complex ligates ubiquitin to cyclin B, in a process involving the action of E1 and E2-C. **(c)** Ubiquitinylated cyclin B is rapidly degraded by a constitutively acting 26S proteasome and thus, cdc2 is converted to the inactive, monomeric form. **(d)** The cyclosome complex is converted back to the inactive, interphase form by phosphatase action.

glycine residue of ubiquitin and ε-amino groups of lysine residues in these substrate proteins. In addition, the formation of multi-ubiquitin chains has been shown to occur[34], in which a molecule of ubiquitin is linked to Lys48 (Ref. 35) of another ubiquitin, which in turn is linked to the protein substrate. Such multi-ubiquitinylated proteins are degraded by a large 26S ATP-dependent protease complex, now called the 26S proteasome, discovered by Rechsteiner and colleagues[36]. We found that the 26S proteasome is formed by the ATP-dependent assembly of three components, called conjugate-degrading factors CF-1, CF-2 and CF-3 (Ref. 37). One of these, CF-3, was identified as the previously known 'multi-catalytic protease' or the 20S proteasome[38,39], suggesting that the 20S proteasome is the catalytic core of the 26S proteasome complex.

The last step in the pathway is the regeneration of free and re-usable ubiquitin, a process carried out by ubiquitin carboxy-terminal hydrolases or isopeptidases. We have described two isopeptidases that are involved in this process: one that disassembles multi-ubiquitin chain remnants following the action of the 26S proteasome[40], and another that is associated with the 26S proteasome and presumably releases ubiquitin from peptides derived from the protein substrate[41].

The above studies on the basic biochemistry of the ubiquitin system were all carried out with enzymes from reticulocytes, using artificial protein substrates. More recently, I became interested in the question of how does the ubiquitin system carry out the specific and programmed degradation of cyclin B, a major mitotic cyclin. Cyclin B is the positive regulatory subunit of protein kinase cdc2. The active cyclin B–cdc2 complex (also called M-phase promoting factor, or MPF) causes entry of cells into mitosis, and after a short lag, activates the system that degrades its cyclin B subunit. The degradation of cyclin B inactivates cyclin B and allows cells to exit from mitosis. Here again, I used biochemical approaches to study the mechanisms of cyclin degradation. In this case, the quest for a cell-free system has led me to *Spisula solidissima*, a large clam that produces large numbers of oocytes. Luca and Ruderman[42] established a cell-free system from clam oocytes that faithfully reproduces cell-cycle stage-specific cyclin degradation. In this work, I was helped first by Bob Palazzo and Leonard Cohen, and then by a collaboration with Joan Ruderman. It was first established that cyclin degradation is carried out by the ubiquitin system[43,44].

Fractionation of the clam oocyte system[45] revealed two novel components involved in cyclin degradation: a cyclin-selective ubiquitin-carrier protein E2-C (Refs 45, 46), and a cyclin–ubiquitin ligase associated with an ~1500 kDa complex that we termed the cyclosome[1]. By similar fractionation–reconstitution work in *Xenopus* extracts, King *et al.*[2] subsequently isolated a similar complex, named the Anaphase Promoting Complex (APC). Cyclosome/APC is inactive in the interphase, but becomes active in cyclin–ubiquitin ligation towards the end of mitosis. The inactive, interphase form of cyclosome/APC can be activated *in vitro* by MPF[1]. The active, mitotic form of cyclosome/APC can be inactivated *in vitro* by treatment with an okadaic acid-sensitive phosphatase[47]. It thus seems that the degradation of cyclin B is regulated by reversible phosphorylation of the cyclosome complex. Our current working hypothesis on the mode of regulation of cyclin degradation is illustrated in Fig. 3. The mode of action of the cyclosome complex, its possible additional substrates and the mechanisms that regulate its activity are of great interest for future research.

Looking back on nearly 20 years of work on the ubiquitin system, it seems that the main lesson is the continued importance of using 'classical' biochemistry in modern biological research. In his book *For the Love of Enzymes*, Arthur Kornberg divided the history of biomedical research into four main periods: first, the time of the microbe hunters, which was followed by the vitamin hunters, followed by the enzyme hunters, followed by today's gene hunters. But, Kornberg also pointed out that the times of enzyme (or protein) hunting are far from being over. Molecular genetics is most powerful when combined with biochemistry. Without any knowledge of the biochemical functions of gene products, molecular genetics by itself cannot solve basic biological problems. In the case of the ubiquitin system, without the biochemical work, the genes of most of its components would now probably be open reading frames of unknown function in databases of genome projects. At best, some would be known

as genes required for protein degradation by an unknown mechanism.

So, if you have a problem that cannot be solved by molecular biology, do not hesitate to enter the cold room! And in all cases, look out for a good cell-free system. This should not be interpreted that I am trying to convince anybody to abandon molecular biology. In the ubiquitin field, molecular genetic work of other investigators, working in yeasts and other organisms, have not only confirmed the biochemical findings, but also greatly expanded our understanding of the mechanisms, scope and functions of the ubiquitin proteolytic pathway.

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