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Ubiquitin-Independent Proteasomal Degradation

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Abstract

Most proteasome substrates are marked for degradation by ubiquitin conjugation, but some are targeted by other means. The properties of these exceptional cases provide insights into the general requirements for proteasomal degradation. Here the focus is on three ubiquitin-independent substrates that have been the subject of detailed study. These are Rpn4, a transcriptional regulator of proteasome homeostasis, thymidylate synthase, an enzyme required for production of DNA precursors and ornithine decarboxylase, the initial enzyme committed to polyamine biosynthesis. It can be inferred from these cases that proteasome association and the presence of an unstructured region are the sole prerequisites for degradation. Based on that inference, artificial substrates have been designed to test the proteasome's capacity for substrate processing and its limitations. Ubiquitin-independent substrates may in some cases be a remnant of the pre-ubiquitome world, but in other cases could provide optimized regulatory solutions.

Introduction

Most degradation by the proteasome depends on ubiquitin conjugation. However, for a significant subset of proteins turnover is independent of ubiquitin conjugation. Examining these exceptional cases can illuminate the general prerequisites for proteasome capture, processing and degradation.

It is not so easy to clearly determine whether ubiquitin modification mediates degradation of a particular substrate [1]. Ubiquitination is found in contexts unrelated to degradation, so demonstrating the presence of ubiquitin adducts is not a sufficient criterion. If associated with turnover, ubiquitin conjugates are usually present only transiently. Fleeting low abundance conjugates may be hard to document, unless mutants or drugs are used to impair degradation or deubiqitination. If the lysine targets of modification are known these can be modified to test functionality. Similarly, if the E2 and E3 enzymes required for conjugation are known, their activities can be modulated experimentally and the effects of these manipulations on turnover measured. If there is sufficient information, one can hope to use purified components to carry out conjugation and test its effect on proteolysis by proteasomes. There are but few substrates for which all these things have been done convincingly. The difficulty of such a project- showing that a particular protein is rapidly degraded as a result of ubiquitin conjugation- implies that ubiquitin-independent cases maybe overlooked or misidentified. As we will see, these questions are further complicated

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by the fact that some proteins depend on both ubiquitin-mediated and -independent modalities for their degradation.

Several previous reviews or commentaries have cataloged the variety of proteins that have been more or less firmly determined to be degraded by proteasomes in a manner that does not depend on ubiquitin [2–4]. The reader is referred to these. Here we will consider a few examples. These are thymidylate synthase, Rpn4 and ornithine decarboxylase. These have been selected because they have been analyzed by a variety of technical means. The results are clearcut and they offer some mechanistic understanding of how proteins are degraded without ubiquitin. These examples provide an opportunity to consider the elements relevant to analysis of ubiquitin-independent recognition and degradation by the proteasome.

Rpn4

Rpn4 is a transcriptional activator of proteasome genes in budding yeast. Because it is both a very short-lived proteasome substrate and activates proteasome production, it participates in a negative feedback circuit that stabilizes the level of proteasomes [5, 6]. Rpn4 exemplifies a protein that is degraded through both ubiquitin-dependent and -independent pathways. Its two degradation signals are located in different non-overlapping parts of the 531 residues protein. The ubiquitin-dependent degron spans residues 211 to 229, corresponding to an acidic region. Its degradation can be mediated by ubiquitin conjugation at six different lysines, lysine 187 being the preferred site [7]. The N-terminal region of Rpn4 ($Rpn4_{1\text{-}229}$) was found to be degraded rapidly, even if all its lysine residues were mutated, suggesting that this region is degraded in a ubiquitin-independent fashion [8]. Several mutants affected in the ubiquitination pathway do not impair Rpn4 degradation [8], also consistent with the presence of a ubiquitin-independent degron.

Interestingly, although lysine-deficient $Rpn4_{1\text{-}229}$ can be degraded, that is no longer the case if either an N-terminal tag is added or if residues 1–10 are deleted. Such additions or deletions at the N terminus do not prevent degradation if lysine residues are intact [8]. This implies $Rpn4_{1\text{-}229}$ can be degraded via two independent mechanisms, one ubiquitin-dependent, and a second which is ubiquitin-independent and which requires the integrity of a region present at the N terminus. Refinement by deletion analysis of the presumptive N-terminal degron showed that the first 80 residues are sufficient to induce degradation. This N-terminal region can also be transplanted to the N-terminal part of other proteins, such as dihydrofolate reductase (DHFR), and cause them to be degraded [9]. Residues 1–80 are therefore an autonomously active ubiquitin-independent degron.

Both of these degradation mechanisms are proteasome dependent, requiring the 20S and 19S complexes of the proteasome; a 19S mutant is unable to degrade Rpn4. However, although some 19S subunits are known to be responsible for ubiquitinated substrate recognition, no data is available concerning the proteasome subunits responsible for the recognition of a ubiquitin-independent degron. Recently, Ha *et al*. used a crosslinking label transfer technique to show that Rpn2, Rpn5 and Rpt1 are able to interact with the Rpn4 degron, giving a first clue to its molecular recognition mechanisms [9].

Thymidylate synthase

Thymidylate synthase (TS) is an enzyme present in mammalian cells which catalyses the reductive methylation of deoxyuridylate by 5,10-methylene-5,6,7,8-tetrahydrofolic acid to form thymidylate (dTMP) and dihydrofolic acid. It is the only reaction allowing formation of dTMP in mammalian cells and is thus essential for DNA replication. TS is a good example of a non-ubiquitinated proteasome substrate. The regions involved in the degradation signal have been dissected and show how complex such a degron can be.

TS has a half life of 7–10h. When it binds its ligand, the half life increases to 20–24h [10]. TS degradation requires both the 20S and 19S proteasome complexes [11, 12]. Unlike Rpn4, its degradation seems to be only ubiquitin-independent. Mammalian TS, 313 amino acids in size, contains an N-terminal domain, spanning from residue 1 to 28, which is not conserved and has no secondary structures, as shown by X-ray crystallography [13]. Its deletion does not affect the catalytic activity of the enzyme. However, deletion of the first N-terminal residues prevents TS degradation. The 1–30 region is absent in *E. coli* TS and adding it increases enzyme turn-over, the half life decreasing from 48h to 4.5h [14]. The N-terminal region thus acts as a degradation signal.

Removal of lysine residues, essential for ubiquitin addition, in the N- terminal region did not affect its ability to act as a degron, indicating that its function is ubiquitin-independent [14]. Mutagenesis of this domain showed that the proline at position 2 is essential and that adding an N-terminal His tag abolished its ability to act as a degradation signal. Moreover, analysis of TS protein composition showed that proline 2 is free at its N-terminus, as the initiation methionine is not present. N-acetylation also blocks degradation [15]. Thus both the presence of the proline residue and its terminal position are essential.

Although the proline residue plays a critical function, it is not the only determinant of degradation function. Other residues such as those present in the proline rich region 9–15, including two arginines at positions 10 and 11, are also essential [12, 15]. Additionally, a conserved α-helix (residues 31–42) following the N-terminal region was shown to increase the effectiveness of the degron [16]. The helix retains its function when moved with respect to the proline rich region. Furthermore, the endogenous α-helix can be replaced by other α-helices, so it is not sequence-specific. TS also has additional means of degradation that depend on its C terminus, but the mechanism through which this acts is not clear. It is not known which regions are involved in binding of the TS degron to the proteasome. Proline 2 and residues 9–15 seem to be involved in steps subsequent to binding [12], as mutants altering these residues could not be restored to function by addition of a domain which binds the proteasome.

Ornithine decarboxylase

Ornithine decarboxylase (ODC) is the initial and rate limiting enzyme for biosynthesis of polyamines in eukaryotes. These small molecules are biologically ubiquitous, and are essential for viability. Cellular polyamines are determined by both biosynthesis and catabolism, and also by exchange with extracellular pools. The interactions among these processes are complex, and under physiologic conditions can mediate either homeostasis of polyamine pools or their expansion in response to increased cell requirements. Interestingly, turnover is the predominant mechanism for controlling activity of multiple key enzymes of the pathway. ODC is degraded by the proteasome, and this is its predominant means of regulation [17, 18]. Excess cellular polyamines induce production of the protein antizyme 1 (AZ1). As will be described, AZ1 accelerates the proteasomal degradation of ODC. The relationship among free polyamine pools, AZ1 production and AZ1-induced ODC destruction constitutes a feedback loop that stabilizes polyamine abundance.

The very rapid disappearance of ODC activity in rodent liver in response to puromycin [19] provided an early clue to its great lability (half-life of 11 minutes). The structural basis for its rapid turnover emerged from a study of the function of a 37 amino acid C-terminal region of mouse ODC (termed cODC) This part of the protein is not required for enzymatic activity [20], but proved to be both necessary and sufficient for the rapid turnover of ODC. Expression in cultured cells revealed mouse ODC to disappear with a half life of about an hour, but a truncated protein deprived of cODC was completely stable [21]. A comparative

study of mammalian ODC and that of the parasite *Trypanosoma brucei* (TbODC) confirmed cODC as the degron. ODC and TbODC are highly homologous throughout their length, but the homology ends where cODC begins. In mammalian cells TbODC is stable and ODC is not. Grafting cODC to the C terminus of TbODC converted it to an unstable protein [22]. Numerous subsequent studies have shown that cODC has autonomous function; grafting it to the C terminus of stable proteins, e.g., GFP [23], is sufficient to increase its turnover.

Although turnover of mammalian ODC does not require AZ1, partnering the two greatly enhances ODC degradation. In its native and enzymatic form, ODC is a homodimer. AZ1 dissociates ODC: :ODC to form the enzymatically inactive ODC: :AZ1 heterodimer. The binding site for AZ1 lies outside of cODC; replacing residues 117 to 140 within the 461-amino-acid mouse ODC sequence with the equivalent region of trypanosome ODC disrupted both antizyme binding and *in vivo* regulation by polyamines [24]. However, cODC becomes more accessible to a specific antibody in ODC: :AZ1 than in ODC: :ODC [25]. So cODC might be more or less buried in the ODC: :ODC complex and the disruption of that complex by AZ1 binding, exposes cODC. The greater accessibility of cODC probably explains in part the ability of AZ1 to promote degradation. However, it is likely that this is not the whole story. Other isomers of antizyme can also form a heterodimer with ODC, but they are much less effective in targeting it for degradation [26]. Furthermore, a C-terminal fragment of AZ1 (residues 106–212) binds to and inhibits the activity of ODC, can heterodimerize with it, but does *not* promote ODC degradation [27]. This suggests that there are elements in the N-terminal half of AZ1 functionally important for directing ODC turnover.

In *Saccharomyces cerevisiae* important regulatory features are retained: ODC turnover is independent of ubiquitin and controlled by a distant ortholog of mammalian antizyme, synthesis of which also depends on polyamine-induced translation frameshifting [28]. However, in this case the unstructured degron is positioned at the N terminus rather than the C terminus [29].

The identification of the mammalian ODC degron [21] raised a host of questions. What is the responsible protease? What is the basis of its interactions with the degradation system? What are the functional elements of the ODC degron? Some answers to these questions soon appeared. Experimental analysis required gaining access to reagents for quantitative analysis of this labile, elusive and low abundance protein. The generation of cell lines that overproduced ODC and the cloning of its cDNA were important elements in this process [30–32], as was an increasingly refined understanding of the ubiquitin proteasome system.

Analysis of *in vitro* degradation by fractionated reticulocyte lysates gave an important early clue the proteasomes were responsible, but ubiquitin was not. Fractions containing proteasomes but devoid of ubiquitin or ubiquitin conjugated enzymes were proficient in ODC degradation [33]. In *ts85* cells with a thermo-sensitive ubiquitin conjugated E1 enzyme, ODC activity decayed rapidly at the nonpermissive temperature, implying that conjugation had no role in degradation of the enzyme [34]. Biochemical experiments with purified proteasome revealed that the 26S complex (but not the 20S form) degrades ODC, that ATP is required and that AZ1 greatly accelerates the process [35]. Subsequent experiments with purified proteasomes [36] revealed that AZ1 increases the affinity of ODC for the proteasome, but does not accelerate its degradation. Importantly, ubiquitin conjugated substrates, or ubiquitin chains, competed for degradation of ODC, implying a common element in the recognition or processing of these distinct classes of substrates. Targeted mutagenesis revealed an important functional role for a cysteine residue lying near the middle of the 37 residue of the ODC degron [37]. Mutating Cys441 to Ala or to Ser greatly impaired degradation both *in vitro* and *in vivo*.

Substrates of proteasome contain two essential functional motifs [38]. One provides affinity for the proteasome- in most cases ubiquitin conjugates fulfill that role. The second is an unstructured region, of sufficient size and favorable geometry, which initiate invasion and subsequent processive translocation into the proteasome. Analysis of the properties of the cODC degron revealed that it offers both these properties in a compact package. In substrates stabilized by Cys441 mutations, degradation could be restored by providing an alternative means of tethering to the proteasome [39]. It had previously been reported that a stable protein could be fused to proteasome constituents, acting as a mechanism for proteasome delivery [40]. Adapting such an approach, chimeric proteins were made and tested consisting of the nonessential Rpn10 protein fused to proteins with various forms of cODC or alternative sequences [39]. Rpn10 fusion provided a means of proteasome delivery that restored degradation of proteins that have been made stable by Cys441 mutation. This implied that in cODC the essential Cys provides a proteasome tethering function. The converse experiment was also revealing: For substrates provided with the artificial Rpn10 proteasome tether, an unstructured C-terminal region unrelated to the cODC sequence (but of similar size) could act to initiate degradation. Both algorithmic predictions of secondary structure and crystallographic models [41] of ODC are consistent with the conclusion that its C-terminal degron is disordered. The cODC degron provides two functions- tethering which requires the essential Cys, plus a disordered region for proteasomal entry, which is broadly independent of sequence.

Artificial substrates designed to probe how proteasomes work

A general rule can be inferred from the specific cases described- those of Rpn4, thymidylate synthase and ODC: Only two things are needed for a protein to be degraded by proteasomes: 1. A means of association and 2. An unstructured region of sufficient size. This understanding has lead to the design of a series of artificial proteasome substrates that can be used to study certain biochemical functions of the protease [42, 43]. These substrates contain Rpn10 at the N-terminus as a proteasome association domain and an extension corresponding to the Cys441Ala mutated cODC at the C-terminus. In between these two domains, proteins are positioned that are more or less easy to unfold by application of mechanical force. By systematically varying the mechanical properties of these folded domains, the unfolding ability of proteasomes can be tested. The kinetics of degradation of these chimeric substrates revealed the unfolding and translocation capacities of proteasomes. As might be expected from the uniform presence of the Rpn10 association element, the series of related substrates share a similar affinity for yeast proteasomes, with K_m values clustered around 200 nM. However, the time required to degrade each substrate varied widely, and depended on the mechanical stability of the domain present between Rpn10 and the extension. The mean time for degradation ranged from 5 min in the case of the DHFR protein, which is loosely folded, to 40 min in the case of the tightly folded I_{27} domain of titin. Addition of methotrexate to DHFR has the effect of stabilizing its structure, and the time needed to unfold Rpn10-DHFR-extension increased to 14 min. Similarly, the single-residue Val13Pro mutation, which destabilizes the I_{27} titin domain, decreased the degradation time of Rpn10- I_{27}-extension from 40 min to 8 min. These data substantiate the conclusion, long expected but not previously tested systematically, that unfolding can be rate-limiting for degradation by proteasomes.

Based on data from taxonomically distant but homologous proteases (bacterial [44, 45], archaeal [46]) the motive force of substrate unfolding and translocation centers on a Tyr residue present in a loop (ArΦ loop) and found in each of the six homologous ATPase proteins (Rpt1 to 6) of the proteasome. The ATPases are arranged as a hexameric ring which is docked to the 20S alpha heptameric ring. ATP binding and hydrolysis impels the loops to move and to pull on substrates, unfolding and translocating them to the 20S proteolytic

chamber. Genetic data demonstrate that each of these ATPases is essential and has distinct and specific functions [47]. Accordingly, homologous Tyr to Ala mutations were made in the ArΦ loop of the individual proteasome Rpt proteins. The mutations had different effects depending on which ATPase was mutated. *In vivo*, the strongest phenotypic effects were seen in the rpt1 and rpt6 mutants, growth of which was highly impaired, especially under stress, either by culture at elevated temperature or induced by inclusion of the arginine analog canavanine in the medium. In the case of rpt3, the mutation affected the growth cycle; cells proliferated more rapidly but divided at a smaller size. *In vitro* degradation assays using the various purified mutant proteasomes were performed with the artificial Rpn10-tethered substrates previously described. In comparison with proteasomes with a wild type ArΦ loop, mutation of Rpt3, 4 and 5 caused defects attributable to a reduced pulling force on the substrate. Surprisingly, the Rpt1 and Rpt2 mutants degraded the substrate about 50% more efficiently than wild type proteasomes. Moreover, they were also more efficient in hydrolyzing ATP; their activity was respectively 150% and 50 % greater than that of wild type. It is possible that the ArΦ loop Tyr residues may constrain loop motion and that mutation to Ala relieves these constraints, allowing the loops to move more rapidly. If so, in the case of the rpt1 and rpt2 mutants, increased loop motion might augment productive interactions with substrates.

How broad is the class of Ubiquitin-independent degradation?

Proteomics studies have questioned the prominence of protein turnover in determining global steady-state protein abundance [48]. Although transcriptional and translational controls demonstrably are of great importance, it is necessary to consider that selection and detection bias can influence such conclusions. Proteins that turn over rapidly are intrinsically low in abundance, and may escape the scrutiny of broad-brush proteomics approaches. Whatever the global role of degradation in establishing the cell's protein repertoire, turnover is pre-eminently suited as a regulator of cellular transitions, as only fast turnover can lead to rapid adjustments from one level of protein expression to another [49]. Just as there may be detection bias in identifying transient proteins, there may be technical impediments in determining the relative importance of ubiquitin-dependent and -independent mechanisms for their turnover. Ubiquitin conjugation provides a specific signature and a set of well-honed tools for case finding. It is easier to catch crooks whose fingerprints are already in the files. Ubiquitin-independent events leave fewer clues behind.

An interesting case in point relates to co-translational and immediate post-translational turnover. A third of proteins die young, an observation made more than 30 years ago [50] and subsequently rediscovered [51, 52]. More than a decade ago, it was demonstrated that a significant pool of proteins are ubiquitinated and degraded during their translation [53]. More recently, using quantitative proteomics to evaluate the ubiquitinome, Harper, Gygi and colleagues [54] concluded that a substantial fraction of ubiquitinated proteins require ongoing protein synthesis, a result consistent with the rapid turnover of a cohort of newly synthesized proteins *via* ubiquitination and proteasome degradation. However, it remains to be determined whether an additional tranche of newly synthesized proteins are degraded by means that do not require ubiquitin. A plausible model of turnover of newly-made proteins is that they experience a kinetic race for folding to a native state and entry into a relevant cellular complex. In this view, proteins are at greater risk for degradation before acquiring their native state. Any protein that is transiently or more durably disordered can become a loser in this race. Knowing which and what fraction of these undergoes ubiquitin conjugation before being led to the chopping block will require the use of assays that do not depend on the presence of ubiquitin. Otherwise, such investigations prejudge the conclusion. In light of these considerations, it is of interest to discuss the category of disordered proteins and their participation in proteasomal degradation.

Disordered regions as a degron: A new class of the IDP family

Disorder is a recurrent theme in the discussion of proteasome substrates. In the three degrons that have been presented in detail, Rpn4, TS and ODC, disordered regions are certainly present, but they are not generic and have special features related to their functionality. This is true of the initial 10 residues of Rpn4, the initial proline of TS and the Cys residue found in the middle of the ODC C-terminal degron. In recent decades there has been increasing study and understanding of proteins that are natively disordered. These investigations have radically changed our vision of the protein “structure-function” dogma [55]. Intrinsically disordered proteins (IDPs) are either fully or partially unstructured. In the latter case, they can be organized in different domains, structured or not, each of them linked to a biological function. The presence of disorder confers several properties [56]. While IDPs are disordered when isolated, they often perform their biological function when interacting with other proteins through the process of folding-upon-binding. The interaction between IDPs and their partners is specific but often has a low affinity. These properties, which are linked to their flexibility, allow IDPs to interact with different partners and thus to possess different functions [56]. Bioinformatics analysis indicates that 6–33% of proteins in bacteria and 35–51% in eukaryotes are disordered [57, 58]. They are involved in many cellular functions such as signal transduction, regulation of cell division, transcription and translation, chaperone action, transport and regulation of the assembly or disassembly of large multi-protein complexes. Their significance in cellular regulation is suggested by the fact that IDPs represent 60–80% of the proteins involved in signaling [58]. However, their role in degradation is poorly understood.

Proteins that undergo degradation independent of ubiquitin share a common feature, they belong to the IDP family [2]. ODC, Rpn4, TS, p53, p21, c-Jun, α-synuclein and others possess intrinsically disordered regions (IDR) and are degraded independently of ubiquitin. Those which are degraded by 26S proteasomes and contain a portable autonomous degron, e.g. ODC, Rpn4 and TS, must offer some means of association with the proteasome regulatory complex. How do these undertake specific interactions that mediate proteasome association? Some IDPs fold upon binding to their interaction partners. These degrons may be capable of conformational modifications associated with binding. However, in that case the structural modification would be transient as, after binding to the proteasome, domain unfolding is presumably essential to initiate entry. Alternatively, the specific structural features of these degrons could interact directly in ways that do not depend on induced or spontaneous changes in their conformation.

Additionally, it has been suggested that IDPs could be degraded be 20S alone [59, 60]. This is clearly the case *in vitro* [61–63]. The ability of 20S proteasomes to degrade an IDP *in vitro* could be proportional to the extent of disorder [64] within the protein but this has not been shown *in vivo*. Degradation by 20S alone is clearly not the case for Rpn4 and TS [65], although ODC has been documented to undergo degradation by 20S proteasomes in an alternative pathway regulated by a flavoenzyme [66]. One of the main functions of the 19S complex is to unfold proteins. Exceptionally, p21 can be degraded, *in vivo*, by the 20S proteasome core particle [62]. But p21 is a highly disordered protein, and unfolding is not needed. For most IDPs, an IDR represents only a part of the protein and the folded domain still needs to unfold to enter the 20S catalytic core. These examples show that degradation by 20S is not a general rule for IDPs. Even if no general degradation mechanism has been found for IDPs, the degron function that disordered regions bring to proteins constitutes a novel class within the IDP members.

Why ubiquitin-independent degradation?

Proteasomes have a longer evolutionary history than ubiquitin. 20S proteasomes are found in all three biological super-kingdoms [67], in all archaea and eukaryotes, and in some actinobacteria. Ubiquitin and its system of conjugating and deconjugating enzymes is confined to eukaryotes, and evolved much later [68]. In the ubiquitin-free realms, proteasomal degradation is necessarily ubiquitin-independent. The relevant question then becomes: Given the complexity, prevalence and versatility of the ubiquitin marking system, why have eukaryotes not evolved to use it *exclusively* as their way to designate proteasome substrates?

Two classes of explanation seem plausible

1. Ubiquitin-independent degradation may be an historic remnant. For some proteins, turnover that does not invoke ubiquitin may simply be good enough to have been retained from the world of pre-ubiquitin biology. An archaic regulatory remnant is especially likely to resist replacement if it is a part of an interlocking multi-component system of regulation. (Ubiquitin itself exemplifies this principle. Along with histones, ubiquitin is among the most slowly evolving proteins [69], presumably because it engages in multiple important interactions.) ODC seems to fit this case. It is the initial and rate limiting enzyme in the biosynthesis of polyamines. Homeostasis and the adjustment of polyamine pools to changing cellular needs is complex and engages multiple interlocking feedback loops that mediate production, interconversion, destruction and transport of polyamines [70]. Many of these adjustments of activity are dominated by proteolytic turnover rather than allostery or synthesis. A key regulator of ODC degradation, antizyme, is controlled by polyamine-dependent modulation of the translational frameshifting efficiency of antizyme mRNA [71]. It is difficult to imagine stepwise evolution of this contraption. Where to begin? Better perhaps to just leave it in place.
2. Ubiquitin-independent degradation may offer an alternative mechanism that supplements and provides partial independence of ubiquitin pool mis-regulation. Consider the case of Rpn4, which is both a proteasome substrate and a positive regulator of proteasome synthesis. Slow Rpn4 turnover leads to more Rpn4, faster transcription of genes that encode proteasomes, more proteasomes, faster Rpn4 turnover and the restoration of steady state. Recall that Rpn4 turnover can be directed by two degrons, one that depends on ubiquitin and a second that does not. If ubiquitin homeostasis and proteasome homeostasis constituted identical cellular states, these two degrons would be fully redundant. But that is not the case- cells can experience “ubiquitin stress” and “proteasome stress” under distinct conditions [72]. If Rpn4 degradation were limited to dependence on ubiquitin conjugation, inappropriate regulatory responses would become available. If ubiquitin pools were depleted or diverted to non-degradative use, Rpn4 levels would rise, driving proteasome synthesis but failing to correct the salient metabolic problem. An experimental test of this hypothesis is available. Mutant forms of Rpn4 have been described which retain ubiquitin-dependent regulation and transcriptional function, but are not subject to ubiquitin-independent degradation [8]. It would be of interest to determine whether cells expressing such a mutant in place of wild type Rpn4 have phenotypes that are elicited by ubiquitin stress.

Acknowledgments

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Highlights

Multiple criteria jointly test ubiquitin dependence/independence of degradation.

For a few ubiquitin-independent substrates such criteria have been met.

These cases are informative as to the general prerequisites for proteasome degradation.

Ubiquitin-independent substrates may be a remnant of the pre-ubiquitome world.

Other could provide optimized alternative regulatory solutions.