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Back to the Future with Ubiquitin

Review

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Two papers published in 1984 by the Varshavsky laboratory revealed that the ubiquitin/proteasome pathway is the principal system for degradation of short-lived proteins in mammalian cells, setting the stage for future demonstrations of this pathway's many regulatory roles. This perspective discusses the impact of those papers and highlights some of the subsequent insights that have led to our current appreciation of the breadth of ubiquitin-mediated signaling.

Introduction

Over a thousand papers published in the year 2003 alone cited "ubiquitin" as a keyword, compared to fewer than a hundred such papers in 1984. The dramatic difference reflects the efforts of many laboratories, whose collective findings have shown that nearly every aspect of eukaryotic cell biology carries a connection to ubiquitin. As a result, today it is easy to frame a career around this remarkable signaling molecule. But one's motives had to be purer in 1984—despite the novelty of ubiquitin's role as a degradation signal, it was impossible to gauge the generality and significance of the eponymous proteolytic pathway.

In the accompanying supplement, *Cell* republishes two seminal papers that provided major insights into what we now call the ubiquitin/proteasome pathway. In this work, Varshavsky and coworkers exploited their discovery of a temperature-sensitive defect in ubiquitin conjugation to reveal for the first time the enormous scope of ubiquitin-dependent proteolysis in mammalian cells (Ciechanover et al., 1984; Finley et al., 1984). In the accompanying supplement, the authors themselves comment on how their findings influenced the growth of the ubiquitin field. Here, I offer an independent perspective, beginning with the 1984 papers and proceeding to some of the developments that, in my view, have most notably altered our view of ubiquitin from that which prevailed twenty years ago.

Background: The Ubiquitin/Proteasome Pathway of Protein Degradation

Breaking a peptide bond is a difficult proposition—the uncatalyzed hydrolysis of one bond in a polypeptide chain is estimated to occur with a half-life of several hundred years under physiological conditions (Wolfenden and Snider, 2001). The kinetic stability of proteins is biologically desirable—one wouldn't want these workhorses of the cell to undergo spontaneous fragmentation—but it creates a problem when proteins need to be eliminated for purposes of regulation or quality control. This problem is solved by proteases, which often use a combination of acid, base, and nucleophilic catalysis to facilitate the attack of water on peptide bonds. Proteases are rarely energy-dependent, however, because the reaction that they catalyze is thermodynamically favorable.

The discovery of the ubiquitin/proteasome pathway emerged from efforts to understand why intracellular proteolysis, measured as the release of amino acids from intact cells, requires metabolic energy (Simpson, 1953). Key elements of the answer became clear in the early 1980's as a result of the pioneering biochemical studies of Hershko and coworkers. These investigators found that energy, in the form of ATP, is needed to modify proteolytic substrates with ubiquitin, a highly conserved 76 amino acid polypeptide that is joined to a substrate lysine side chain through an isopeptide bond to ubiquitin's C terminus (Ciechanover et al., 1980; Hershko et al., 1980). Ubiquitination occurs through sequential steps catalyzed by activating (E1), conjugating (E2), and ligase (E3) enzymes (Hershko et al., 1983). The presence of multiple substrate-linked ubiquitins recruits the 26S proteasome, a 2.5 MDa complex that uses energy derived from ATP hydrolysis to unfold the substrate polypeptide chain and translocate it into an interior chamber (Baumeister et al., 1998). Having arrived at this site, the substrate is hydrolyzed by a nucleophilic mechanism to produce small peptides. Ubiquitin is spared from degradation through its release from the substrate (or a substrate fragment) by deubiquitinating enzymes (Hershko et al., 1980). Thus, there are two independent reasons why ATP is required for intracellular proteolysis: to activate ubiquitin's C terminus in preparation for conjugation and to support the proteasome's substrate unfolding and translocation activities (Figure 1A) (Baumeister et al., 1998; Pickart, 2001).

Much of what is stated in the preceding paragraph was already known in outline form by the early 1980's, although E1 was the only enzyme that had been thoroughly characterized. As shown by Hershko, Rose, and coworkers, E1 activates ubiquitin by using ATP to synthesize ubiquitin C-terminal adenylate, which then serves as an enzyme bound substrate for the formation of an E1-ubiquitin thiol ester (Figure 1B) (Haas et al., 1982). The latter ubiquitin is passed to an E2 cysteine residue and from there, in an E3-dependent manner, to the substrate (Figure 1C) (Hershko et al., 1983). Although the true properties of the proteasome were beyond the wildest imagination of researchers working at that time, it was clear that the ubiquitin-recognizing protease was a complex, ATP-dependent entity (Hershko et al., 1984b).

But the most vexing feature of this pathway was the lack of a biological context. The elegant mechanistic framework discussed above was developed from experiments conducted in rabbit reticulocyte extracts. Despite certain hints (Hershko et al., 1982), it was unclear if nucleated mammalian cells harbored the same pathway. The biological purpose of the pathway was even more uncertain. Although misfolded and truncated polypep-

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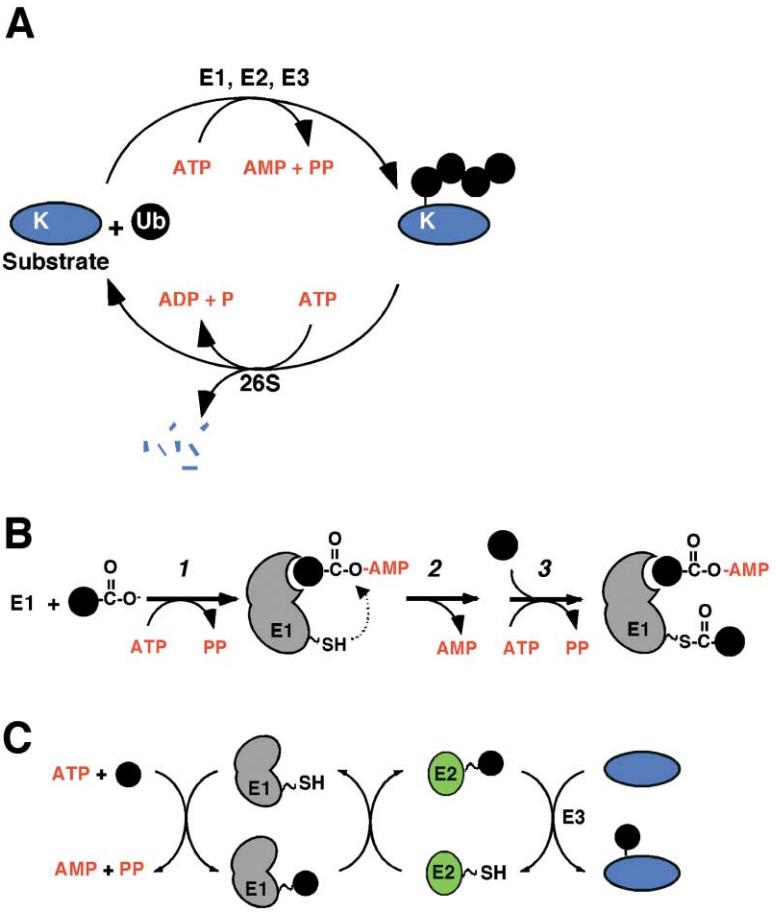

Figure 1. Components and Mechanisms in the Ubiquitin/Proteasome Pathway

(A) Overview of pathway showing how ATP is used in its conjugative (top) and degradative (bottom) phases. *E1*, *E2*, and *E3* are ubiquitin activating, conjugating, and ligase enzymes, respectively; *K* denotes a substrate lysine residue.

(B) E1-catalyzed reaction. Step 1, ubiquitin adenylate formation; step 2, transfer of ubiquitin from adenylate to cysteine (product not shown); step 3, second round of adenylate formation to yield fully loaded enzyme.

(C) The ubiquitin conjugation cascade. Elaboration of a polyubiquitin chain (data not shown) often involves the same E2/E3 complex, but can also involve a different complex (Hoege et al., 2002). Certain E3 enzymes form ubiquitin thiol esters during catalysis of substrate ubiquitination (Figure 2 below).

tides were known to be targeted in a selective manner for ubiquitination and proteasome degradation (Hershko et al., 1982), no normal protein was yet known to be eliminated by this interesting mechanism. Accordingly, the pathway was viewed by many as an intracellular garbage disposal. This function did not inspire broad interest among biologists.

One final player needs to be introduced to appreciate these classic papers. Histone 2A (H2A) was the first protein shown to be modified by ubiquitin through an isopeptide linkage (Goldknopf et al., 1975). It is the most abundant ubiquitinated protein in most nucleated mammalian cells, comprising 10%–20% of the total conjugate pool; histone H2B is also subject to ubiquitination (Jason et al., 2002). Because H2A is a long-lived protein, its status as a natural substrate of ubiquitination shed no direct light on the purposes of ubiquitin-dependent proteolysis. Nonetheless, ubiquitinated H2A played an important part in the work discussed below.

ts85 Cells, Ubiquitination, and Proteolysis

The mouse mammary carcinoma cell line called ts85 was discovered based on its phenotype of temperature-sensitive arrest in the G2 phase of the cell cycle, but the molecular basis of this interesting phenotype was unknown (Mita et al., 1980). Varshavsky and coworkers were intrigued by the rapid disappearance of ubiquitinated H2A that occurred when ts85 cells were shifted to the nonpermissive temperature (Marunouchi et al., 1980). Reasoning that this event could be explained by a failure in ubiquitin conjugation, they set out to evaluate this possibility in a systematic manner.

In the first republished paper, Finley and coworkers show that extracts of ts85 cells grown at a restrictive temperature display a marked defect in ubiquitin conjugation when compared to extracts of cells grown at a permissive temperature (Finley et al., 1984). Neither the parental cells nor temperature-insensitive revertant cells (both of which had normal cell cycles) displayed this property, indicating that inhibition of ubiquitination is tightly correlated with the defect in cell cycle progression. Inhibition was similar in assays of ubiquitin conjugation to lysozyme (a model substrate recognized by an E3 enzyme in the extract), unidentified cellular proteins, and H2B (Ciechanover et al., 1984; Finley et al., 1984). Knowing as we do today that there are diverse substrate-specific E3s, the global character of the observed defect affords virtual proof that the failure occurs at an early step in the conjugation cascade. To prove that the very first enzyme was the labile factor, the researchers affinity-purified E1. They found that the homogeneous enzyme from ts85 cells, but not E1 from the parental cells, rapidly lost ubiquitin-activating capacity at high temperature as measured in assays of ubiquitin thiol ester formation (Finley et al., 1984). The activity of E1 disappeared with similar kinetics to activity in

ubiquitin-substrate conjugation (Ciechanover et al., 1984; Finley et al., 1984), further supporting a causal relationship between the two defects and suggesting that inactivation of E1 underlies temperature-sensitive cell cycle arrest. Years later, with the advent of routine cDNA cloning, it was shown that the defect in cell cycle progression is indeed rescued following transfection of a wild-type E1 cDNA (Ayusawa et al., 1992).

Recognizing that this conditional defect in ubiquitin conjugation could be exploited for purposes of functional discovery, Ciechanover and coworkers asked a simple question: how are rates of intracellular proteolysis affected when the activity of the ubiquitin/proteasome pathway is drastically reduced through the thermal inactivation of E1? Pulse-chase experiments revealed that the turnover of abnormal or truncated polypeptides was inhibited by more than 80% when ts85 cells were shifted to the restrictive temperature, concomitant with a profound inhibition of the ubiquitination of these polypeptides (Ciechanover et al., 1984). This outcome agreed with earlier indications that misfolded proteins are selectively recognized for ubiquitin tagging (Hershko et al., 1982), but it provided a decisive demonstration that the ubiquitination of these species in nucleated cells correlates with their degradation. In the most important experiment, the turnover of short-lived normal proteins was found to be inhibited by more than 90% at the restrictive temperature. The turnover of these polypeptides at the permissive temperature was accompanied by their transient appearance in the ubiquitin conjugate pool, and was ATP-dependent but insensitive to lysosomotropic agents—all as expected for turnover in the ubiquitin/proteasome pathway. (The identification of the proteasome as the relevant protease did not occur for several more years, however.)

Enduring Lessons

Data published in these papers represented the first evidence that the ubiquitin/proteasome pathway is the principal mechanism for turnover of normal short-lived proteins in mammalian cells. Subsequent research has confirmed this conclusion in several different ways. For example, cell-permeable inhibitors of the proteasome ablate the turnover of short-lived proteins in mammalian cells (Rock et al., 1994) and mutations in (yeast) proteasome subunit genes elicit a similar effect, in some cases concomitant with cell cycle arrest (Ghislain et al., 1993; Gordon et al., 1993; Heinemeyer et al., 1991; Seufert and Jentsch, 1992). Confirming an important role of ubiquitin conjugation, deletion of the *UBC4* and *UBC5* E2 genes of *Saccharomyces cerevisiae* greatly inhibits the turnover of short-lived and abnormal proteins (Seufert and Jentsch, 1990). These two E2s act in concert with many E3s; in this respect they resemble E1, which provides activated ubiquitin for all conjugation processes. (In most cases, the deletion of E2-encoding genes elicits rather selective effects because of the pronounced specificities of E3s, as discussed below.)

The studies of Varshavsky and coworkers also provided the first clue that ubiquitination regulates the cell cycle. The argument that proteolysis is the ubiquitin-dependent process that underlies this regulation, although inferential in 1984, was decisively validated by the later discovery that the turnover of mitotic cyclins is ubiquitin-dependent (Glotzer et al., 1991; Hershko et al., 1991). The recognition that the ubiquitin/proteasome pathway plays a central role in cell cycle progression led to a series of key findings that proved to be relevant not only for this function, but also more generally. In particular, the defining member of a large family of multisubunit E3s, called SCF complexes (*S*kp/*C*ullin/*F* box), was discovered through investigations of how ubiquitin-dependent proteolysis regulates the G1/S transition (Feldman et al., 1997; Skowyra et al., 1997). Another, distantly related E3, called the APC (*A*naphase *P*romoting *C*omplex), regulates the metaphase-to-anaphase transition and exit from mitosis (reviewed in Jackson et al., 2000; Peters, 2002). The substrates targeted by these ligases are activators and inhibitors of cyclin-dependent kinases (CDKs). The role of the ubiquitin/proteasome pathway in regulating cell cycle progression is reviewed in detail elsewhere in this issue (Murray, 2004 [this issue of *Cell*]). Studies of SCF substrate susceptibility to ubiquitin tagging also provided the first, and still some of the most notable, examples of how phosphorylation regulates E3/substrate interactions, as discussed in the article by Murray and in excellent earlier reviews (Deshaies and Ferrell, 2001; Jackson et al., 2000; Peters, 2002).

Somewhat ironically, this first genetic experiment in the ubiquitin/proteasome field involved mammalian cells (Ciechanover et al., 1984; Finley et al., 1984). However, the same year saw the cloning of the first ubiquitin pathway gene in *S. cerevisiae* (Ozkaynak et al., 1984), ushering in a long period in which budding yeast dominated molecular genetic investigations of ubiquitin-dependent signaling. Not only have these investigations illuminated these processes; they have also generated powerful tools in the form of plasmids and yeast strains (Hochstrasser, 1996). Nonetheless, mammalian cell lines like ts85 remain useful today because in contrast to the situation with proteasomes, there are still no cell-permeable inhibitors of ubiquitination. Even though a thermolabile E1 enzyme is a rather blunt instrument, cells harboring it can be used to show that a given event relies on ubiquitin conjugation. Such cell lines have figured importantly in studies of ubiquitin-dependent processes that are proteasome-independent (discussed below), including endocytosis and protein trafficking (for example, Strous et al., 1996). These cell lines have also proved useful for demonstrating the ubiquitin independence of other events (Shringarpure et al., 2003).

Do the 1984 papers hold any surprises for today's reader? There was at least one for the author. Finley et al. combined equal volumes and parental and ts85 cell extracts and found that the mixture displayed 50% of the parental extract's ubiquitination activity at several temperatures (Finley et al., 1984). The authors argued that if the heat-labile component had been a regulatory factor such as a kinase, the active factor in the parental extract should have acted catalytically on its targets in the ts85 extract and complemented the ubiquitination defect (Finley et al., 1984). In fact, from today's point of view it is rather surprising that the active E1 in the parental extract did not produce exactly this outcome. E1 is a far more efficient enzyme than most downstream conjugating factors and is often considered to afford

ubiquitin activation activity in excess of that required by subsequent reactions (Pickart, 2001). The result obtained by Finley et al. therefore raises the possibility that in contrast to current views, the E1 step could be rate-limiting for certain ubiquitination events.

Then and Now

The two papers discussed above led to a new worldview; not only was the ubiquitin/proteasome pathway a major proteolytic mechanism in the average mammalian cell, but it was also likely to regulate cell cycle progression. These conclusions are so well accepted today that it is difficult to appreciate the magnitude of their impact at the time the two papers appeared. Succinctly put, this work forced biologists as well as biochemists to respect the ubiquitin/proteasome pathway.

In what other ways has our view of ubiquitination changed since 1984? A comprehensive discussion would greatly exceed the scope of this article but a few developments are noteworthy, especially when viewed through the lens of the state of knowledge in 1984.

Ubiquitination Regulates Lysosomal Proteolysis

Researchers studying ubiquitin in the mid-1980's held it as a tenet of faith that the ubiquitin/proteasome pathway had no point of intersection with lysosomal proteolysis. This commandment reflected the fact that agents which disrupt lysosomal functioning, have no effect on the ATP-dependent turnover of short-lived and abnormal proteins (see Ciechanover et al., 1984). In direct contradiction of this formerly strict rule, we now know that ubiquitination is sometimes required for lysosomal proteolysis. This could not be detected in the 1984 study because only a small fraction of short-lived proteins is targeted to lysosomes. However, later studies of individual endocytosed proteins revealed that a subset of these molecules must be conjugated to ubiquitin as a trigger for internalization from the plasma membrane (Hicke and Riezman, 1996; Kolling and Hollenberg, 1994). In fact, endocytosis is just one of many protein trafficking steps that depend on ubiquitin conjugation, as recently reviewed elsewhere (Aguilar and Wendland, 2003; Hicke and Dunn, 2003). The discovery that retroviruses subvert certain ubiquitin-dependent trafficking events in order to achieve budding offers exciting new possibilities for therapeutic intervention (Garrus et al., 2001).

Truly, a System

If a ubiquitin researcher placed in cryostorage in 1984 were to be thawed out today, there is little doubt about what he or she would find most remarkable: the complexity of it all. In the mid-1980's, we knew of one E1, several E2s, and one E3 (Hershko et al., 1983). Although this collection of enzymes already seemed too small to account for the burgeoning biology, no one could have predicted the system's actual breadth. Several factors have combined to produce this knowledge, including detailed investigations of specific ubiquitination/turnover events, newly identified associations with human disease, genome sequencing projects, and bioinformatics. The recognition that E3s are organized into a small number of families was particularly important (Deshaies, 1999; Jackson et al., 2000; Joazeiro and Weissman, 2000; Pickart, 2001). From a mechanistic standpoint, E3s fall into two groups: those that utilize a covalent mechanism (HECT domain E3s) and those that do not (most notably RING-domain E3s) (Figure 2). The mammalian RING-domain family is very large and it is likely that a substantial fraction of its members are E3s. Some consist of just one (multidomain) subunit (Lorick et al., 1999), whereas others are multiprotein complexes in which each subunit is a member of a distinct protein family, with the (small) RING subunit acting to recruit the E2 (Seol et al., 1999) (see also below). There are approximately fifty E2s and more than seventy deubiquitinating enzymes in humans, while the 26S proteasome is composed of at least 64 protein subunits, which are encoded by 32 independent genes (Baumeister et al., 1998). (This review largely ignores the fascinating topic of proteasomes.) Altogether, several percent of the human genome is likely to be devoted to the ubiquitin pathway, taking into account both proteolytic and nonproteolytic functions (Semple, 2003). In 1984, even the most ardent ubiquitin supporter would have rejected this number, which also applies in plants and yeast (Semple, 2003; Vierstra, 2003).

Any perspective on the ubiquitin/proteasome pathway must remark on the hierarchical nature and regulatory potential afforded by this multiplicity of conjugation factors. Each E3 enzyme recognizes a restricted set of substrates (discussed below) and is served by one or a few E2s. These properties are a reflection of the modular construction of E3s—the substrate and the E2 enzyme bind to separate sites, with members of a given E3 family sharing a conserved E2 binding domain. Originally inferred from functional studies, these molecular properties are now documented by atomic-resolution structures of E3s complexed with their cognate E2s (Brzovic et al., 2003; Huang et al., 1999; Zheng et al., 2000) and substrates (discussed below).

Dedicated substrate/E3 pairings permit independent regulation of the ubiquitination of distinct substrates. In some cases, E3 specificity may be further modulated through the association of one E3 with different E2s (Chen et al., 1993). Having many E2s might also control the flow of activated ubiquitin to the cognate E3s of different E2s. This mechanism, if operative, would be most important if the activity of E1 is limiting. Finally, certain RING-domain E3 families take the modular construction idea to an extreme. As first shown for the SCF E3s in the context of cell cycle regulation, the E2- and substrate binding functions can be delegated to separate polypeptides, which are brought together through adaptor-dependent interactions with a scaffold protein called a cullin (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997). The existence of substrate binding (F boxes; SOCs boxes), cullin, and adaptor protein families (Skps, Elongins), in conjunction with functional data, shows that E3 specificity can be reprogrammed by changing the identity of the substrate recognition subunit (Deshaies, 1999).

Substrate Selection and Its Regulation

The finding that most short-lived proteins are degraded in the ubiquitin/proteasome pathway (Ciechanover et al., 1984) raised a pressing question about specificity.

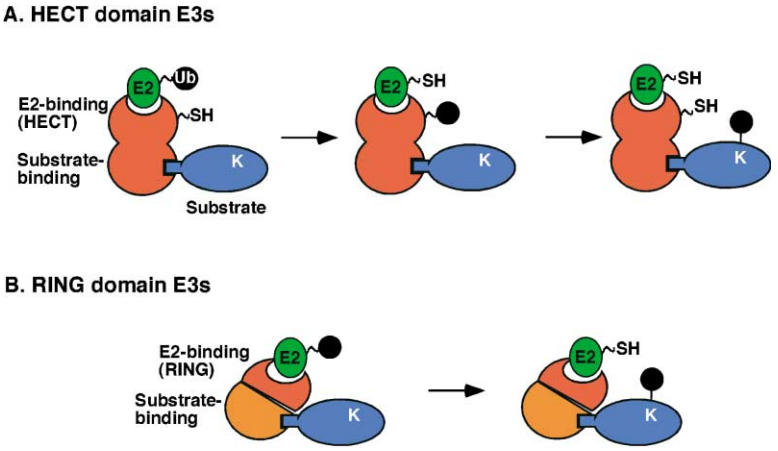

Figure 2. Major E3 Classes

(A) HECT domain E3s (*H*omologous to *E*6AP *C*-*T*erminus) bind cognate E2s via the conserved HECT domain and transiently accept ubiquitin at a cysteine residue in this region; a different region of the same polypeptide chain binds the substrate (blue) through an element in the degron (square).

(B) RING-domain E3s (*R*eally *I*nteresting *N*ew *G*ene) are scaffold proteins that use the RING domain (red) to bind the E2 and a different domain (orange) to bind the substrate. In SCF and other multisubunit RING-domain E3s, the RING and substrate binding domains occur in separate polypeptides (text).

Did all such substrates share a common recognition determinant? This was unlikely a priori. The existence of many E3s solves this problem in principle, but fails to show how selective recognition is practiced. Elucidating E3/substrate interactions has been an enduring goal of researchers over the last two decades.

The first E3 to be characterized was the one that recognized the denatured lysozyme substrate used in early biochemical studies. Called E3α, it seemed to require that model substrates carry a free α-amino group (Hershko et al., 1984a). In 1986, studies by Varshavsky and coworkers in yeast unexpectedly uncovered the complete relationship between the identity of the N-terminal amino acid and substrate stability, called the N-end rule (Bachmair et al., 1986). Further investigations of this mechanism showed that the orthologous yeast E3 (Ubr1) recognized this determinant, which together with a lysine residue subject to ubiquitination, was termed an N-degron (Varshavsky, 1997). These studies provided the functional definition of a degron (an element that is both necessary and sufficient for substrate ubiquitination) and established the modular organization that ultimately proved to apply to all E3s. The N-end rule is also biologically important, as dramatically shown by the essentiality of one of its components for cardiovascular development in the mouse (Kwon et al., 2002).

New degrons continue to be reported at a regular rate. In one interesting recent example, the specificity subunit of a cytosolic SCF E3 was found to recognize N-linked high-mannose oligosaccharides (Yoshida et al., 2002). (In a turn of events that would amaze the time-traveling researcher mentioned above, it was discovered in the 1990's that misfolded proteins of the endoplasmic reticulum (ER) are ejected from that compartment and degraded by the cytosolic ubiquitin/proteasome pathway in a process known as ERAD (*ER*-*A*ssociated *D*egradation) (Kostova and Wolf, 2003). Since proteins can only acquire these sugars in the ER interior, having the glycan as a component of the degron may be a clever way to achieve uniform targeting of a subset of proteins originating in that compartment. Surprisingly, however, we still do not fully understand the one example of selective targeting that was known in 1984, namely that of misfolded polypeptides. Certain E3s recognize inappropriately exposed hydrophobic surfaces (Johnson et al., 1998), whereas other E3s coopt chaperones as their specificity factors (Cyr et al., 2002), but it is uncertain if these targeting mechanisms are the whole story. A burgeoning area of research suggests that inadequate clearance of misfolded proteins by the ubiquitin/proteasome pathway may contribute to neurodegenerative diseases such as Parkinson's and Huntington's, giving new impetus to studies of misfolded protein degradation (Berke and Paulson, 2003; Giasson and Lee, 2003).

Studies of degron recognition in physiological substrates have revealed a level of regulatory sophistication that would have been unimaginable in 1984. E3/degron interactions can be modulated by posttranslational modifications (among other mechanisms) that serves to link ubiquitination to other cellular events (reviewed in Deshaies, 1999; Deshaies and Ferrell, 2001; Laney and Hochstrasser, 1999; Peters, 2002) (Figure 3). Most famously, CDK-catalyzed phosphorylation triggers the proteolysis of CDK regulators at appropriate points in the cell cycle (see Deshaies and Ferrell, 2001; Peters, 2002 and Murray, 2004 [this issue of *Cell*]). Another recent example is the oxygen-dependent hydroxylation of a specific proline residue in *H*ypoxia *I*nducible *F*actor-1α (HIF-1α), which triggers recognition by a cullin-based E3 that has the *V*on *H*ippel *L*indau (VHL) tumor suppressor protein as its specificity subunit; the ensuing degradation of HIF-1α shuts off a hypoxic program of gene expression (Ivan et al., 2001; Jaakkola et al., 2001). Structural studies of this E3 show that the hydroxyproline residue of HIF-1α binds to a region of VHL that is frequently mutated in a hereditary cancer syndrome (Hon et al., 2002; Min et al., 2002; Stebbins et al., 1999).

Structurally Distinct Ubiquitin Modifications Impart Diversity in Signaling

In 1984 we knew that histones could be modified with a single ubiquitin. Although the purpose of this modification was mysterious, it definitely did not signal proteolysis. Substrates destined for proteasomes, on the other hand, were decorated with many ubiquitins and this high stoichiometry seemed to be important for productive degradation (Chin et al., 1982; Hershko et al., 1984b; Hough and Rechsteiner, 1986). Later work proved that these multiple ubiquitins must be linked together in a specific type of polyubiquitin chain to order to guarantee

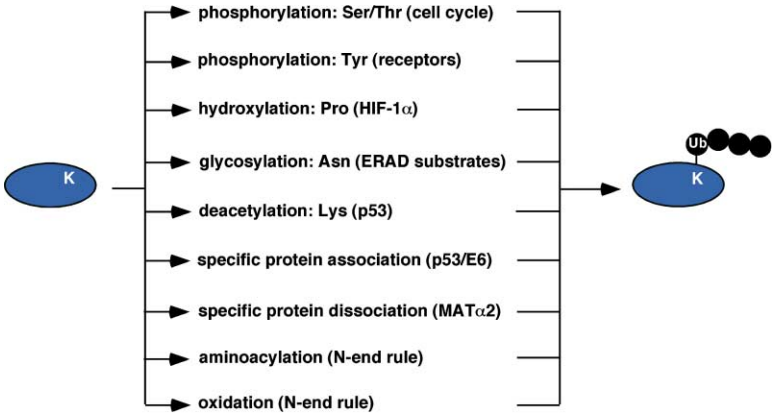

Figure 3. Mechanisms for Modulating Substrate Recognition by E3s

Shown are posttranslational modifications and other mechanisms known to regulate the recognition of cognate substrates by different E3s. For discussions of phosphorylation-based recognition, see Deshaies, 1999; Jackson et al., 2000; Joazeiro and Weissman, 2000; Murray, 2004; for deacetylation, see Brooks and Gu, 2003; for aminoacylation, see Kwon et al., 2002; Varshavsky, 1997; for oxidation, see Kwon et al., 2002; for other examples, see the text.

efficient targeting to proteasomes (Chau et al., 1989; Finley et al., 1994). In contrast, ubiquitin-dependent protein trafficking usually requires the ligation of just one ubiquitin to the substrate (Gregory et al., 2003; Hicke and Dunn, 2003). Thus, mono- and polyubiquitination are associated with different functional outcomes.

A different type of polyubiquitin chain, linked through ubiquitin-K63 instead of K48, is generated during the autoubiquitination of TRAF family signal-transducing E3s, apparently leading to the activation of a specific cytosolic kinase and ultimately to the expression of NF-κB target genes in mammals (Deng et al., 2000; Kovalenko et al., 2003; Trompouki et al., 2003; Wang et al., 2001). The same type of atypical chain regulates ribosome function in the cytosol (Spence et al., 2000) and is necessary for a conserved pathway of DNA damage tolerance in the nucleus (Hofmann and Pickart, 1999; Spence et al., 1995). Exactly how these noncanonical polyubiquitin chains signal downstream events is unclear, but they do not evoke substrate proteolysis. They could be recruitment signals for unidentified factors or they might modulate the properties of the target protein to which they are attached. Still, their properties indicate that different polyubiquitin chains can be associated with distinct signaling outcomes. Indeed, ubiquitin-dependent DNA damage tolerance presents a remarkable example of how signal structure can regulate downstream effects. Depending on the circumstances, the DNA polymerase processivity factor PCNA is modified at a single site by monoubiquitin, a K63-linked polyubiquitin chain, or the ubiquitin-like protein SUMO (Hoege et al., 2002). The chain signals error-free replicative bypass of DNA lesions (Hoege et al., 2002), whereas monoubiquitin and SUMO may signal bypass by distinct translesion polymerases (Stelter and Ulrich, 2003).

Being a protein, ubiquitin offers its downstream signal-transducing components more abundant and sophisticated recognition opportunities than are afforded by conventional covalent modifiers; polymerization further expands these possibilities. This mechanism is not unique to ubiquitin. Oligo- and polysaccharides richly embody the principle of structure-based recognition and polyphosphate chains have unique signaling properties (Wang et al., 2003). The recent detection of all seven possible ubiquitin-ubiquitin linkages in the yeast proteome suggests that new signaling functions of polyubiquitin chains remain to be discovered (Peng et al., 2003).

Parallel Universes

From structurally distinct ubiquitin modifications, it is only a small step to a remarkable recent development—structurally distinct ubiquitins (so to speak). We now know that ubiquitin defines a family of structurally related signaling proteins which share a common biochemical mechanism of isopeptide tagging. The interferon-induced ISG15 protein was the first such protein to be discovered (Loeb and Haas, 1992); other examples followed in short order. The functional range of individual family members varies widely, as reviewed elsewhere (Muller et al., 2001; Schwartz and Hochstrasser, 2003). Nedd8/Rub1, for example, seems to function only as an activator of cullin-based E3s, whereas SUMO modifies numerous cellular proteins and may signal several different fates for its substrates.

Histone Ubiquitination: Somewhat Less Mysterious

Why are histones subject to ubiquitination? Studies conducted between 1984 and 2000 suggested several possible answers, none of which appeared to be definitive, probably because the modification can serve several functions (Jason et al., 2002). A recent advance came from work in budding yeast, which revealed that site-specific ubiquitination of histone H2B promotes site-specific methylation of histone H3, with an ultimate readout of transcriptional silencing (Sun and Allis, 2002). This is only one of several newly discovered roles for ubiquitination in transcriptional regulation (Conaway et al., 2002).

Forward to the Future

Although many features of ubiquitin biology stand in clearer relief today than in 1984, the intensity of effort focused on the pathway has also served to spotlight features that we do not yet (or still do not) understand. Because a full discussion of these interesting questions would require a separate review, only a few are mentioned here.

Deubiquitination: The End at the Beginning

While there is a clear rationale for having many E2s and E3s, we still lack a satisfactory explanation for the multiplicity of deubiquitinating enzymes. Many of these enzymes belong to a large cysteine protease family, the *Ub*iquitin processing *P*roteases (Amerik et al., 2000). A few UBPs play important roles in regenerating ubiquitin from proteolytic intermediates (providing the fodder for new ubiquitination events) and another handful have been implicated in a specific biological process (Amerik et al., 2000). But most of them are functionally uncharacterized. One attractive hypothesis proposes that certain UBPs are target-protein specific, but so far only a few enzymes definitively conform to this paradigm (Cohen et al., 2003; Li et al., 2002). Meanwhile, additional families of deubiquitinating enzymes continue to be discovered. A small zinc-dependent family (JAMM/MPN$^+$) includes a proteasome subunit that removes polyubiquitin chains from substrates during proteolysis (Verma et al., 2002; Yao and Cohen, 2002). A much larger cysteine protease family (OTU) includes a known negative regulator of the inflammatory response (Balakirev et al., 2003). However, it was CYLD, a member of the UBP family that is also a tumor suppressor, that was recently shown to repress NF-κB activation, possibly by removing K63-linked polyubiquitin chains from TRAF E3s (Kovalenko et al., 2003; Trompouki et al., 2003).

How Do E3s Work?

In the prevailing view, RING-type E3 enzymes are bridging factors that bring the E2 enzyme with its activated ubiquitin into the vicinity of the substrate, and then hope for the best. However, while induced proximity can provide large catalytic rate enhancements, such effects require an exact placement of the reactants (Fersht, 1984). It is unclear that E3s can meet this requirement, given the tens of angstroms that are inferred to separate the bound E2 and substrate molecules based on recent crystal structures (Orlicky et al., 2003; Zheng et al., 2002). Although the E2 enzyme provides catalytic assistance to RING E3s (Wu et al., 2003), additional mechanisms presumably come into play and must be characterized in order to interdict the chemical step of ubiquitin conjugation for purposes of research or therapy (Nalepa and Harper, 2003).

After Ubiquitination, Then What?

The hundreds (thousands?) of ubiquitin-modified proteins present in a cell at any point in time need to be individually recognized in a manner that correctly translates the information contained in each ubiquitin signal into appropriate downstream events. How is this achieved? As yet we know little about signal recognition that does not involve proteasomes. The recent discovery of several families of ubiquitin binding proteins is thus an exciting development (Aguilar and Wendland, 2003; Buchberger, 2002; Hicke and Dunn, 2003). Some members of these families are already known to function in specific signaling pathways and studies of their molecular recognition properties should aid greatly in understanding how ubiquitin signals are transduced.

Ubiquitination and Human Disease

An increasing number of inherited diseases has been found to be caused dysfunctions in ubiquitination, offering a challenge for the present and an opportunity for the future. Typically, a mutation in an E3 enzyme or its cognate substrate results in substrate stabilization, leading to deleterious consequences. Stabilization of HIF-1α by mutations in VHL (see above) may conform to this paradigm. The inappropriate destabilization of a cellular protein can also be a pathologic event (Scheffner et al., 1990). These are just two examples among many, as recently reviewed elsewhere (Schwartz and Ciechanover, 1999). Unfortunately, matching an interesting E3 with its cognate substrate (or vice versa) is often a difficult proposition (Giasson and Lee, 2003; Huang et al., 1999). The same difficulty applies in the analysis of deubiquitination, dysfunctions of which occur in several known diseases (see above). In contrast to the biochemical fractionation approaches that predominated in 1984, enzyme/substrate matching today is frequently achieved by protein interaction screening. New technologies, including RNA interference, are also being deployed in the service of this goal (for example, Brummelkamp et al., 2003). Finally, global inhibition of the ubiquitin-proteasome pathway may be an exacerbating factor in certain diseases (Bence et al., 2001; Berke and Paulson, 2003; Song et al., 2003), but in other cases it can have a therapeutically beneficial effect. An active site inhibitor of the proteasome was recently approved for treatment of multiple myeloma and is being tested for efficacy against other cancers (Adams, 2002). Ultimately, E3 enzymes that regulate cell cycle progression and cell proliferation may provide important new therapeutic targets in cancer and other diseases (Nalepa and Harper, 2003).

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Review TRENDS in Cell Biology Vol.17 No.4

The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies

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The N-end rule states that the half-life of a protein is determined by the nature of its N-terminal residue. This fundamental principle of regulated proteolysis is conserved from bacteria to mammals. Although prokaryotes and eukaryotes employ distinct proteolytic machineries for degradation of N-end rule substrates, recent findings indicate that they share common principles of substrate recognition. In eukaryotes substrate recognition is mediated by N-recognins, a class of E3 ligases that labels N-end rule substrates via covalent linkage to ubiquitin, allowing the subsequent substrate delivery to the 26S proteasome. In bacteria, the adaptor protein ClpS exhibits homology to the substrate binding site of N-recognin. ClpS binds to the destabilizing N-termini of N-end rule substrates and directly transfers them to the ClpAP protease.

Introduction

Intracellular protein degradation plays an essential role in many physiological processes by removing either damaged polypeptides or proteins that harbor specific destruction tags. Whereas the proteases contained within compartments dedicated to biomolecule destruction, such as the lysosome and the plant vacuole, hydrolyze proteins in a rather non-specific manner, protein degradation in the cytosol of pro- and eukaryotic cells exhibits a high degree of specificity that is used to protect cellular proteins from unwanted degradation and to subject proteins with signaling functions to regulated proteolysis. The selective removal of regulatory proteins, such as transcription factors or signal transduction proteins, represents an efficient and rapid strategy to control checkpoints for many cellular processes, including cell growth, division, differentiation and programmed cell death. Here, we describe and compare the strategies that are used by eukaryotes and prokaryotes in regulated proteolysis via the N-end rule pathway. The N-end rule defines the stability of proteins according to the nature of their N-terminal residues. Amino acids are classified as stabilizing and destabilizing residues, which serve as recognition determinants for protein degradation. Novel findings highlight the ancient origin of the N-end rule pathway and indicate common mechanisms in regulated protein degradation between eukaryotes and prokaryotes, despite fundamental differences in key factors involved in the various proteolytic systems.

Destructive machines: common architecture and mechanisms of ATP-dependent proteases

In eukaryotic cells, degradation of cytosolic and nuclear proteins is mainly mediated by the 26S proteasome, a large protein machine composed of two different complexes. The 20S core complex forms a hollow cylinder that is composed of four heptameric rings, consisting of α- or β-subunits. It harbors the proteolytic active sites in its interior chamber, which is only accessible through narrow pores at either end [1]. This self-compartmentalization ensures processive substrate hydrolysis to short oligopeptides by providing high concentrations of active sites and, through the sequestration of these sites, prevents unregulated protein degradation. These advantages, however, come at an energetic price because substrates need to be unfolded and translocated before they can reach the proteolytic chamber. This task is fulfilled by the 19S complex, which is located at either end of the proteolytic 20S complex. The base of each 19S complex contains six AAA+ (ATPase associated with diverse cellular activities) proteins that promote the ATP-dependent unfolding and threading of substrates into the proteolytic chamber of the 20S proteasome (see Figure Ia in Box 1).

Proteolysis of either misfolded or specifically tagged proteins in the cytosol of prokaryotes is mediated by proteasome-like machines that also consist of an ATPase module (e.g. ClpA, ClpX) and a proteolytic component that is either covalently attached (e.g. Lon) or diffusible (e.g. ClpP) [2]. The peptidase ClpP from *Escherichia coli*, which does not exhibit sequence homology to the α- and β-subunits of the eukaryotic 20S complex, forms, however, a structure of similar architecture. It consists of two heptameric rings that form a barrel-shaped proteolytic core with the active sites hidden in an interior chamber. Access to these sites is controlled by narrow pores that do not allow the passage of folded polypeptides. ATP-fueled substrate unfolding and translocation is mediated by various Hsp100 proteins (e.g. ClpA), which associate with either end of the ClpP core. Hsp100 proteins are members of the class of heat-shock proteins and represent a subgroup of the large AAA+ protein family. During substrate processing, they fulfill the very same function as their distant cousins present in the 19S cap of eukaryotic proteasomes (see Figure Ib in Box 1).

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Box 1. Principles of regulated proteolysis in eukaryotes and prokaryotes: comparison of substrate selection and processing

In eukaryotic cells (Figure Ia), substrates (green) are mainly recognized by E3 ligases that mediate, in conjunction with associated E2 ubiquitylating enzymes, the covalent labeling of bound substrates with ubiquitin (Ub). The initial Ub attachment is followed by the formation of a substrate-linked polyubiquitin chain, which is recognized by components of the 19S cap complex of the proteasome. AAA+ proteins present in the base of the 19S complex mediate the ATP-driven unfolding and translocation of bound substrates into the core of the proteolytic 20S complex. Ubiquitin is spared from degradation through its release from the substrate by deubiquitylating enzymes. In prokaryotic cells (Figure Ib), substrates (red) can be directly recognized by extra domains (e.g. N-domain) of AAA+ proteins (e.g. ClpA). Alternatively, substrates (green) are initially recognized by adaptor proteins that deliver their bound cargo following binding to N-domains of the cognate AAA+ partner protein. AAA+ proteins form ATP-dependent proteolytic machines upon complex formation with peptidases (e.g. ClpP) and mediate the unfolding and translocation of bound substrates into the proteolytic chamber of the associated peptidase.

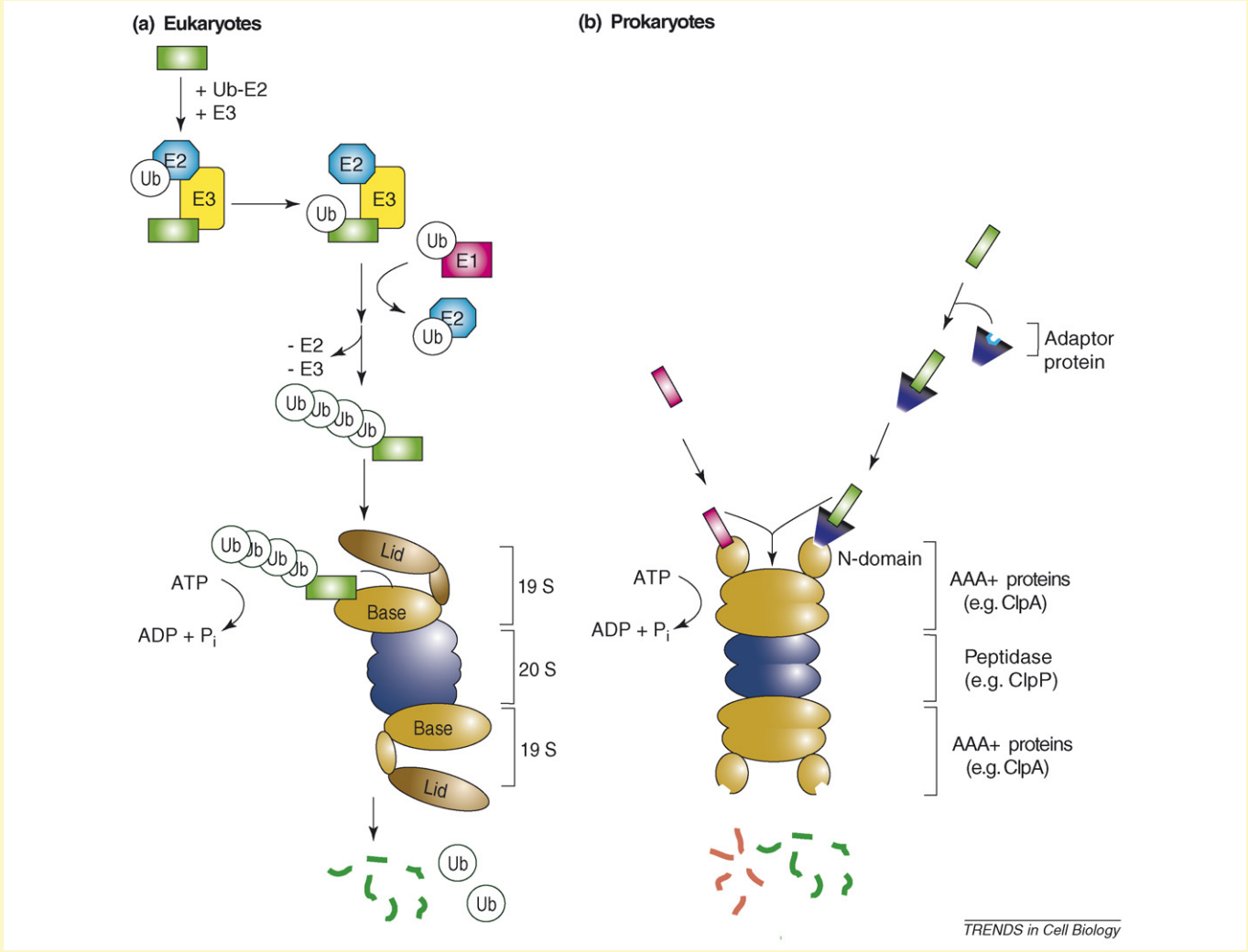

Figure I. Regulated proteolysis in **(a)** eukaryotes and **(b)** prokaryotes. Pi, inorganic phosphate.

Selection of unstable proteins: different strategies in Eukaryota and Prokaryota

Each proteolytic system must exhibit a high degree of substrate specificity to prevent uncontrolled degradation of bulk proteins and to ensure its regulatory function in signal transduction pathways. Unstable proteins harbor specific degradation signals, termed degrons, that are recognized by components of the proteolytic systems and subsequently delivered to hydrolyzing proteases. Although this basic strategy is shared by eukaryotes and prokaryotes, the underlining mechanisms for substrate selection turns out to be entirely different.

In Eukaryota, the conserved protein ubiquitin (Ub) plays an essential role as a secondary signal for protein degradation and is covalently attached to target proteins [3]. Short-lived regulatory or misfolded proteins are conjugated to Ub through the action of three enzymes: E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (Ub-protein ligase). A ubiquitylated substrate bears a covalent linkage between an internal Lys residue and a Ub moiety. The selectivity of ubiquitylation represents the crucial step in substrate selection and is mainly determined by E3 enzymes (see Figure Ia in Box 1). Each E3 enzyme recognizes a restricted set of substrates and is served by one (or a few) E2 enzyme. Their function as specificity factors is reflected in the large number of E3 enzymes with more than 500 distinct Ub ligases in mammals. The E3-dependent recognition of the various primary

degradation signals leads to the enzymatic addition of a branched poly-Ub chain that serves as a secondary signal for targeting of the substrates to the proteasome. This step allows the 19S complex of the 26S proteasome to integrate the signals from different degrons and to interact with a large variety of substrates. Ubiquitin itself is spared from degradation through its release from the substrate by deubiquitylating enzymes that are present in the 19S complex of the proteasome.

Regulated proteolysis in Prokaryota also involves specific degrons and recognition proteins; this process, however, is entirely independent of ubiquitin, which is absent in bacteria. The absence of ubiquitin conjugation in prokaryotes is remarkable and points to an entirely different strategy through which these cells select substrates for degradation. Here, substrate specificity is mediated by the Hsp100 components of the proteolytic machinery. Hsp100 proteins gain functional diversity with the presence of additional domains that are missing in other family members. Such extra domains (e.g. N-domain) either directly interact with substrates or serve as binding platforms for adaptor proteins that recognize specific substrates and transfer them to their cognate Hsp100 partner protein (see Figure Ib in Box 1). The multiplicity of E3 enzymes in Eukaryota is replaced in prokaryotic cells by a smaller number of recognition determinants, which, however, still target a large variety of substrates for degradation [4].

The N-terminus matters: lessons from the N-end rule pathway

In 1986, Varshavsky and coworkers reported that different genetic constructs of β-galactosidase proteins from *Escherichia coli* exhibited very different half-lives when produced in *Saccharomyces cerevisiae*, ranging from more than 20 h to less than 3 min [6]. The stability of the model proteins was dependent on the nature of their N-terminal amino acid residues, which allowed for a classification of amino acids as either stabilizing or destabilizing residues. The resulting degradation signals, termed N-degrons, were the first characterized degrons in eukaryotes, defining the half-lives of proteins according to the nature of their N-terminal residues. The N-end rule pathway is present in all organisms examined so far, including the Gram-negative model bacterium *E. coli* [5], the yeast *S. cerevisiae* [6], the plant *Arabidopsis thaliana* [7] and mammalian cells [8]. The signals for substrate degradation via the N-end rule are similar in these organisms, but also show distinct differences. In mammalian and yeast cells, the N-degron comprises an N-terminal destabilizing residue of either type 1 (Arg, Lys, His) or type 2 (Phe, Leu, Trp, Ile, Tyr) and an accessible Lys residue for ubiquitylation (Figure 1b,c) [9]. In plants, the N-end rule includes basic and aromatic residues [7,10,11]. In *E. coli*, only aromatic amino acids and Leu of the eukaryotic type 2 N-degron (except Ile) represent the destabilizing residues (Figure 1a,c) [5].

The characteristics of the N-degrons raise the issue of how destabilizing N-termini are generated. Newly synthesized proteins contain N-terminal Met (fMet in prokaryotes), which is a stabilizing residue in all organisms according to the N-end rule. Therefore, an N-degron of an N-end rule substrate can only be produced from a pre-N-degron. N-terminal Met is removed cotranslationally from the majority of newly synthesized proteins by methionine aminopeptidases (MetAPs). This processing step, however, only occurs if the residue at position 2, destined to be N-terminal after cleavage, has a small side chain (e.g. Gly, Cys, Ala, Ser) representing a stabilizing residue (Figure 1d) [12]. This specificity of MetAPs is conserved from prokaryotes to eukaryotes, indicating a coevolution of the N-end rule pathway and the specificity of MetAPs, which is tailored not to create N-end rule substrates, enabling the N-end rule pathway to be operative in regulated proteolysis. Hence, it is either the removal of stabilizing N-terminal residues and/or the generation of novel destabilizing N-termini that function as the regulated entry point to the N-end rule pathway and, therefore, need to be tightly controlled.

How can pre-N-degrons be converted into functional N-degrons? N-degrons can be buried in the interior of a protein sequence and rendered accessible only after endoproteolytic cleavage (Figure 1e). Processing proteases can convert pre-N-degrons into N-degrons either directly through the generation of novel N-termini harboring primary destabilizing residues [1] or the creation of secondary/tertiary destabilizing amino acids (see below) [14]. A special way to enter the N-end rule pathway has been reported for some unstable viral proteins that are synthesized as part of stable polyproteins. The large precursor protein is processed into its individual components harboring novel N-terminal residues, which could represent N-degrons. Degradation of such generated proteins via the N-end rule pathway has been demonstrated for HIV-1 Integrase and the RNA polymerase (nsP4) of the Sindbis α-virus [15,16].

A second strategy to create destabilizing residues relies on the enzymatic modification of certain N-termini that function as entry points into the N-end rule pathway (Figure 1f). The N-end rule pathway is indeed organized hierarchically in the sense that, in addition to destabilizing N-terminal 'primary' residues, 'secondary' and 'tertiary' destabilizing residues are also found, which are initially located N-terminally as well. These secondary and tertiary destabilizing residues differ from the primary ones in that they are not directly mediating protein degradation but, instead, are converted to primary destabilizing residues through an enzymatic cascade. A tertiary destabilizing residue is first converted to a secondary destabilizing residue, followed by the addition of a primary destabilizing residue, thereby labeling the protein for degradation. In yeast and mammalian cells, for example, Asn and Gln represent tertiary destabilizing residues that can be converted by N-terminal amidases (e.g. NTA1) into the secondary destabilizing residues Asp and Glu [17,18]. Secondary residues, in turn, function through their conjugation to Arg by the *ATE1*-encoded arginyl-tRNA protein transferase (R-transferase). Arg is finally directly recognized as a primary destabilizing residue and targets the substrate to Ub-dependent protein degradation (Figure 1f) [19]. Interestingly, the hierarchical N-end rule organization is evolutionarily conserved, although the enzymatic reactions that mediate the conversion of secondary destabilizing residues into primary ones differ. Thus, in *E. coli*,

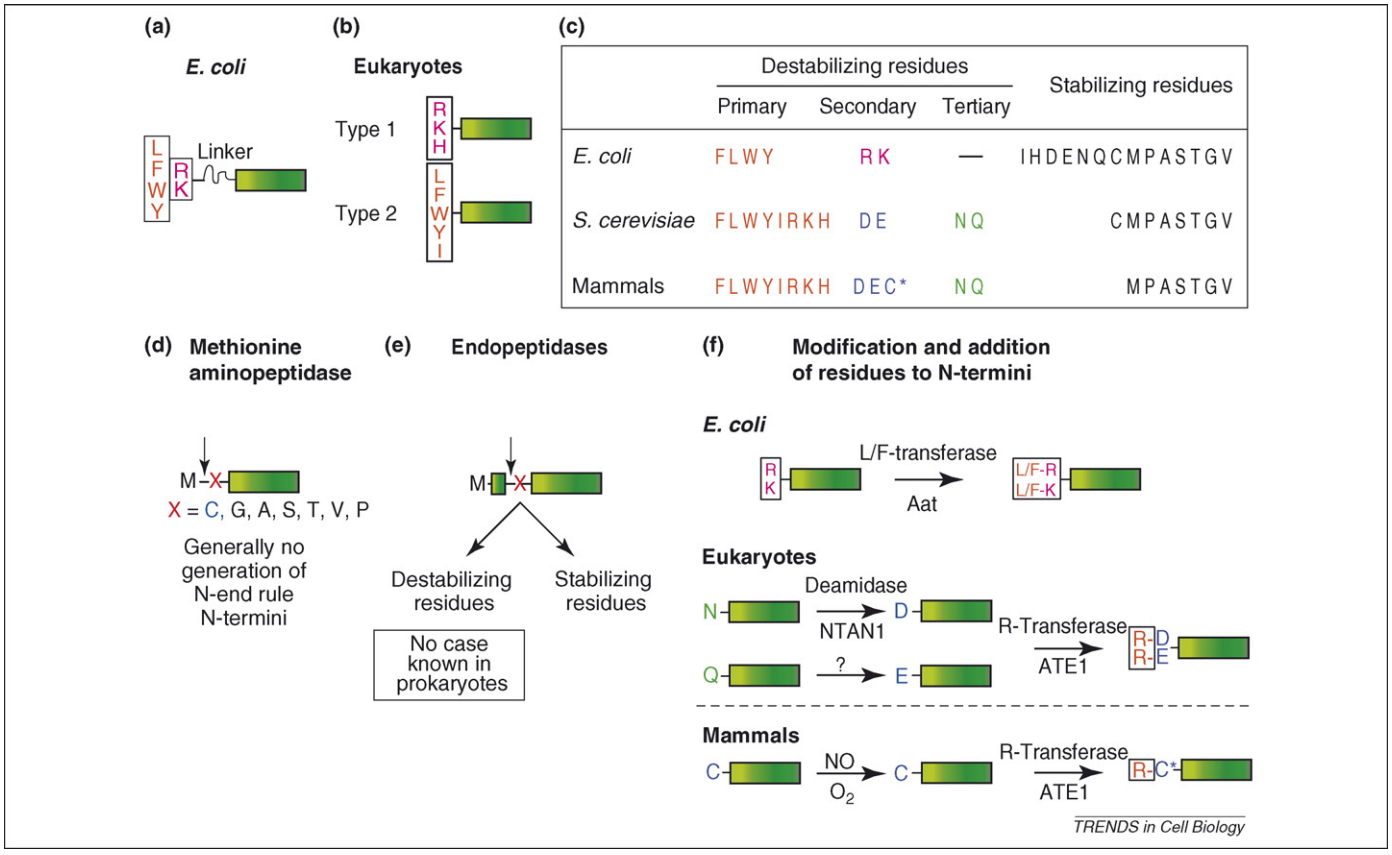

Figure 1. The N-end rule: code and generation of N-degrons. **(a–c)** Comparison of the N-end rule determinants in different model organisms. **(a,c)** In *E. coli*, large hydrophobic (Leu, Phe, Trp, Tyr) and basic residues (Arg, Lys) represent primary and secondary destabilizing residues, and both contribute to the N-degron code. In addition the N-end rule substrate harbors a flexible linker that separates the N-terminal degradation signal from the substrate moiety is required for transfer to and degradation by the ClpA/ClpP protease. **(b,c)** In eukaryotes, basic residues (Arg, Lys, His) and large hydrophobic residues (Leu, Phe, Trp, Tyr, Ile) function independently as type 1 and type 2 primary destabilizing residues. **(c)** Table summarizing the characteristics of individual amino acids according to the present N-end rule. C* indicates an oxidized Cys residue. **(d)** Newly synthesized proteins harbor a stabilizing N-terminal Met and do not function as N-end rule substrates. Methionine aminopeptidases can remove Met from the N-terminus if the adjacent residue has a small side chain, generating proteins that still harbor stabilizing N-terminal residues with the exception of Cys, which can act as a secondary destabilizing residue in mammals. **(e)** Endoproteolytic cleavage events generate protein fragments with novel N-termini including both, stabilizing and destabilizing residues. **(f)** Enzymatic cascades convert tertiary and secondary destabilizing residues into primary ones. Tertiary destabilizing residues (e.g. Asn) can be converted by N-terminal amidases (NTAN1) into secondary destabilizing residues. Secondary residues function through their ATE1-mediated conjunction to Arg in eukaryotes or through the Aat-mediated attachment of Leu/Phe in bacteria. Oxidized Cys (C*), generated in the presence of nitric oxide or oxygen, acts as a secondary destabilizing residue in mammalian cells and is converted into primary destabilizing Arg via R-transferase. Note: amino acids are represented in the figure using the IUPAC one-letter code. Red, primary destabilizing residues; purple, prokaryotic secondary destabilizing residues; blue, eukaryotic secondary destabilizing residues; green, tertiary destabilizing residues; black: stabilizing residues.

Arg and Lys function as secondary destabilizing residues and recruit primary destabilizing residues (Phe, Leu) by conjugation by a leucyl/phenylalanyl-tRNA protein transferase (L/F-transferase or Aat) (Figure 1f) [5]. Interestingly, the human pathogen *Vibrio vulnificus* encodes for a second L-transferase (Bpt). In contrast to *E. coli* Aat, the *V. vulnificus* Bpt L-transferase is homologous to eukaryotic ATE1 (R-transferase) and exhibits a 'hybrid' specificity: like ATE1, it recognizes Asp or Glu as secondary destabilizing residues but adds a Leu instead of an Arg residue [20]. Accordingly, the eukaryotic pathogen *Plasmodium falciparum* harbors the transferase ATEL1, which is homologous to prokaryotic L/F-transferases but has the same activity as eukaryotic R-transferases. The recently determined crystal structure of *E. coli* L/F-transferase suggests a rationale for the change in enzymatic specificity by pointing to differences in the aminoacyl-tRNA binding pockets of *E. coli* L/F-transferase and *P. falciparum* ATEL1 [21]. This switch in enzymatic activities suggests that the already established primary destabilizing residues of the corresponding N-end rule dictated the evolution of the enzymatic cascade that act on secondary destabilizing residues.

N-recognin and ClpS: the N-degron specificity factors

Which components mediate the recognition of N-degrons? Hershko and colleagues described UBR1 as the first characterized E3 enzyme that directly interacts with primary destabilizing residues [22]. Later, more E3 ubiquitin ligases that recognize N-degrons were identified and collectively termed N-recognins. The mouse genome encodes for seven N-recognins (UBR1–7) that share a Zinc finger-like domain, termed the Ubr box [23]. The contribution of most of these N-recognins to the N-end rule pathway is still largely unknown. Evidence for overlapping functions of UBRs is provided by the analysis of knockout mice. Double-knockout $Ubr1^{-/-}/Ubr2^{-/-}$ mutant mice, in contrast to the corresponding single knockouts, are inviable. Rescued fibroblasts of such double-mutants still retain a functional N-end rule pathway, albeit with a lower activity that is mainly dependent on the N-recognin UBR4 [23]. Together, these findings

point to common and specific functions of N-recognins that potentially act on overlapping but also different substrate pools. A molecular basis for varying substrate specificities of N-recognins is provided by genetic studies that led to the identification of two distinct binding sites for primary destabilizing residues within these E3 ligases [24]. The type 1 site mediates the recognition of basic residues (Arg, Lys, His) and is present within the conserved Ubr box of all N-recognins. The type 2 site interacts with bulky hydrophobic and aromatic residues (Leu, Ile, Phe, Tyr, Trp) and is only present in a subset of N-recognins (e.g. UBR1), providing a rationale for diverse substrate specificities [23]. Interestingly, *A. thaliana* encodes the E3 ligase PRT1, which recognizes aromatic residues (Trp, Tyr, Phe) as N-degrons. PRT1 is distinct from N-recognins and potentially recognizes N-degrons via its so-called ZZ domain. Surprisingly, PRT1 can functionally replace Ubr1 of yeast, although it is not evolutionarily related to N-recognins [7,25].

Bacteria neither encode N-recognin nor PRT1 homologs despite the existence of a functional N-end rule pathway. Intriguingly, a bioinformatics analysis revealed a limited homology between the type 2 binding site of the N-recognin UBR1 and ClpS, an specific adaptor protein of the ClpA/ClpP protease in *E. coli* [26,27]. This region of homology is enriched in acidic and hydrophobic residues, providing a complementary surface for the interaction with N-degrons (Figure 2). *E. coli* mutants lacking either the Hsp100 protein ClpA or its adaptor protein ClpS are deficient in degrading N-end rule model substrates *in vivo* [5,28]. ClpS directly interacts with N-end rule model substrates, qualifying ClpS as the primary recognition determinant of the N-end rule in *E. coli* [28]. The function of ClpS in bacterial N-end rule degradation and its limited homology to eukaryotic N-recognins suggest that, despite obvious differences in the degradation pathway, the first decisive step of substrate recognition is conserved between bacteria and eukaryotes and underline the ancient origin of the N-end rule pathway.

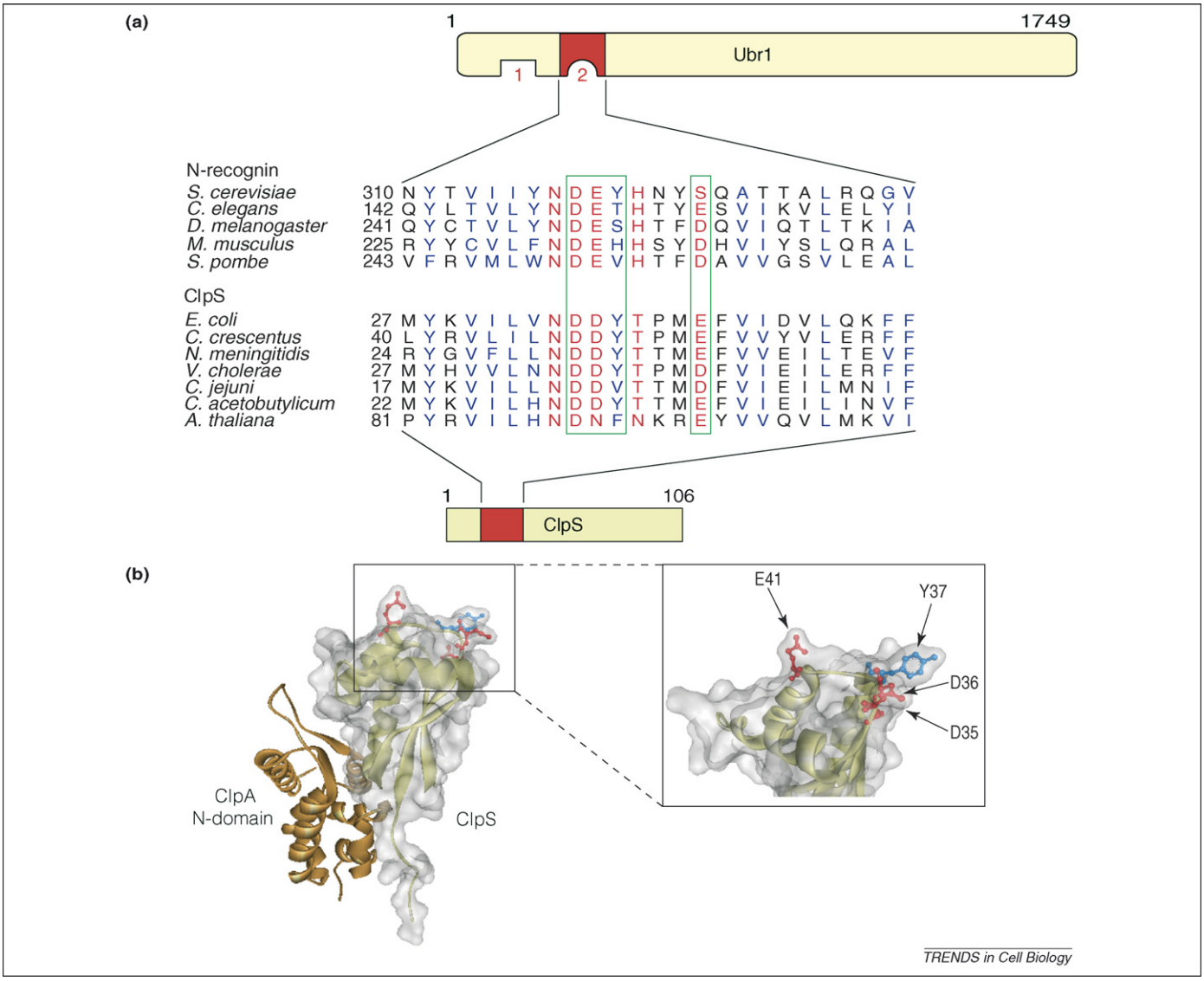

Figure 2. Eukaryotic N-recognin UBR1 and the bacterial adaptor protein ClpS serve as N-degron specificity factors by sharing a limited homology in the substrate binding site for N-end rule substrates. **(a)** Sequence alignment of the putative type 2 N-end rule substrate recognition site of N-recognins and the N-end rule binding site of ClpS homologs. Color-coding: blue, conserved hydrophobic residues; red, conserved charged residues; green box, surface-exposed residues in ClpS. **(b)** Space-filling model of ClpS in complex with the N-domain of ClpA. The detailed view of the ClpS structure highlights surface-exposed, conserved residues that mediate N-degron recognition.

Whereas, in eukaryotes, the specificity of the N-end rule pathway was mainly determined through genetic studies, the molecular features of the N-degron in *E. coli* were characterized by the direct determination of the substrate specificity of ClpS using combinatorial peptide libraries [28]. ClpS only binds to peptides with primary destabilizing residues (Phe, Tyr, Trp, Leu) at the N-terminus with a free α-amino group. Secondary destabilizing residues (Arg, Lys), positioned next to a destabilizing N-terminal residue, strengthen this interaction, thereby, directly contributing to the formation of N-degrons of *E. coli*, an additional role to that they already play as a target for L/F-transferases. A third requirement for N-end rule degradation in *E. coli* involves the handover of N-end rule substrates from the ClpS adaptor to its Hsp100 partner protein, ClpA. Substrate transfer to and subsequent processing by ClpA require the additional presence of an unstructured linker between the N-degron and the folded moiety of the substrate protein. This feature adds an additional layer to the high selectivity of the bacterial N-end rule, causing a further restriction of the potential substrate pool and enabling the pathway to regulate specifically biological processes. Remarkably, ClpS plays a dual function in the control of the substrate flow to the ClpA/ClpP proteolytic machinery. Indeed, ClpS does not only confer the specificity for N-degrons, it also inhibits the degradation of non-N-end rule substrates by ClpA/ClpP, thereby activating the protease on demand. ClpS homologs are present in many bacterial species as well as in plant chloroplasts. Residues that are crucial for the interaction with N-degrons are conserved within ClpS proteins, suggesting that the determinants of the *E. coli* N-end rule are also operative in these organisms.

Substrates and physiological functions of N-end rule pathways

Owing to its early discovery, the N-end rule pathway was initially believed to represent the major pathway that determines the half-life of proteins in eukaryotic cells. It is now known that the pathway is operative in regulated proteolysis. This change in opinion was initiated by the puzzling finding that mutations in the machinery that recognizes N-degrons had, at the first glance, no severe phenotype. At present, however, the list of substrates and processes regulated by the N-end rule is growing constantly.

S. cerevisiae SCC1, a subunit of cohesin, represents one of the first identified *in vivo* substrates of the N-end rule pathway [13]. Cohesin is a large protein complex that establishes the cohesion of sister chromatides during DNA replication. At the onset of anaphase, the protease ESP1, termed separase, is activated and cleaves SCC1, thereby generating an unstable C-terminal SSC1 fragment that bears a destabilizing primary residue at its N-terminus. Stabilization of the C-terminal SCC1 fragment in *S. cerevisiae ubr1*Δ mutants is linked to an increased frequency of chromosome loss, demonstrating a function of the N-end rule during mitosis in yeast cells. In *Drosophila melanogaster*, the N-end rule pathway plays a crucial role in controlling apoptosis [14]. DIAP1, a key inhibitor of apoptosis, is cleaved by activated caspases, generating an N-terminal truncated fragment bearing a tertiary destabilizing residue (Asn), which is converted into a primary destabilizing residue via the deamidation/arginylation branch of the N-end rule pathway. Remarkably, the conversion of DIAP1 into an N-end rule target was found to be essential for its anti-apoptotic activity, suggesting a potential codegradation of associated pro-apoptotic factors [29].

Recently, the first mammalian substrates of the N-end rule were identified encompassing regulators of G protein signaling (RGS4, RGS5 and RGS16) [30,31]. Interestingly, these substrates harbor an N-terminal Cys residue that was known to function as a secondary destabilizing residue in mammalian cells but not in yeast. Oxidation of the N-terminal Cys was a prerequisite for its ATE1-mediated arginylation, resulting in the exposure of a classical primary destabilizing residue. Notably, oxidized Cys structurally mimics Asp, the classical recognition determinant of R-transferases, providing a rationale for its function as a secondary destabilizing residue. Cys oxidation requires nitric oxide as well as oxygen, but also necessitates the presence of a basic residue at position 2 of the substrate (thereby restricting this branch of the N-end rule pathway to a limited set of substrates). It has been suggested that, via this mechanism, N-degrons can serve as nitric oxide sensors, thereby potentially controlling multiple signal transduction pathways [30]. The identified substrates (RGS4, RGS5 and RGS16) act as important negative regulators of G-protein-mediated cardiovascular signaling and, in striking correlation, the embryos of *Ate1*$^{-/-}$ knockout mice, deficient in substrate arginylation, die as a result of defects in cardiovascular development [32]. This severe phenotype points to an important role of the N-end rule pathway in mammalian cells, a role that is further substantiated by the recent analysis of mutant mice lacking individual or multiple N-recognins. *Ubr1*$^{-/-}$ mice exhibit pancreatic insufficiencies, similar to the Johanson–Blizzard syndrome that is caused by mutations of *UBR1* in humans [33]. *Ubr2*$^{-/-}$ mice exhibit a gender-dependent lethality. Whereas most *Ubr2*$^{-/-}$ females die during embryogenesis, male *Ubr2* $^{-/-}$ are viable but infertile [34]. An even more severe phenotype was obtained when both N-recognins-encoding genes, *UBR1* and *UBR2*, were deleted [35]. These double knockouts die as early embryos, exhibiting severe defects in neurogenesis and cardiovascular development.

The phenotypes that result from the absence of individual components of the N-end rule pathway vary strongly in their severity amongst different organisms. Although *S. cerevisiae ate1*Δ mutant cells deficient in substrate arginylation have no obvious phenotype, *A. thaliana* plants lacking ATE1 display a delayed leaf senescence and *Ate1*$^{-/-}$ mice even die as embryos [19,32,36]. Similarly, the physiological function of the prokaryotic N-end rule is still enigmatic. *E. coli* cells lacking ClpS have no reported phenotype and *in vivo* substrates of this N-end rule pathway remain to be identified.

Conclusions

Recent progress in the analysis of the N-end rule pathway demonstrates that prokaryotes and eukaryotes share

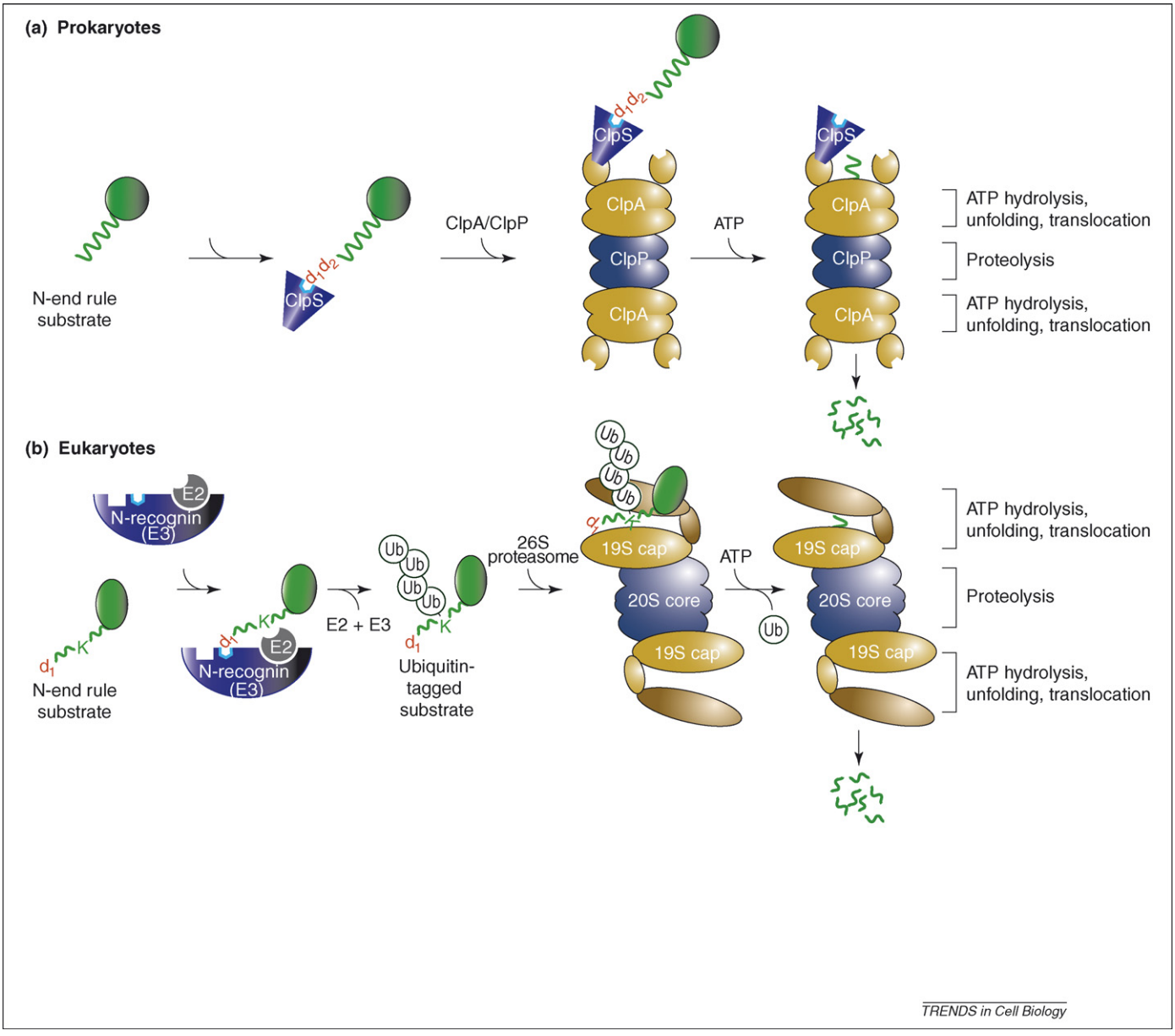

Figure 3. The N-end rule pathway in prokaryotes and eukaryotes. **(a)** In bacteria, bulky hydrophobic residues (Phe, Leu, Trp, Tyr) function as primary destabilizing residues (d1) and basic residues (Arg, Lys) as secondary destabilizing residues (d2) for the recognition of N-end rule substrates. The adaptor protein ClpS binds directly to target proteins containing primary or primary and secondary residues at the N-terminus and serves as a functional homolog of eukaryotic N-recognin. ClpS-bound substrates are directly delivered to the AAA+ chaperone ClpA via a specific interaction between ClpS and the N-terminal extra domain of ClpA. Efficient transfer of N-end rule substrates from ClpS to ClpA requires the presence of an unstructured linker region between the N-terminal destabilizing residues and the substrate moiety, allowing ClpA-mediated ATP dependent unfolding and translocation into the proteolytic chamber of ClpP where degradation occurs. **(b)** In eukaryotes, primary destabilizing residues (d1, type 1: Arg, Lys, His; type 2: Phe, Leu, Trp, Tyr, Ile) of the substrate are recognized by the E3 ligase N-recognin that contains distinct binding sites for type 1 and type 2 N-end rule substrates. N-recognin associates with an ubiquitin-conjugating enzyme (E2) and targets an internal Lys (K) residue of the N-end rule substrate for ubiquitylation by E2. The multiubiquitylated substrate is recognized by the 19S cap complex of the proteasome and subsequently unfolded and translocated by AAA+ proteins into the 20S core for proteolysis.

common principles in regulated proteolysis despite the involvement of different players. The selection of N-degrons is based on similar recognition determinants and is mediated by either N-recognins (E3 ligase) in eukaryotes or by the prokaryotic adaptor protein ClpS. Both specificity factors exhibit only a limited homology that is restricted to the site of substrate interaction; however, both fulfill the same function by linking substrate recognition to processive substrate hydrolysis via either the ubiquitin/proteasome system in Eukaryota or, more directly, by direct transfer to proteasome-like machineries in Prokaryota (Figure 3).

The processes that are controlled by the N-end rule in different organisms are merely beginning to be unraveled and only a few *in vivo* substrates have been identified. The actual number of substrates can be expected to be much higher as based on the known substrate specificities of endopeptidases, which can generate protein fragments bearing destabilizing N-terminal residues [13,29] and recent direct approaches aimed to estimate the number of mammalian N-end rule substrates [37]. The N-end rule pathway has not lost its glamour and will still surprise with new twists and turns.

Acknowledgements
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Current Opinion in Structural Biology

Paradigms of protein degradation by the proteasome

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The proteasome is the main proteolytic machine in the cytosol and nucleus of eukaryotic cells where it degrades hundreds of regulatory proteins, removes damaged proteins, and produces peptides that are presented by MHC complexes. New structures of the proteasome particle show how its subunits are arranged and provide insights into how the proteasome is regulated. Proteins are targeted to the proteasome by tags composed of several ubiquitin moieties. The structure of the tags tunes the order in which proteins are degraded. The proteasome itself edits the ubiquitin tags and drugs that interfere in this process can enhance the clearance of toxic proteins from cells. Finally, the proteasome initiates degradation at unstructured regions within its substrates and this step contributes to substrate selection.

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Introduction

Cellular protein concentrations are controlled through their rates of synthesis and degradation. In the cytosol and nucleus of eukaryotic cells, most of this degradation is by the ubiquitin proteasome system (UPS). At the center of the UPS is a single proteolytic machine, the proteasome, which controls the concentrations of hundreds of regulatory proteins and clears misfolded and damaged proteins from the cell. Thus, the proteasome has to be able to degrade any protein but do so while avoiding the accidental destruction of the rest of the cellular proteome. Here we review recent advances in our understanding of how the proteasome selects its substrates. Just as protein synthesis is regulated at many different levels, it is becoming increasingly clear how protein degradation is also.

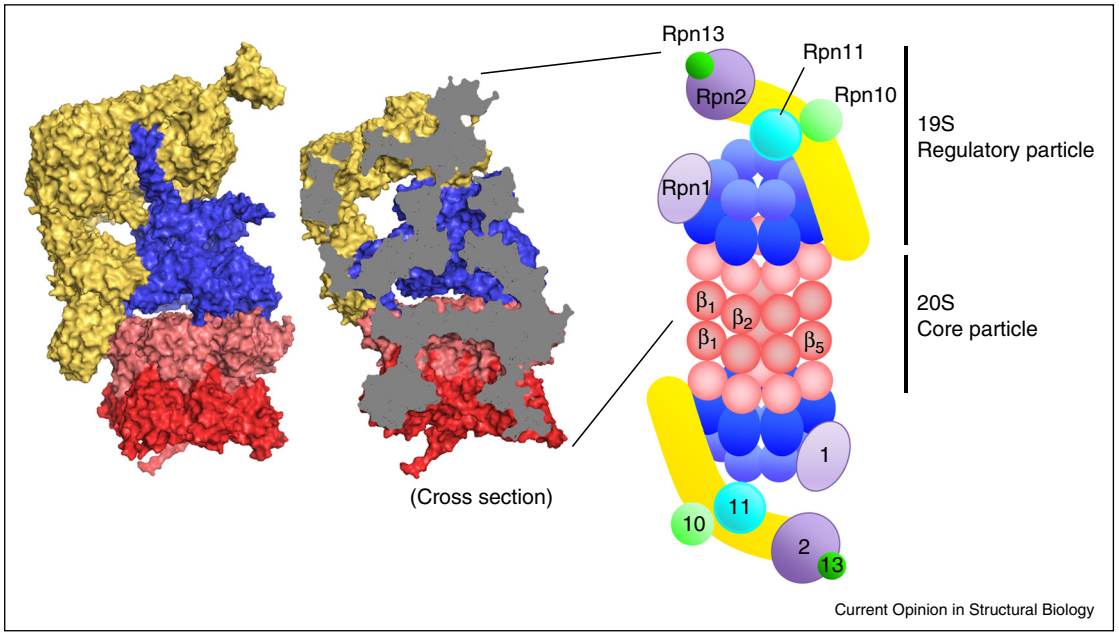
The basic principle of proteasome substrate selection is well understood [1,2]. The proteasome is a large particle of ~33 different subunits that add up to a molecular weight of approximately 2.5 MDa. It combines three different proteolytic sites with broad and complementary sequence preferences to allow it to degrade many different amino acid sequences. The proteasome particle controls the activity of these sites by encapsulating them inside its structure and controlling access to them. Most proteins are targeted to the proteasome by the covalent attachment of ubiquitin molecules. The proteasome recognizes the ubiquitin signal and initiates degradation at an unstructured region in the protein. The substrate is then unfolded and translocated to the proteolytic sites in an ATP-dependent reaction. However, many questions remain. For example, the proteasome is able to extract individual subunits from complexes without degrading their binding partners, the proteasome degrades ubiquitinated proteins in a specific order and ubiquitin signals target proteins to processes that do not involve degradation. We do not know how the proteasome makes these distinctions. At the same time, some proteins that lack ubiquitin signals are degraded by the proteasome. Over the last few years, new proteasome structures and biochemical investigations have brought new insights into these questions.

Proteasome

The proteasome particle is functionally and structurally divided into two parts. Its core is formed by a cylindrical 20S particle composed of four heptameric rings that are stacked onto top of each other. The inner two rings each consist of seven related β-subunits that are arranged to form a large internal cavity and three of the subunits in each ring contain a proteolytic site that faces the internal cavity. A ring of seven related α-subunits on each side flanks the β-rings and substrates have to enter the proteolytic cavity formed by the β-rings through a pore at the top of the α-ring. The pore is too narrow to allow folded proteins to pass through it. In free core particle, access to the pores is further hindered by the N-termini of the α-subunits so that even unfolded peptides are degraded only poorly.

The core particle is activated by regulatory particles or caps that bind to the ends of the core particle and induce conformational changes that open the pores. Four different caps are known and the best understood of them is the19S regulatory particle. It consists of 19 subunits that add up to a molecular weight of ~900 kDa. The complex of one or two of these caps with the 20S core particle is called the 26S proteasome and this seems to be the most common form of the proteasome in cells. The subunits of the 19S cap recognize substrates, unfold and translocate

Figure 1



Structure of the 26S proteasome. Molecular surface of the 19S activator particle bound to the 20S core particle (PDB 4C0V) (left). The 20S core particle is composed of two central β rings (dark red) and one α ring (light red) at each end. The 19S regulatory particle, which contains AAA ATPase subunits (blue) and non-ATPase subunits (yellow), caps each end of the 20S. Cross section reveals the degradation channel that connects the proteolytic chamber in the 20S core particle to the entrance into the 19S activator (middle). Structures are produced by PyMOL. Schematic drawing of the 26S proteasome indicates the approximate locations of the enzymatic activities and binding platforms on the 19S activator cap (right). α (light red) and β (dark red) subunits of the 20S particle, ATPase domain (dark blue) and OB domain (light blue) of ATPase subunits, backbone of lid subparticle (yellow), docking subunits Rpn1 (light purple) and Rpn2 (dark purple), ubiquitin receptors Rpn10 (light green) and Rpn13 (dark green), and DUB metallo-protease subunit Rpn11 (sky blue).

them into the core particle for degradation into short peptides.

Structure of the 26S proteasome

The structure of the 26S proteasome proved difficult to determine, perhaps because a number of accessory factors associate with the particle non-stoichiometrically or because the structure undergoes conformational changes. In a major breakthrough, a series of studies published over the last two years describe the structure of the 19S cap bound to the core particle at high resolution by combining cryo-electronmicroscopy, crystallography, biochemical data and computer modeling [3••,4••,5••,6••,7••,8••,9••,10••] (Figure 1).

The heart of the 19S cap is a ring of six ATPase subunits (Rpt1–Rpt6), which make up the motor that feeds substrates to the proteolytic sites. The subunits form a long channel at their center that runs through approximately two-thirds of the 19S particle and ends in a ring of the AAA+ domains at the C-terminal end of the ATPase subunits. The very C-termini of the AAA+ domains dock into the 20S core particle and trigger pore opening. Two large subunits that serve as interaction platforms bind to the ATPase ring, Rpn1 to the outside of the ring, and Rpn2 to the top of the ring. Rpn1 provides the binding sites for a series of non-stoichiometric proteasome subunits called UbL-UBA proteins, which serve as additional ubiquitin receptors and we will discuss these briefly later, and Rpn2 organizes the two ubiquitin receptors Rpn10 and Rpn13 subunit near the outer end of the 19S cap. No single one of these receptors is essential in yeast [11••] so that it seems that the different receptors work together to form a versatile binding platform to capture proteasome substrates (Figure 3). The cap also contains a pair of JAMM or MPN domain metallo-protease subunits called Rpn11 and Rpn8. Only Rpn11 is enzymatically active and it cleaves entire ubiquitin chains off the substrates as these are degraded. Rpn11 is located near the entrance of the substrate channel formed by the ATPase subunits so that it is well placed to interact with substrate protein feeding into the proteasome. Thus, the activities required for protein degradation are ordered sequentially along the long axis of the proteasome particle [2] (Figure 1).

The remainder of the cap is formed by seven scaffolding subunits that form a clamp that binds to the side of the cap reaching all the way from the end of the proteasome particle, where it interacts with Rpn2 and the ubiquitin receptor Rpn10, via the ATPase subunits, down to the α-ring of the

core particle. The clamp subunits complete a network of interactions that seems to stabilize the proteasome particle and may allow allosteric regulation and coordination between the activities on the proteasome particle. Biochemical experiments have shown that substrate and ATP binding can affect gating of the substrate channel and proteolytic activity [12–14]. Comparison of the proteasome structures in the presence of ATP but without substrate, in the presence of ATP and with substrate bound, and in the presence of a slowly hydrolysable ATP analog reveal substantial conformational changes in the proteasome structure [9••,10••]. For example, substrate or ATP analog binding switches the cap from a presumably inactive conformation in which the substrate channel is discontinuous to a conformation in which the channel is properly aligned through the entire proteasome particle and the active site of Rpn11 swings in and out of alignment with the channel entrance. The ATPase subunits switch between arrangements in which they form a spiral or a planar ring but it is not clear to what extent these changes reflect motor action that drives substrate into the proteasome or switches between resting and active states.

Alternative proteasome activators

Recently, Barthelme and Sauer found that the chaperone Cdc48 can also form a complex with the 20S core particle and support the degradation of substrate proteins [15••,16]. Cdc48, called p97 or VCP in animals, is a cytosolic chaperone distantly related to ATPase subunits in the 19S regulatory particle and involved in the degradation of a subset of proteasome substrates by a poorly defined mechanism. For example, it is part of the quality control process for endoplasmic reticulum proteins (ERAD) where it is required for the translocation of misfolded proteins from the ER to cytosolic proteasomes [17]. It now appears that Cdc48/p97/VCP may be directly involved in degradation by serving as an alternative proteasome cap, perhaps to unfold different subsets of proteins than the 19S cap. Proteasome with Cdc48 caps would resemble the archaeal proteasome and the analogous bacterial AAA+ proteases. These proteases fulfill similar functions as the eukaryotic proteasome and share the same overall architecture [18].

Two other further types of proteasome caps are known, called the 11S particle and the PA200 activator. These caps neither recognize ubiquitin nor hydrolyze ATP and their role seems to be to degrade a specific subset of substrate and some unstructured proteins [1,19].

Ubiquitination

Ubiquitination system

Most proteins are targeted to the proteasome by ubiquitin tags or degrons. Ubiquitin is attached to the target proteins through the sequential action of a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). In most cases, ubiquitin forms an isopeptide bond through its C-terminal carboxy group (Gly76 of ubiquitin) with the ε-amino group of lysine residues in the substrate, and more rarely with the N-terminus of the polypeptide chain or the side chain of a cysteine residue in the substrate protein [20–22]. Typically ubiquitin is attached to more than one residue in the target proteins and in many cases, a second ubiquitin is then attached to a lysine residue in the first ubiquitin and so on to create polyubiquitin chains. In addition, cells contain large numbers of deubiquitinating enzymes (DUBs) that remove ubiquitin chains again [23].

Ubiquitin signals

Thousands of proteins are ubiquitinated in yeast cells, but almost half of the ubiquitinated proteins are not targeted to the proteasom for degradation [24] and it is not clear how the cell differentiates between the different ubiquitin signals. The canonical view is that ubiquitin chains linked through Lys48 of ubiquitin target to the proteasome and biochemical experiments show that chains of at least four ubiquitin moieties are required for proper recognition [20,25]. Modification with a single ubiquitin molecule or through polyubiquitin chains linked through other Lys residues such as Lys63 and even linear ubiquitin chains play roles in cellular processes that do not involve the proteasome such as the regulation of chromatin structure, membrane trafficking and signal transduction. However, the distinctions are not strict and Lys63-linked polyubiquitin chains [26,27] and even monoubiquitin tags [28–30] can target some substrates to the proteasome for degradation. Purified proteasome binds the Lys63-linked polyubiquitin chain with almost the same affinity as the Lys48-linked polyubiquitin chain [31•] and so specificity may come from accessory proteins. For example the ESCRT complex involved in membrane trafficking binds Lys63-linked polyubiquitin chains better than Lys48-linked chains whereas the UbL-UBA proteins that can serve as non-stoichiometric ubiquitin receptors for the proteasome have the opposite preference [31•]. Therefore, a Lys48-linked polyubiquitin chain has a greater chance to be delivered to the proteasome than the Lys63-linked polyubiquitin chain. A different possibility is that physical properties of the substrate proteins themselves, such as their stability against unfolding [32] and the presence of initiation sites for the proteasome [33••,34] contribute to specificity as processes such as membrane trafficking or the formation of signaling complexes do not require protein unfolding and do not involve initiation.

Dynamic regulation of ubiquitination

Ubiquitination is not a simple switch that turns degradation on and off, but rather an adjustable signal that fine-tunes degradation and can determine the order in which proteins in a regulatory pathway are degraded. For example, the progression of cells through the cell division cycle requires the degradation of regulatory proteins in

the correct sequence. Degradation can be ordered by timing the ubiquitination event and many E3s recognize their substrates only when their interaction site is first phosphorylated by a kinase [22]. Degradation order is also controlled by the nature of the ubiquitin modification and, during the cell cycle, regulators that acquire long ubiquitin chains are degraded before regulators that are ubiquitinated with multiple shorter chains [35•,36•]. The regulators are ubiquitinated by the same E3 but for the early substrates ubiquitination is more processive than for the late substrates probably because the substrates have different dissociation rates, from the E3 [35•,36•].

Ubiquitin tags on proteins can grow and shrink even while bound to the proteasome through the action of E3 and DUB enzymes associated with the proteasome. In yeast, the DUB Ubp6 and in mammalian cells the Ubp6 homolog Usp14 and the DUB Uch37 bind to the 19S proteasome cap [1]. These DUBs trim ubiquitin chains from the distal end of the chain in steps of one or a few ubiquitin moieties at a time and thus limit the time that a substrate remains associated with the proteasome [37,38••]. Hence, proteins that are difficult to degrade because they cannot be unfolded or because they lack good initiations sites would dissociate from the proteasome after it tried to degrade them for a limited time, freeing up the proteasome for a different substrate and preventing it from clogging up. On the flipside, inhibitors of proteasome DUB Usp14 show promise as drug for the treatment of neurodegenerative diseases by increasing the proteasome's ability to degrade resistant substrates, presumably by increasing their interaction with the proteasome [38••]. Small molecular inhibitors of proteasomal DUBs are also tested in cancer therapy but here the drugs affect degradation differently and lead to the accumulation of ubiquitinated proteins [39] so that the biological effect may be similar to that of the proteasome inhibitors already used to treat multiple myeloma [40].

E3s also bind the proteasome [41]. In particular, the E3 Hul5 associates with Ubp6 on the 19S activator of the proteasome where it counteracts the activity of Ubp6 by increasing the length of polyubiquitin chains [42••]. Ubiquitin chain editing may serve to fine-tune degradation rates or to make protein targeting more robust by buffering fluctuations in ubiquitin chains and substrate stability. Another possibility is that ubiquitin ligation on the proteasome makes degradation more processive to avoid the formation of partially degraded protein fragments [43] by re-ubiquitinating long proteins as the proteasome runs along their polypeptide chain [44].

A second component to the proteasome targeting code?

Initiation of degradation

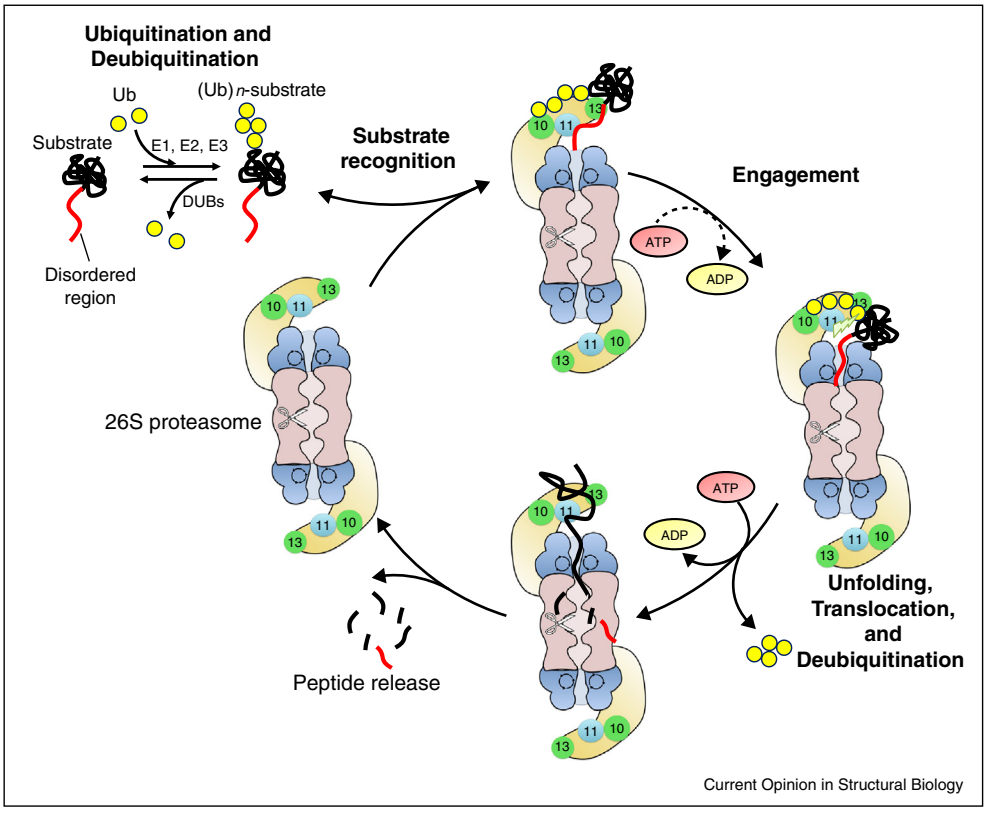
The proteasome recognizes and binds its substrates through their polyubiquitin tag but initiates degradation at a disordered region in the substrate [33••,45] (Figure 2). Once the substrate is engaged at the initiation site, the proteasome proceeds along the polypeptide chain from there to unfold and degrade the entire protein sequentially [32]. The initiation region is reminiscent of the linear targeting signals found in substrates of the archaeal and bacterial analogs of the proteasome [18]. Bacterial AAA+ proteases recognize their linear degrons through loops that line the pore at the center of the ring of ATPase subunits and it seems likely that the proteasome recognizes its initiation sites similarly [46]. In the proteasome, the equivalent loops line the degradation channel at a position some 30–60 Å from the entry pore. The diameter of the pore is too narrow to allow folded proteins to pass through it so that a disordered polypeptide tail would have to be at least 20–30 amino acids long to be able to reach the ATPase loops. This length requirement agrees roughly with the results of *in vitro* degradation experiments with model proteasome substrates, where proteins become degraded rapidly by purified yeast proteasome once they contain an unstructured tail of approximately 30 amino acids in length [33••,45,47].

The requirement of unstructured initiation regions may also be reflected in the global stability profiles of proteins. At least 30% of eukaryotic proteins contain intrinsically disordered regions (IDRs) and these are involved in various cellular activities [48,49]. There is some evidence from bioinformatics studies that proteins that contain disordered regions have on average shorter half-lives than proteins lacking these regions [50,51] but so far the evidence for this relationship is not consistent. Other studies do not find these correlations [52–54] and there is some evidence that ubiquitination sites of proteasome substrates are preferentially located in unstructured regions [55,56]. Even when the a protein lacks an unstructured region, ubiquitination itself may induce the local unfolding near the ubiquitinated residue, which, in turn, could create an initiation site for the proteasome [57].

Degradation of protein complexes

Ubiquitin tag and initiation site do not have to be located on the same polypeptide chain but can work together in *trans* so that a ubiquitinated subunit in a complex can target a binding partner for degradation [34]. The ubiquitinated subunit serves as an adaptor that binds to the proteasome and presents the bound protein for proteolysis. Presumably, UbL-UBA proteins function in this manner to serve as non-stoichiometric ubiquitin receptors for the proteasome [1,58]. These proteins bind to the proteasome through their UbL (ubiquitin-like) domains and to ubiquitinated proteins through their UBA (ubiquitin associated) domains and stimulate degradation of the ubiquitinated protein while the UbL-UBA proteins themselves escape degradation. The mechanism behind this unexpected stability of UbL proteins has been investigated for yeast Rad23 [59–61]. These experiments

Figure 2



Schematic representation of the degradation cycle of the ubiquitin proteasome system. Proteins are targeted to the proteasome by a two-part degradation signal or degron. It consists of a disordered region within the substrate and a reversibly attached polyubiquitin tag (Ub_n). Polyubiquitin tag is attached by a E1–E2–E3 ubiquitination cascade and this process can be reversed by DUBs (top left). The proteasome recognizes its substrates at the ubiquitin tag through ubiquitin receptors (Rpn10 and Rpn13; green) (top) and initiates degradation at the unstructured region (right). Once the proteasome has engaged its substrate, it unravels the protein by translocating it into a central cavity in the core particle, where the protein is proteolysed (bottom). The polyubiquitin tag is cleaved off by the intrinsic DUB Rpn11 (skyblue) as unfolding and degradation begins.

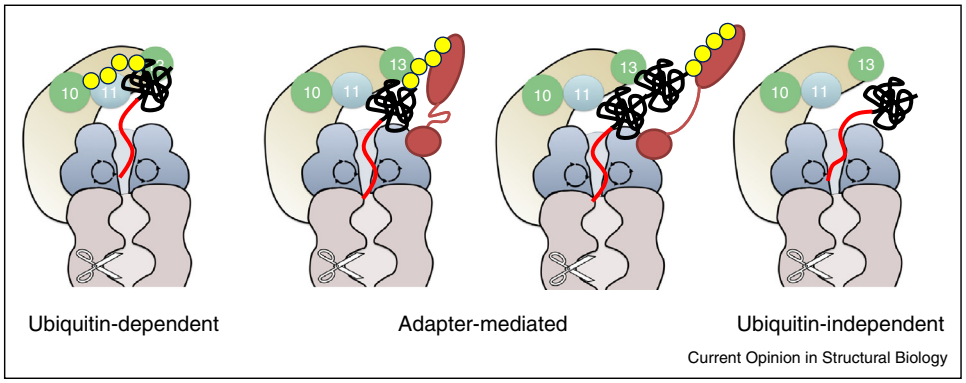
showed that Rad23 escapes degradation because it lacks an effective proteasome initiation site [60,61].

The flipside of this mechanism is also observed and the proteasome is able to remodel protein complexes by degrading only the ubiquitinated subunit and leaving other proteins in the complex intact [62,63]. This remodeling activity is important in many regulatory processes. For example, during cell cycle regulation in yeast, the proteasome extracts the cyclin-dependent kinase inhibitor Sic1 from its complex with cyclin and cyclin-dependent kinase to degrade solely Sic1 [64]. Shortly afterwards, the cyclin is ubiquitinated and then degraded to release intact but inactive kinase [65]. Since the proteasome is able to degrade proteins that are bound to the proteasome indirectly it is unlikely that ubiquitination by itself specifies target selection. Presumably, the proteasome instead determines which subunit is degraded by where it initiates degradation. Once the polypeptide chain of a subunit is fed into the degradation channel, the proteasome proceeds along that chain and hydrolyzes the protein sequentially [32]. The most likely initiation site for the proteasome is probably the unstructured region closest to the entrance to the degradation channel. Indeed, biochemical experiments show that initiation regions must be placed at the appropriate distance from the ubiquitin tag for a protein to be degraded, presumably so that the proteasome can bind the ubiquitin tag and engage the initiation region simultaneously [47]. Thus, under some circumstances, the proteasome may select substrates at the initiation step.

Ubiquitin-independent substrates

A range of proteins is degraded by the proteasome without being ubiquitinated [66] and the best understood example is ornithine decarboxylase (ODC) [67,68]. Degradation of ODC requires ATP as well as an accessory protein called antizyme and begins a 37 amino acid long unstructured region at the C terminus of ODC [68]. To some extent, this ODC tail can function as a transferable degradation signal and induce the degradation of some proteins. One plausible explanation for the ubiquitin-independent

Figure 3



The proteasome recognizes substrates in three different modes; ubiquitin-dependent (left), adapter-mediated (middle), and ubiquitin-independent (right) modes. In all three modes, an intrinsically disordered region in the substrate is recognized by the ATPase motor to allow the proteasome to initiate degradation. This aspect of proteasomal degradation resembles the targeting mechanisms predominant with the bacterial and archaeal analogs of the proteasome. Ubiquitin tags can be either recognized by the two intrinsic proteasome receptors Rpn10 and Rpn13 (left), or by non-stoichiometric proteasome subunits that serve as substrate adaptors such as UbL-UBA proteins (middle). The UbL-UBA proteins might bind substrates by themselves (second right) or together with the intrinsic substrate receptors (second from left) and facilitate degradation of by positioning the disordered region properly. Finally, some substrates may be recognized only by their initiation sites.

degradation is that the unstructured regions themselves have bind sufficiently tightly to the ATPase ring loops so that ubiquitin is not required for proteasome association (Figure 3). Thus, this targeting mechanism can be taken as a variation of the conventional proteasome degron in which the ubiquitin tag component is missing and which resembles the degrons observed in the archaea and bacteria [18].

Several other proteasome substrates including p21/Cip1, c-Jun, c-Fos, p53, p73 IκBα, T-cell antigen receptor chain α, Fra-1, and Hif-1α, can also be degraded in an ubiquitin-independent manner [69–71]. The mechanisms of these processes are not well understood and it is possible that these proteins are degraded by isolated 20S core particle in the absence of ATP [69], though *in vivo* perhaps more likely by 20S core particle activated by alternative caps [70] or even by 26S proteasome [71]. The proteins in this group of ubiquitin-independent proteasome substrates are largely unstructured, but their degradation can still be regulated. The best understood example of this regulation is given by NQO1 [72,73•]. NQO1 is largely unstructured and can be degraded by 20S proteasome *in vitro*. Binding of NQO1's cofactor FAD stabilizes the protein's structure and inhibits its proteasomal degradation. Quite interestingly, FAD binding to NQO1 also stabilizes other ubiquitin-independent proteasome substrates, setting up a regulatory circuit controlled by the availability of FAD and thus the metabolic state of the cell.

System-wide studies of the UPS

The mechanisms described above are largely derived from investigations of the behavior specific proteins *in vitro* or in the cell. Over the last five years, high-through studies have begun to provide a system-level picture of how the UPS regulates protein concentrations. Improvements in mass spectroscopy technology and in the strategies for sample preparation are making it possible to define the proteins that are ubiquitinated in the cells and the nature of their ubiquitin modifications [74–76]. So far, the sets of ubiquitinated proteins identified in different studies overlap only partially suggesting that current experiments do not yet capture all ubiquitinated proteins [74]. The studies still provide valuable insights, for example by describing the wide range of polyubiquitin chains made in cells [27] and the fraction of nascent proteins that are ubiquitinated as part of protein quality control surveillance [77,78].

Other approaches measure the stability and turnover rates of a large fraction of the proteins in cells. The first experiments used the tagged protein collection in yeast and followed their degradation by cycloheximide shut-off and Western blotting [79] and later measurements in mammalian cells use SILAC [80] or fluorescent protein fusions [52,81]. These studies show that protein halftimes in eukaryotic cells range over at least two orders of magnitudes and thus that the protein concentrations are indeed adjusted by the balance of synthesis and degradation. Combining protein stability measurements with the ubiquitination databases, or with chemical inhibition of protein ubiquitin ligases provides increasing depth to our understanding of the regulation of cellular protein stability [50–53,82].

Summary

As we begin to understand the mechanism of the UPS in increasing biochemical detail it is becoming clear that the

regulation of degradation is far richer than the binary decision between degradation or no degradation. Just like protein synthesis is tuned by a myriad of processes, we are discovering new ways in which their degradation is tuned. Recent structural and biochemical discoveries have provided a range of novel paradigms that govern proteasome action and new experimental strategies make it possible to observe protein ubiquitination and degradation system-wide. It will be interesting to see whether and how they are used in the cell.

Acknowledgements

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Review

The 26S proteasome complex: An attractive target for cancer therapy

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ABSTRACT

The 26S proteasome complex engages in an ATP-dependent proteolytic degradation of a variety of oncoproteins, transcription factors, cell cycle specific cyclins, cyclin-dependent kinase inhibitors, ornithine decarboxylase, and other key regulatory cellular proteins. Thus, the proteasome regulates either directly or indirectly many important cellular processes. Altered regulation of these cellular events is linked to the development of cancer. Therefore, the proteasome has become an attractive target for the treatment of numerous cancers. Several proteasome inhibitors that target the proteolytic active sites of the 26S proteasome complex have been developed and tested for anti-tumor activities. These proteasome inhibitors have displayed impressive anti-tumor functions by inducing apoptosis in different tumor types. Further, the proteasome inhibitors have been shown to induce cell cycle arrest, and inhibit angiogenesis, cell–cell adhesion, cell migration, immune and inflammatory responses, and DNA repair response. A number of proteasome inhibitors are now in clinical trials to treat multiple myeloma and solid tumors. Many other proteasome inhibitors with different efficiencies are being developed and tested for anti-tumor activities. Several proteasome inhibitors currently in clinical trials have shown significantly improved anti-tumor activities when combined with other drugs such as histone deacetylase (HDAC) inhibitors, Akt (protein kinase B) inhibitors, DNA damaging agents, Hsp90 (heat shock protein 90) inhibitors, and lenalidomide. The proteasome inhibitor bortezomib is now in the clinic to treat multiple myeloma and mantle cell lymphoma. Here, we discuss the 26S proteasome complex in carcinogenesis and different proteasome inhibitors with their potential therapeutic applications in treatment of numerous cancers.

Contents

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1. Introduction

The 26S proteasome complex is a non-lysosomal proteolytic machine in eukaryotes [1,2]. It consists of a 20S core particle (CP) and a 19S regulatory particle (RP). The 20S CP confers the proteolytic activities of the proteasome, whereas the 19S RP shows an ATP-dependence and specificity for ubiquitin protein conjugates. The 20S CP resembles a cylinder composed of four rings (two α and two β rings) [1,3]. These rings are flush with each other, giving the 20S CP a seven-fold symmetry. Each α ring is composed of seven different α components (α1–α7). Similarly, seven different β components (β1–β7) form a β ring. Three of the seven β-components in the β ring are catalytically active, and are named by their substrate specificities: chymotrypsin-like (β5), trypsin-like (β2), and post-acidic or caspase-like (β1). The chymotrypsin-like activity cleaves proteins after hydrophobic residues, while the trypsin and caspase-like activities cleave after basic and acidic residues, respectively [4,5]. The substrate protein is translocated into the catalytic chamber of the 20S CP with the help of the 19S RP. The substrate protein is targeted to the 26S proteasome via its polyubiquitylation (Fig. 1). The ubiquitin chains are added to the protein substrate by three enzymes: ubiquitin-activating E1, ubiquitin-conjugating E2, and ubiquitin-ligase E3 (Fig. 1). E1 ubiquitin activating enzyme binds with a ubiquitin molecule, passes the ubiquitin to E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase enzyme enables the linking of C-terminal glycine residues of ubiquitin to lysine (K) residue on the substrate protein (Fig. 1).

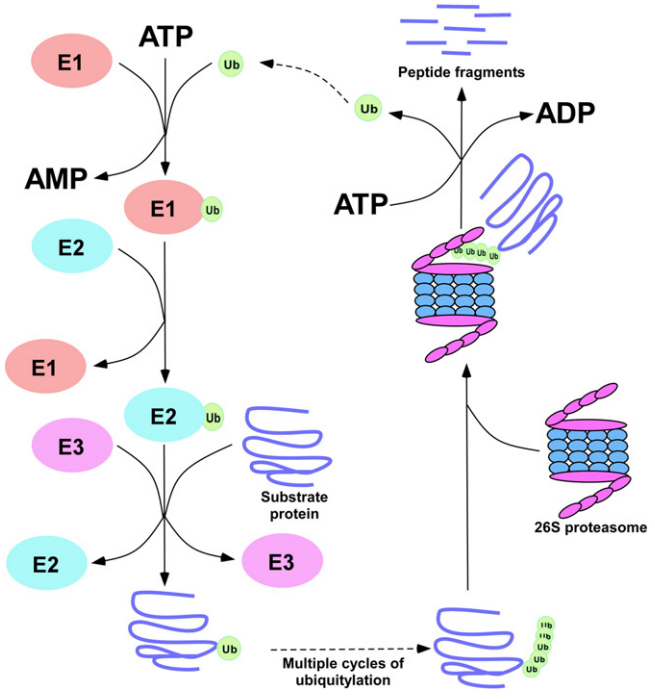

Fig. 1. The schematic diagram showing ubiquitylation of substrate protein and its subsequent degradation by the 26S proteasome complex. A ubiquitin activating enzyme (E1) first forms a thio-ester bond with ubiquitin (a highly conserved protein with 76 amino acids) in an ATP-dependent manner. Ubiquitin then binds to a ubiquitin conjugating enzyme (E2). Subsequently, the carboxy-terminus of ubiquitin forms an isopeptide bond with a K residue on the substrate protein in the presence of a ubiquitin ligase enzyme (E3). Multiple ubiquitylation cycles result in polyubiquitylation of the substrate protein. The 26S proteasome complex recognizes, unfolds and degrades polyubiquitylated-substrate protein into small peptides. Ub, ubiquitin.

Polyubiquitylation occurs through the linkage on one of the seven K residues of ubiquitin. The 19S RP recognizes the K48-linked polyubiquitylated-substrate protein, unfolds it, and finally feeds it into the catalytic chamber of the 20S CP for proteolysis in an ATP-dependent manner [6–8]. Further, the 19S RP cleaves off the ubiquitin from the substrate protein, and recycles it for future use. While K48-mediated polyubiquitylated-form of the substrate protein is recognized and degraded by the proteasome, K63-linked polyubiquitylation is not targeted for degradation by the 26S proteasome complex, but plays a crucial role in cellular signaling.

The proteasome complex is found in both the cytoplasm and nucleus of eukaryotic cell where they regulate the ebb and flow of proteins involved in progression through the cell cycle, inflammatory mechanisms, antigen presentation, signal transduction, apoptosis, and other key regulatory cellular processes. Through these processes, the 26S proteasome complex plays a crucial role in maintaining normal cellular functions. The proteasome exists in two isoforms: the constitutive proteasome or the 26S proteasome, and the immunoproteasome [9]. While the constitutive proteasome is found in most cells, the immunoproteasome is tissue-specific and abundant in immune-related cells. The immunoproteasome is formed in response to cytokine signaling. The immunoproteasome differs from its more common counterpart in that it contains a variation of the normal β-components. The β1, β2 and β5 components of the constitutive proteasome are replaced by β1i (LMP2), β2i (MECL1 or LMP10) and β5i (LMP7) in the immunoproteasome. The immunoproteasome also has an 11S regulatory structure or PA28 instead of the 19S RP of the 26S proteasome. Stimulation from the γ-interferon (IFN-γ) can instigate the switch of constitutive β-components to the immuno β-components. Similarly, tumor necrosis factor-alpha (TNF-α) has also been shown to induce the expression of immuno β-components and 11S regulatory cap to form the immunoproteasome. Such modifications help the immunoproteasome to generate antigenic peptides in a major histocompatibility complex (MHC) class I-mediated immune response [10].

2. The 26S proteasome complex in different cellular events and carcinogenesis

The 26S proteasome regulates many cellular functions, the most prominent of which includes the advancement through mitosis, growth, chemotaxis, antigen presentation, angiogenesis, apoptosis, and the expression of several genes which in turn regulate other processes. These mechanisms influenced by the 26S proteasome are some of the processes altered or deregulated in cancers. The most prominent substrates and related molecules of the 26S proteasome involved in cellular processes and carcinogenesis are discussed below.

2.1. Nuclear factor-kappaB (NFκB)

NFκB was originally discovered as a regulator for the expression of the kappa light-chain gene in murine B-lymphocytes [11]. Later on, NFκB has been found in nearly all animal cell types. NFκB is a transcription factor, and is involved in the activation of the genes encoding for cytokines, chemokines, growth factors, cell-adhesion molecules, and surface receptors [12–15] (Fig. 2). Through transcriptional regulation of a number of genes, NFκB controls various immune and inflammatory responses. Further, it suppresses apoptosis, and induces angiogenesis, cell proliferation and migration (Fig. 2), and

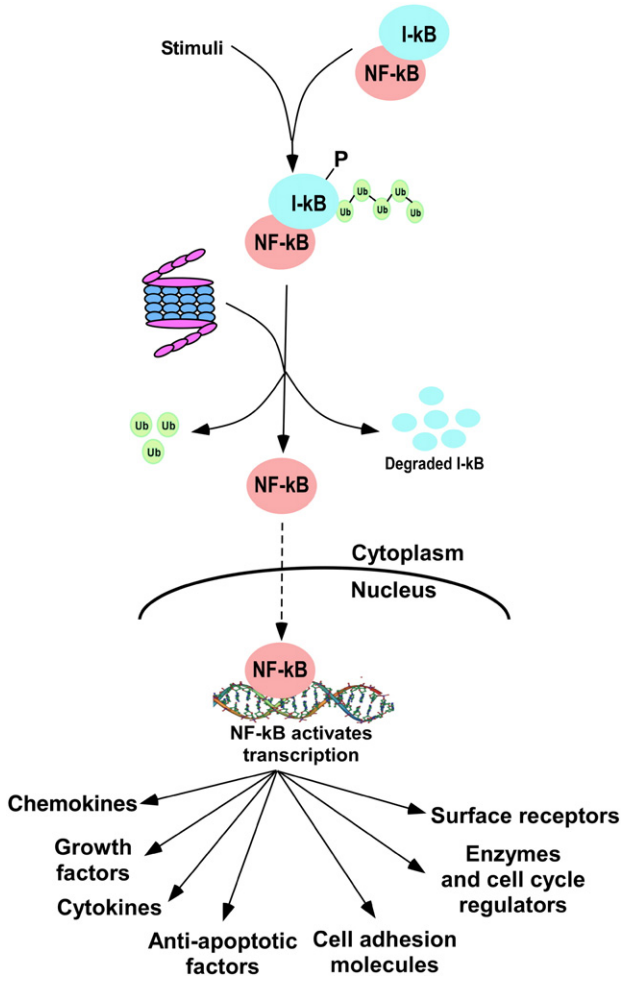

Fig. 2. The schematic diagram showing the regulation NFκB functions by the 26S proteasome complex.

thereby plays a crucial role in tumorigenesis [16–18]. NFκB is a heterodimer of p50 and p65. The 26S proteasome is involved in generating p50 from the precursor protein p105. p50 then binds to p65 and becomes the active dimer or NFκB. In the cytoplasm, IκB binds to NFκB and inhibits the translocation of NFκB to the nucleus for gene activation (Fig. 2). External stimuli (e.g. ionizing and ultraviolet irradiation, pathogens, stress, free radicals, and cytokines) induce phosphorylation of IκB (Fig. 2). This phosphorylation triggers polyubiquitylation of IκB for degradation by the 26S proteasome complex (Fig. 2). The proteasomal degradation of IκB promotes the translocation of NFκB to the nucleus to switch on the transcription of its target genes (Fig. 2). Thus, the 26S proteasome complex plays a pivotal role in regulating the function of NFκB and associated key intra- and inter-cellular events. Therefore, misregulation of NFκB function would lead to various types of cancers. Incidentally, several cancers like breast cancer, myeloma, prostate cancer, and leukemias show constitutive activity of NFκB [14,19–24]. This confers chemoresistance and increased aggression in phenotypes through the continued expression of factors associated with anti-apoptosis, angiogenesis, cell growth/proliferation, and metastasis [14,24–26]. In addition to its involvement in cancer, NFκB is also linked to inflammatory and autoimmune diseases, septic shock, viral infection, improper immune development, processes of synaptic plasticity and memory as well as neurodegenerative and heart diseases [27–29].

2.2. Apoptosis

Cancer is characterized by an uncontrolled growth and spread of abnormal cells. Thus, the induction of apoptosis would promote the killing of abnormal cancer cells. However, cancer cells often have a disregulation of apoptotic signaling pathways, leading to the suppression of apoptosis. Such an aberrant regulation of apoptosis provides a survival advantage to the cancer cells and therefore resistance to chemotherapy. Intriguingly, the key factors involved in controlling the apoptosis are regulated by the 26S proteasome complex [9,30]. For example, the levels of the pro-apoptotic factors such as p53, Bax, and NOXA are increased following inhibition of the proteolytic function of the 26S proteasome. Further, the inhibition of the proteasome activity has been shown to downregulate the anti-apoptotic factors such as Bcl-2 and IAP (inhibitor of apoptosis) proteins. Therefore, inhibition of the proteolytic function in cancer cells would promote apoptosis by upregulating the functions of the pro-apototic factors and suppressing the anti-apoptotic factors, hence killing cancer cells. Indeed, inhibition of the proteolytic function of the 26S proteasome has been shown to enhance apoptosis in a number of cancer cells [9] (Fig. 3).

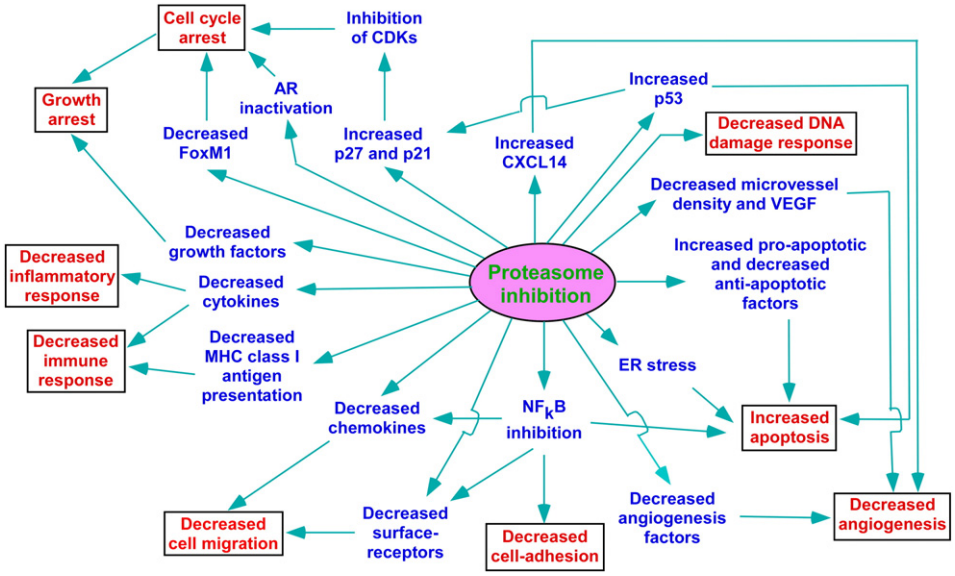

Fig. 3. The schematic diagram showing the effects of the proteasome inhibition on different pathways, contributing to cancer prevention.

As mentioned above, p53 is a pro-apoptotic factor that plays a critical role in apoptosis. Normally an unstable protein, its enhanced levels result in the initiation of the apoptosis cascade. Down-regulation of 26S proteasomal degradation causes the pro-apoptotic accumulation of p53, and hence induction of apoptosis (Fig. 3). Activation of an E3 ubiquitin ligase, MDM2, ubiquitylates p53 and subsequently leads to its proteasomal degradation [31]. This downregulation of p53 activity leads to tumor progression and drug resistance. Further, in high-risk HPV (human papilloma virus)-related cancer cases, the E6 oncoprotein and E6-AP (E6 associated protein, an E3 ubiquitin ligase) bind to p53, and ubiquitylate it for subsequent 26S proteasomal degradation [32,33]. Thus, the 26S proteasome complex plays a key role in regulating the level of p53, and through it apoptosis and cancer progression. Therefore, the inhibition of the proteolytic function of the 26S proteasome in cancer cells would augment the level of p53, and would eventually impair cancer progression by inducing apoptosis (Fig. 3). Unsurprisingly, the inhibition of the proteasome activity has been shown to induce p53-dependent apoptosis in renal cancer cell lines [34], colon cancer [35], melanoma, and multiple myeloma [36]. Proteasome inhibition has also been demonstrated to activate the downstream target genes of p53 such as Bax, p21, PUMA (p53 upregulated modulator of apoptosis) and Fas ligand [37].

2.3. Cell cycle

Regulation of cell cycle progression is a pivotal step in controlling carcinogenesis. In general, rapid cell cycle progression leads to cell proliferation and uncontrolled growth, ultimately developing transformed cancerous cells. Cyclins and cyclin dependent kinases (CDKs) tightly control the progression of cell cycle. However, the functions of several cyclins, CDKs, and their interplay are regulated by the proteolytic activity of the 26S proteasome complex, conferring proteasomal regulation of cell cycle progression in a number of ways. For example, CDK inhibition by tumor suppressor p27 downregulates cyclins D and E, and subsequently negatively controls the cell cycle progression through G1/S phase [38] (Fig. 3). The 26S proteasome complex is involved in the degradation of p27, and thus promotes cell cycle progression (Fig. 3). The E3 ubiquitin ligase, Skp-2 (S-phase kinase protein 2) targets p27 for ubiquitylation for its 26S proteasomal degradation. Due to this, low levels of p27 would lead to rapid cell cycle progression and hence oncogenesis. In fact, low levels of p27 are observed in various malignancies such as lymphoma, breast, lung, colon, prostate, ovarian, and brain cancers [39]. Further, high levels of Skp-2 contributing to enhanced proteasomal degradation of p27 have been demonstrated in several cancers including non-small cell lung carcinoma [40]. Similarly, the 26S proteasome is also involved in the regulation of CDK-inhibiting protein p21 levels, hence controlling cell cycle progression [41–43] (Fig. 3).

Like cyclins D and E, cyclins A and B are also regulated by the 26S proteasome. The anaphase-promoting complex/cyclosome (APC/C) serves as an E3 ubiquitin ligase, and ubiquitylates both cyclins A and B, marking them for degradation by the 26S proteasome complex [44–46]. The 26S proteasomal degradation of cyclins A and B guarantees that the cell completes mitosis and can enter the next cell cycle. In fact, cyclin B is rapidly degraded by the proteasome as the cell exits mitosis [47–49]. Clearly, an alteration of the proteasomal degradation of cyclins A and B, or their enhanced level would be correlated with a number of cancers. Indeed, cyclin B has been found to be overexpressed in numerous cancer cell lines [50].

Further, the 26S proteasome complex has been shown to regulate cell cycle progression via an oncogenic transcription factor, Forkhead Box M1 (FoxM1). FoxM1 induces the expression of the genes that are involved in cell cycle progression (Fig. 3). It is expressed at a low level in normal cells. However, its overexpression can lead to a rapid cell cycle progression. In fact, FoxM1 has been shown to be overexpressed in numerous cancers such as non-small cell lung carcinoma [51] breast cancer [52], colorectal cancer [53], glioblastomas [54], pancreatic carcinomas [55] and squamous cell carcinomas [56]. Additionally, the inhibition of the proteolytic activity of the 26S proteasome has been demonstrated to suppress the expression of FoxM1 and its transcriptional activity in cancer cell lines [57,58] (Fig. 3). Thus, the proteasome plays an important role in regulation of oncogenesis via controlling the expression and activity of FoxM1.

2.4. Endoplasmic reticulum (ER) stress

Following the translation of mRNA at the ER, proteins are folded into their functional forms. When proteins are not folded properly, they are directed to 26S proteasomal degradation. If misfolded or unfolded proteins are not degraded by the proteasome, they form aggregates and lead to the ER stress. The ER stress triggers unfolded protein response (UPR) to reduce the accumulation of unfolded proteins and restore the ER function. When protein aggregation or ER stress persists, especially in cancerous cells with high rates of protein synthesis, the UPR signaling switches from the pro-survival to pro-apoptotic. Consequently, the 26S proteasome complex also plays an important role in regulating the ER stress and cell survival. Therefore, inhibition of the proteasomal function in cancer cells would promote apoptosis and have an anti-tumor function (Fig. 3). In fact, the inhibition of the proteolytic activity of the 26S proteasome has been shown to induce pro-apoptotic ER stress in multiple myeloma [59], pancreatic [60], head and neck cancer [61], and non-small cell lung carcinoma [62].

2.5. Androgen receptor (AR)

AR is a ligand-dependent transcription factor, and belongs to the family of nuclear receptors. It plays an important role during differentiation and growth of the prostate and accompanying urogenital structures [24,63]. In presence of its ligand androgen, it binds to the promoters of a set of genes and regulates their expression [24,63]. With the ability to upregulate or downregulate under certain conditions, AR influences the expression of many genes. Some include: keratinocyte growth factor, probasin, prostate specific antigen (PSA), p21, Kallikrein, ornithine decarboxylase, and the AR gene itself. When the strict regulation of AR slips, it causes tumorigenesis, especially prostate cancer. Androgen plays a crucial role in the development of prostate cancer by activating AR. Prostate cancer is the second leading cause of cancer death among American males. Due to this, great importance is placed on androgen and its part in the development of prostate cancer. When AR is inactivated in AR-dependent prostate cancer, those cells can no longer replicate DNA or enter S phase, causing cell death [24] (Fig. 3). Interaction between calmodulin (CaM) and AR can cause CaM-dependent protein kinases to phosphorylate AR, thus manipulating its molecular stability and nuclear localization via the 26S proteasome [24]. Proteasome inhibitor MG-132 arrests AR interaction with co-regulators ARA70 or TIF2, as well as preventing nuclear translocation of AR, repressing AR transactivation [24,63]. Further, the inhibition of the proteolytic activity of the 26S proteasome by bortezomib reduces the basal level of AR, and subsequently induces the apoptosis of androgen-dependent human prostate cancer LNCaP cells [24]. However, some prostate cancers grow in the absence of androgen, named androgen-independent AR-positive prostate cancer. Interestingly, decreasing active AR levels in these cells still inhibits growth [24]. Together, these studies have implicated the 26S proteasome complex in regulating AR expression and function, and hence carcinogenesis. Thus, proteasome inhibition has anti-tumor effects via modulating the activity of AR. Likewise, proteasome inhibition has also been shown to regulate GR (glucocorticoid receptor) and ER (estrogen receptor) target genes in MCF-7 breast cancer cells [64]. Further, Alarid and colleagues [65] have recently provided a new link of proteasomal function in estrogen signaling in breast cancers, by demonstrating the repression of ER gene expression in response to proteasome inhibition.

2.6. Chemokines

Chemokines are a family of chemotactic cytokines, and cause cells to move along a chemical gradient. This mechanism is very important in terms of angiogenesis, cell migration, and metastasis, all playing enormous roles in cancer [66–69]. The role of chemokines in metastasis is the outcome of modified presentation of G-protein coupled receptors. When the production of the chemokine receptor CXCR4 is upregulated in cancer cells (e.g., metastatic breast cancer, ovarian cancer and malignant melanoma cells), the cell migration is increased towards tissues that constitutively express the cognate chemokines ligand CXCL12, like liver, bone marrow, lymph nodes, and lung. To the contrary, the chemokine CXCL14/BRAK exhibits tumor suppressing activity but its expression is often modified in several cancers including prostate as well as squamous cell carcinomas of the head, neck, and cervix [69–71]. The ubiquitin–proteasome system is responsible for degrading chemokine CXCL14 in cancer and other immortalized cells, but the proteasome does not degrade CXCL14 in normal epithelial cells [69]. Treatment with proteasome inhibitor lactacystin resulted in the expression of CXCL14 in cancer cell lines that had previously shown impaired CXCL14 expression. Also, when LNCaP prostate cancer cells were treated with proteasome inhibitor MG-132, polyubiquitylated CXCL14 was found. These results demonstrate that cancer cells prevent their own CXCL14 expression as well as degradation by the proteasome. When overexpressed, CXCL14 inhibits angiogenesis and hence tumor growth [69,72] (Fig. 3). Further, normal and lower levels of CXCL14 would increase the chances of dendritic cells penetrating the tumor, leading to detection of the tumor by the immune system [69,71–73]. Thus, CXCL14 has anti-tumor activities via impairing angiogenesis and enhancing the detection of tumor by the immune system. The absence of CXCL14 in head and neck and prostate cancers favors cancer progression. Therefore, the inhibition of the proteolytic function of the proteasome in the cancer cells would increase the level of CXCL14, and hence would produce anti-tumor effects (Fig. 3).

2.7. Cell-surface receptors

Cell-surface receptors, growth factors and their signaling pathways play important roles in carcinogenesis. Cell-surface receptors have been demonstrated to be ubiquitylated and degraded by the 26S proteasome complex. Proteasomal degradation of the cell-surface receptors is very relevant in cancer chemotherapy. For example, tyrosine kinase receptors are degraded by the 26S proteasome, and such degradation provides anti-tumor activity to herbimycin A which inhibits multiple tyrosine protein kinases [74]. Similarly, protein kinase C (PKC) inhibitor downregulates PKC via 26S proteasomal degradation [75,76]. Other cell-surface receptors such as T-cell antigen receptor (TCR) and platelet-derived growth factor (PDGF) are also degraded by the 26S proteasome. These receptors are ubiquitylated in response to ligand binding for proteasomal degradation [77,78]. Thus, the 26S proteasome complex plays an important role in regulating the stabilities of the cell-surface receptors, and hence their signaling pathways.

2.8. AP-1

The proto-oncogene products c-JUN and c-FOS interact to form the transcription factor, AP-1 (activator protein 1). These two proto-oncoproteins form AP-1 as a heterodimer of c-JUN and c-FOS or a homodimer of c-JUN, and are known to be degraded by the 26S proteasome complex [79–84]. The delta region, a 27 amino acid long segment of c-JUN, enables ubiquitylation and proteasomal degradation of the protein. This segment is missing in v-JUN, the transforming retroviral counterpart of c-JUN. As a result, v-JUN is upregulated, and such an increased stability is very likely to contribute to its oncogenicity [79]. Therefore, an impaired proteasomal degradation of c-JUN can lead to oncogenesis. Moreover, the proteasomal degradation of c-JUN is essential to maintain normal function of AP-1 factors. The AP-1 factors play important roles in handling oxidative stresses [85,86]. AP-1 upregulation has been correlated with drug resistance in several cancer cell lines [87–89].

2.9. DNA repair

The DNA damage and regulation of its repair mechanisms are strongly correlated with carcinogenesis. The proteasome complex plays a crucial role in DNA repair [90]. The inhibition of the proteolytic function would impair DNA repair (Fig. 3), and trigger apoptosis. Therefore, DNA damaging agents have been used to kill cancer cells. Further, the combination of DNA damaging agents with proteasome inhibitor would have synergistic effects in killing cancer cells. Indeed, proteasome inhibitors have shown more effective anti-tumor activities when combined with the DNA damaging agents such as radiation and camptothecin (CPT) [9,90].

2.10. MHC-restricted class I antigens

As mentioned above, MHC-restricted class I antigens are vitally processed by the immunoproteasome and presented on the cell surface for recognization by cytotoxic T lymphocytes. Proteins LMP2, LMP7, and LMP10 are interchanged with the three components of the 20S catalytic core to form the immunoproteasome in the instance of IFN-γ induction [91–95]. Substitution of LMP2, LMP7, and LMP10 yields more types of peptides expressed on the cell surface [96,97]. Thus, the low levels of LMP2, LMP7, and LMP10 can lead to a decrease in MHC class I-restricted peptide presentation, and cause an escape from immune surveillance, leading to cancer. Indeed, very low levels of LMP2, LMP7, and lower antigen presentation are found in 3 small-cell lung carcinoma lines [98]. Further, the mouse T-cell lymphoma cell line SP-3 has been shown to display an underexpression of LMP2 and an impairment of antigen presentation [99]. Oncogenic viruses have been found to down-regulate LMP2 and LMP7 upon viral transformation of the cell [100]. However, the expression of LMP2 and LMP7 has been shown to be increased with an enhancement of antigen presentation in these cancer cell lines by expressing IFN-γ following transfection [99,101]. By expressing fewer peptides, cancerous cells may avoid detection by the immune system. Thus, the immunoproteasome plays an important role in antigen presentation, and its malfunction would lead to the escape from immune surveillance (Fig. 3), and hence cancer. In fact, a low level of immunoproteasome activity is present in certain cancer cells [98–100]. A down-regulation of LMP2 and LMP7 has also been observed in hepatocellular carcinoma [102]. Likewise, a decreased level of 26S proteasomal activity has also been observed in lung cancer stem-like cells *in vitro*[103]. Further, an altered level of the proteasome component MB1 (β5) is found in ovarian cancer [104].

As discussed above, the 26S proteasome is involved in nearly every kind of pathway cancer uses for survival and growth. By understanding these pathways and their relationship with the proteasome, it becomes clear that the manipulation of the 26S proteasome in turn would determine the fate of cancer cells. Undoubtedly, the proteasomal inhibition has been proven to be an attractive anti-cancer tool as discussed below.

3. Proteasome inhibition to treat cancer

When proteasome inhibitors prevent the proteasome from activating NFκB, factors of angiogenesis, survival, and growth are down-regulated while apoptosis is up-regulated in multiple tumor cell lines [16,105–115] (Fig. 3). This effect is also noticed in chemotherapy-resistant cells, additionally due to disruption of proteasomal regulation of caspases and Bcl-2. Further, proteasome inhibition enhances the levels of p21 and p27 [116,117] (Fig. 3). Such enhancement inhibits CDKs and consequently arrests cell cycle, halting the growth of cancer cells (Fig. 3). The inhibition of the proteolytic function of the 26S proteasome has also been shown to impair the

development of new blood vessels from endothelial cells or angiogenesis (Fig. 3) that is a vital factor for tumor growth and metastasis [115,118]. Disruption of angiogenesis by proteasome inhibition also occurs by decreasing microvessel density and the expression of vascular endothelial growth factor (VEGF) [115,118,119] (Fig. 3). Thus, the proteasome inhibition impairs angiogenesis as well as disturbs cellular homeostasis, hence leading to an anti-tumor activity. Overall, these studies demonstrated that the inhibition of the proteolytic function of the 26S proteasome induces apoptosis and cell cycle arrest, and represses angiogenesis as well as metastasis (Fig. 3). In fact, apoptosis and other anti-tumor effects of proteasome inhibition have been observed in various cancer cell lines and xenograft models including lymphoma, leukemia, melanoma, pancreatic, prostate, head and neck, breast, and lung cancers [36,119–126]. Further, cancer cells are more sensitive to the cytotoxic effects of the proteasome inhibition as compared to the normal cells [127,128]. Also, cessation of all proteasomal function is not required to achieve anti-tumor effects [14,129]. Together, these studies have implicated the proteasome inhibition as an attractive way of treating cancer cells (Fig. 3). Therefore, a large number of studies are focused on a variety of proteasome inhibitors for effectively treating cancer.

There is a wide variety of natural and synthetic proteasome inhibitors. These inhibitors are clustered into five groups: peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones, and β-lactones (lactacystin and its derivatives). Small-molecule proteasome inhibitors mimic the peptide substrates of the active sites in the 20S catalytic core subunit of the 26S proteasome complex. Lactacystin is a microbial metabolite isolated from Streptomyces, and is the first compound found to have an inhibiting effect on the proteasome. Lactacystin effectively and irreversibly inhibits the β5-component of the proteasome by selectively modifying N-terminal threonine residues, and also reversibly binds to the β1- and β2-components. MG-132 (Z-Leu-Leu-Leu-aldehyde) and PSI (Z-Ile-Glu-(OtBu)-Ala-Leu-aldehyde) are two of the first proteasome inhibitors synthesized. These are peptide aldehydes that reversibly bind to the β2- and β5 components by forming covalent hemiacetal adducts. At high concentrations, they also inhibit calpains and cathepsins proteases. However, these compounds exhibit low specificity and high metabolic instability, limiting to use as research reagents. The peptide vinyl sulfone proteasome inhibitor has a vinyl sulfone group which is less reactive than the aldehyde group of the peptide aldehyde proteasome inhibitor. The vinyl sulfone group irreversibly binds to the active site. One most potent peptide vinyl sulfone proteasome inhibitor is AdaAhx3-LLL-vs. This inhibitor binds to the active sites of both the constitutive and immunoproteasome with almost equal efficiencies [130,131]. Peptide boronates, epoxomicin (peptide epoxyketone), and lactacystin have shown higher specificity to the proteasome, and therefore show the most promise for drug development. Two of the peptide boronates, MG-262 and bortezomib, form more stable tetrahedral intermediates with N-terminal threonine residues of the 20S CP, lending them a greater efficacy.

In addition to the synthetic and natural proteasome inhibitors, a variety of proteasome inhibiting compounds can also be found in foods. Some of these inhibitors are: apigenin, epigallocatechin-gallate (EGCG), and ajoene. Apigenin is a polyphenolic flavone found in a broad range of fruits and vegetables [132,133]. It has demonstrated chemopreventive properties in several cancer models such as lung, skin, cervical, prostate, and leukemia by scavenging the free radicals, anti-inflammation, and proteasome inhibition [134–141]. It comes as no surprise that frequent ingestion of apigenin and other polyphenolic compounds correlates with a lowered cancer risk and even a suppression of tumor growth [132,133]. Apigenin achieves its proteasomal inhibiting effect by interrupting the chymotrypsin-like activity of the β5-component of the proteasome [133]. In a study using MDA-MB-231 breast cancer cells, Chen et al. [133] found that at the highest concentration tested, apigenin reduced proliferation and viability of cancer cells by 50% after 24 h. Western blot analysis confirmed that apigenin caused 40% proteasome inhibition, a buildup of ubiquitylated-Bax and IκBα, as well as increased caspase-3, caspase-7, and cleaved PARP (poly ADP-ribose polymerase) levels, indicating apoptosis [133,136,141]. In addition to a significant proteasome inhibition, apigenin also seems to induce the expression of death receptor 5 and an apoptosis-inducing TNF-associated ligand in leukemia, prostate, and colon cancer cells without having a toxic effect on normal cells [133,142]. This cancer-targeted toxicity is echoed in animal models *in vitro* and *in vivo*[133]. Like apigenin, EGCG present in green, but not black, tea has been demonstrated to inhibit the proteolytic function of the 26S proteasome. It is a polyphenolic compound, and has an anti-tumor activity [143,144]. Further, ECGC has been shown to attenuate the release of pro-inflammatory cytokines, thereby terminating inflammation [143,144]. Likewise, ajoene is an organo sulfur compound present in garlic [130]. It has been shown to inhibit the trypsin-like activity of the 20S CP of the proteasome complex [145]. It induces apoptosis as well as cell cycle arrest of tumor cells by inhibiting G2/M phase. Consequently, ajoene has cytotoxic effects in tumor cells [146].

Like the proteasome inhibitors found in foods, naturally occurring gallium has also shown anti-neoplastic activity in clinical trials in bladder cancer, lymphomas, and a variety of other malignancies [147–152]. Gallium III complex demonstrates anti-tumor activity via the inhibition of the proteasomal activity of the 26S proteasome [152]. Further, gallium disturbs iron homeostasis by competing with Fe^{3+} for uptake into cells, the mediator for which is the transferrin receptor system that is overexpressed in cancerous cells [152–155]. Chen et al. [152] have demonstrated that a certain gallium complex tested inhibited 81% of proteasomal activity in C4-2B prostate cancer cells. This complex also induces apoptosis, as evidenced by PARP cleavage, TUNEL positivity, nuclei condensation, and activation of caspase-3/caspase-7 [152].

4. Bortezomib: a proteasome inhibitor in the clinic to treat cancer

As mentioned above, the proteasome complex plays crucial roles in many important biological events, and its malfunction is strongly correlated to carcinogenesis. Thus, the proteasome inhibitors have shown a broad spectrum of anti-proliferative and pro-apoptotic activities against haematological and solid tumors. However, many of these proteasome inhibitors have low potency, specificity or stability [156–161]. Therefore, new proteasome inhibitors with greater potency and selectivity were developed. Thirteen boron-containing proteasome inhibitors were synthesized, and subsequently screened for anti-cancer activity using a panel of 60 human tumor cell lines of National Cancer Institute, USA [162]. One compound showed extremely high potency against a wide range of cancer cell lines. This compound is known as bortezomib, velcade, or PS-341 (originally synthesized as MG-341 at a company called Myogenics, and marketed as velcade by Millennium Pharmaceuticals, Inc., Cambridge, MA, USA) (Table 1).

Table 1
26S Proteasome inhibitors with theirs tagets and clinical status.

26S Proteasome inhibitor	Target in 26S proteasome	Clinical status
Bortezomib	Chymotrypsin-like activity	-Approved for MM and MCL.
Carfilzomib	Chymotrypsin-like activity	-Phase III in MM. -Phase I in AML, ALL, and CLL. -Phase Ib/II in solid tumors.
ONX0912	Chymotrypsin-like activity	-Phase I in solid tumors
NPI-0052	Chymotrypsin-like, trypsin-like, Caspase-like activities	-Phase I in MM, solid tumors, refractory lymphoma, and non-small cell lung carcinoma.
CEP-18770	Chymotrypsin-like activity	-Phase I/II in MM.
MLN9708	Chymotrypsin-like activity	-Phase I/II in MM. -Phase I in lymphoma and non-haematological malignancies.

MM, multiple myeloma; MCL, mantle cell lymphoma; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukaemia; and CLL, chronic lymphocytic leukaemia.

Bortezomib is a water-soluble dipeptide boronic acid which contains pyrazinoic acid, phenylalanine and Leucine with boric acid instead of a carboxylic acid.

Bortezomib is a stable proteasome inhibitor that binds covalently and reversibly with the β5 component of the 20S catalytic core subunit of the proteasome forming tetrahedral intermediates on the N-terminal threonine residues [156]. Further, it does not have any known activity against other cellular proteases [163]. Due to these qualities, bortezomib entered into clinical phase I trials [19,20,164–166]. In phase I clinical trials, bortezomib demonstrated an effective proteasome inhibition with fair tolerance levels, and thus moved into phase II clinical trials. Bortezomib had success in this clinical phase with refractory multiple myeloma patients [167], spurring its rapid approval by the Food and Drug Administration (FDA) and European Medicines Agency (EMEA). Interestingly, in the phase III clinical trials in refractory multiple myeloma, the survival rate of the patients treated with bortezomib exceeded that of the patients treated with dexamethasone [168]. Also, this proteasome inhibitor is consistently able to surmount the factors that normally cause treatment resistance including γ-radiation and the chemotherapeutic agent CPT-11 by preventing the activation of NFκB [18,169]. Though accumulation of p53 can initiate apoptosis, bortezomib kills tumor cells independently of p53 levels, even drug resistant multiple myeloma cell lines with mutant p53 [16,170].

Bortezomib specifically shows a high efficacy in multiple myeloma, non-small lung cancer, mantle cell lymphomas, and pancreatic cancer [125,164,167,171,172]. Though not all tumor cell types react similarly to bortezomib, its substantial activity in a variety of cancer cell lines and tumor types in clinical trials propelled it into FDA approval. The FDA first approved bortezomib in 2003 for the third-line treatment of multiple myeloma [173]. Later on, it was approved for the first-line treatment in 2008. It has also been approved in treating mantle cell lymphoma (a fast-growing cancer that begins in the cells of the immune system) in 2006 [174]. As the first FDA approved proteasome inhibitor, bortezomib exhibits around 1000-fold improvement over its aldehyde predecessors and more specificity to the β5 component of the 20S catalytic core of the proteasome. Although bortezomib has shown a significant anti-tumor activity, it is also used to overcome chemoresistance [175–177]. Due to this, bortezomib has been successfully combined with several other agents such as doxorubicin, thalidomide, melphalan, and dexamethasone. Thus, there is a great hope in developing better combinatorial therapy without increasing toxicity in treating numerous cancer patients. Currently, there are a large number of clinical trials going on for combinatorial therapy involving bortezomib against haematological malignancies and solid tumors.

Combinatorial therapies have shown great potential for cancer treatment. The combination of bortezomib with other drugs such as Hsp90 inhibitor, HDAC inhibitor, Akt inhibitor, and lenalidomide have more clinical benefits as compared to bortezomib alone [115,178]. Bortezomib with DNA damaging agent works well with relapsed and/or refractory cancer patients [110,115,179,180]. Hsp90 inhibitor has been shown to overcome bortezomib resistance in mantle cell lymphoma [108,178]. Lenalidomide has been combined with steroids, proteasome inhibitors, mTOR (target of rapamycin) inhibitors, humanized monoclonal antibodies, and Akt inhibitors. Lenalidomide with bortezomib or Akt inhibitor has shown very impressive responses in the cancer patients [115]. Overall, the combinatorial therapies have shown very promising results, and the most successful combination is likely to be approved soon to treat cancer patients.

Although bortezomib has been approved to treat multiple myeloma and mantle cell lymphoma patients, it also sensitizes pancreatic cancer cells to ER stress-mediated apoptosis [30,181–183]. Further, an induction of ER stress is a novel strategy to enhance bortezomib-induced apoptosis in pancreatic cancer cells. The combination of bortezomib with HDAC inhibitor, SAHA (Suberoylanilide hydroxamic acid), entered clinical trials in 2007. Additionally, the combination of bortezomib with HDAC6 inhibitor (more specific) may have better clinical benefits in treating pancreatic cancer or other solid malignancies [30,182].

Though bortezomib kills cancer cells, the cellular mechanisms for clinical efficacy of bortezomib are not clearly known. However, several mechanisms-of-action of bortezomib have been implicated in killing cancer cells, which include disruption of cell adhesion- and cytokine-dependent survival pathways (e.g., NFκB signaling pathway), inhibition of angiogenesis, activation of a misfolded protein stress response (or ER stress), upregulation of pro-apoptotic or downregulation of anti-apoptotic genes. DNA microarray analysis revealed upregulation of genes involved in hypoxia, ER stress/UPR, oxidative stress, apoptosis, and amino acid starvation following proteasomal inhibition [184–194]. Thus, bortezomib seems to kill cancer cells by hypoxic response deregulation in tumor cells, mTOR inhibition, and ER stress-induced apoptosis. Further, bortezomib has been shown to upregulate AP-1 activity and activating transcription factor (ATF) families [192,193,195–199]. ATF4 contributes to apoptosis, thus implicating ATFs in bortezomib-induced apoptosis. Like other cancer therapies, some factors contribute resistance to bortezomib treatment [115]. An increased expression of HSPs reduces the efficacy of bortezomib. For example, HSP27 directly correlates with bortezomib resistance [200], and HSP90 inhibition overcomes bortezomib resistance in mantle cell lymphoma [115,178]. Bortezomib has also been shown to promote IFN-α and TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis in human bladder cancer cells [201]. Therefore, bortezomib manifests its anti-tumor activity via multiple mechanisms.

Bortezomib is metabolized primarily by cytochrome P450 3A4 [202,203]. Though intensely effective in treating many types of cancer, bortezomib is not without its side effects. Bortezomib has a dose limiting toxicity and pain associated with intravenous administration. Patients treated with bortezomib have experienced peripheral neuropathy, pyrexia, adverse gastrointestinal events, myelosuppression, orthostatic hypotension, asthenia, thrombocytopenia, cardiac and pulmonary disorders, and pain [128,167,168,204,205]. Bortezomib is also associated with a high rate of shingles [206]. Further, it has not shown promising results in treating solid tumors [9]. These facts have demanded the need to develop a new generation of proteasome inhibitors. In this direction, several proteasome inhibitors have been developed, and are currently under clinical trials as presented below.

5. Proteasome inhibitors in clinical trials to treat cancer

There are several promising proteasome inhibitors that are currently in clinical trials. These are: carfilzomib (PR-171), ONX0912 (PR-047), marizomib (NPI-0052), CEP-18770, and MLN9708 (Table 1). Several immunoproteasome inhibitors (Table 2) have also been developed, which have shown impressive results in the pre-clinical studies. These inhibitors are described below.

Table 2
Immunoproteasome inhibitors with theirs tagets and pre-clinical results.

Immunoproteasome inhibitor	Target in immunoproteasome	Pre-clinical results
PR-957	Chymotrypsin-like activity	-Inhibits inflammatory response.
PR-924	Chymotrypsin-like activity	-Inhibits tunor growth in animal models without significant toxicities. -Inhibits growth of primary cell lines and primary tumor cells. -Anti-tumor activity against MM.
IPSI-001	Caspase-like activity	-Inhibits haematological malignancies in *in vitro* models. -Inhibits proliferation in myeloma patient samples. -Overcomes other drug resistance.

5.1. Carfilzomib

Carfilzomib (also known as PR-171) is an epoxomicin-based proteasome inhibitor with improved pharmaceutical properties. Proteolix Inc. (California, USA) has developed carfilzomib as a second generation proteasome inhibitor to treat multiple myeloma patients [129]. Carfilzomib irreversibly binds to the catalytic site of the proteasome, and inhibits the chymotrypsin-like activity. Unlike bortezomib, carfilzomib has shown minimal cross-reactivity with the other catalytic sites of the 20S CP. Further, carfilzomib shows minimal reactivity with other protease classes. Thus, carfilzomib has a better selectivity than bortezomib for chymotrypsin-like activity of the 26S proteasome in *in vitro* and *in vivo* studies [129,207,208]. Carfilzomib has also shown better tolerability and dosing flexibility in xenograft models [129,208]. Pre-clinical studies indicate that carfilzomib is active against models of solid tumors, lymphomas, and myeloma [129,208–210]. Carfilzomib inhibits cell proliferation, and induces apoptosis which is associated with activation of JNK (c-Jun N-terminal protein kinase), depolarization of mitochondrial membrane, release of cytochrome *C*, and activation of both intrinsic and extrinsic caspase pathways in patient-derived multiple myeloma cells as well as neoplastic cells from patients with other haematologic malignancies [129,209,210]. The phase I clinical trials of carfilzomib demonstrated that multiple myeloma patients who have relapsed or progressed following a number of therapies (including bortezomib and stem cell transplant) can also achieve durable anti-tumor responses with carfilzomib. Carfilzomib is well tolerated in patients at doses that suppress chymotrypsin-like proteasome activity by >80% in whole blood. The phase II clinical trials of carfilzomib provided promising results in patients with relapsed or refractory multiple myeloma. Currently, clinical phase III trials are ongoing for carfilzomib in multiple myeloma [9,211,212]. Further, carfilzomib is now under clinical phase I trials for acute myeloid leukemia (AML), acute lymphoblastic leukaemia (ALL), and chronic lymphocytic leukemia (CLL) [9,129]. It is also in phase 1b/II trials in solid tumors [118,213]. Like bortezomib, carfilzomib may work better in combination with other therapies. In fact, it has been shown to function better in leukemia and lymphoma in combination with HDAC inhibitors *in vitro* [214,215]. Carfilzomib has also been shown to interact synergistically with HDAC inhibitors in mantle cell lymphoma cells [211]. Furthermore, carfilzomib acts synergistically with dexamethasone, and has shown an increased level of anti-multiple myeloma activity as compared to bortezomib [129].

5.2. ONX0912

Both bortezomib and carfilzomib are administered intravenously. However, an oral proteasome inhibitor could be easily administered in the multi-drug treatment regimens. Proteolix, Inc. has developed an oral analogue, ONX0912 (also known as PR-047) that has N-cap with significant pre-clinical anti-tumor activities [216]. This agent shows an improved therapeutic window over carfilzomib in experimental animal models. It has been demonstrated to reduce tumor progression and prolong survival in animal models of multiple myeloma, non-Hodgkin's lymphoma and colorectal cancer [216–218]. Further, it has been shown to enhance the anti-tumor activity in combination with HDAC inhibitor, lenolidomide and bortezomib [216–218]. This proteasome inhibitor is currently under clinical trials. The clinical phase I trials of this compound are also ongoing in advanced solid tumors [9].

5.3. NPI-0052

NPI-0052 (also known as salinosporamide A or marizomib) is an irreversible second generation proteasome inhibitor, and orally bioactive [219]. It has been developed by Nereus Pharmaceuticals, Inc. (San Diego, CA, USA) [220]. It is a non-peptide, β-lactone compound that is related to lactacystin. It has been derived from the marine bacterium *Salinospora tropica*[221], and possesses anti-tumor activity through caspase-8 activation [222,223]. It stimulates apoptosis predominantly via caspase-8-mediated pathway [222,223]. Thus, NPI-0052 induces apoptosis via mechanisms that are unique from those evoked by bortezomib [108,222,223]. NPI-0052 also differs from bortezomib or carfilzomib in terms of its inhibitory effects on the three major enzymatic activities of the 20S CP. It binds irreversibly to all catalytic sites for proteolysis of the 26S proteasome [223]. At the maximum tolerated dose without apparent toxicity, NPI-0052 shows as high as 90% proteasome inhibition as compared to 70% inhibition by bortezomib [169,219]. The proteasome inhibition by NPI-0052 increases progressively over 24 h, and remained essentially unchanged for 72 h. On the other hand, the proteasome inhibition by bortezomib reaches the maximum level of inhibition at 1.5 h, and then significantly decreases over the next 24 h [219]. Therefore, NPI-0052 appears to be a more effective compound in treating cancer patients. The cellular response to NPI-0052 occurs much earlier than bortezomib. Further, it has shown effectiveness in multiple myeloma cell lines that are resistant to bortezomib [223]. It has also been demonstrated to be significantly effective in pre-clinical studies in Waldenstrom's macroglobulinemia, acute leukemia, CLL, prostate, pancreatic and colon cancers [219,222,224–228]. However, NPI-0052 may be less specific since its analog lactacystin binds to several proteasome subunits as well as inhibits other cellular proteases. Although NPI-0052 blocks a wider range of proteasome activities, it appears to be less toxic to normal cells [223,229]. In mice implanted with human myeloma tumor cells, NPI-0052 was well tolerated and showed prolonged survival as well as significantly reduced the rate of cancer recurrences. Further, the cancer cells were killed more effectively by the combination of NPI-0052 with bortezomib and HDAC inhibitors, MS-275 and valproic acid (VPA) without additional toxicity to normal cells [222]. The clinical phase I trials of NPI-0052 are ongoing in advanced solid tumors, refractory lymphoma and non-small cell lung carcinoma [9].

5.4. CEP-18770

It is a boronic acid-based proteasome inhibitor. Like bortezomib, it is a reversible proteasome inhibitor, and primarily inhibits the chymotrypsin-like activity of the proteasome [118,230]. It is a water soluble and orally bioactive proteasome inhibitor [118,230]. CEP-18770 abrogates the production of VEGF in multiple myeloma cells [118]. Such a decreased level of VEGF production inhibits cell migration and vasculogenesis from the endothelial progenitors [118]. Further, the role of CEP-18770 in angiogenesis is corroborated by its direct inhibitory effect on endothelial cell proliferation, survival, and capillary tubular morphogenesis [118]. CEP-18770 has also been shown to promote apoptosis in human multiple myeloma cell lines [118,230]. It is a potent inhibitor of constitutive and TNF-α-triggered NFκB activation [118]. CEP-18770 has been demonstrated to have a significantly reduced toxicity towards human bone marrow progenitors, bone marrow stromal cells, and normal human intestinal cells as compared to bortezomib [118]. Although CEP-18770 has a significant anti-tumor activity, it is more effective in combination with bortezomib and melphalan in animal tumor models [231]. The clinical phase I trials of CEP-18770 have been completed for solid tumors and non-Hodgkin's lymphoma [9]. Currently, it is under phase I/II clinical trials for multiple myeloma [9].

5.5. MLN9708

It is a small molecule boron-containing peptide inhibitor (Millennium Pharmaceuticals, Inc.). In contrast to bortezomib, MLN9708 is orally bioavailable [232]. Like bortezomib, it inhibits the chymotrypsin-like activity of the proteasome. However, the proteasome dissociation

half-life of MLN9708 is shorter than bortezomib. Further, it has improved pharmacokinetics, pharmacodynamics, and anti-tumor activity in xenograft models [233]. It is biologically inactive. However, it is hydrolyzed quickly in plasma to MLN2238 that is biologically active. It has shown strong anti-cancer activity against numerous cancer cell lines [232,233]. It has also been demonstrated to be effective in human prostate xenograft, colon cancer and lymphoma models [233]. Very recently, Chauhan et al. [232] have demonstrated that MLN9708 has synergistic anti-multiple myeloma activity when combined with bortezomib, HDAC inhibitor, lenalidomide or dexamethasone. This proteasome inhibitor is currently in phase I clinical trials in patients with lymphoma and non-haematological malignancies [9]. Further, clinical phase I/II trials of MLN9708 for multiple myeloma are ongoing [9].

5.6. Immunoproteasome inhibitors

The immunoproteasome is present in immune cells at a lower level. Thus, the inhibition of the immunoproteasome will provide specificity over constitutive proteasome. Such specificity will attenuate the toxicities associated with constitutive proteasome inhibition. Several immunoproteasome inhibitors such as PR-957, PR-924 and IPSI-001 have been developed. Pre-clinical studies of these inhibitors have shown impressive anti-tumor and anti-inflammatory responses. PR-957 (also known as ONX0914) has recently been developed by Proteolix, Inc [234,235]. Like carfilzomib, it is a peptide epoxyketone proteasome inhibitor. It inhibits chymotrypsin-like activity of the immunoproteasome. PR-957 inhibits the functions of IL-1 (interleukin-1), IL-6 and TNF. Further, it blocks the production of IL-23 by activated monocytes and interferon-γ and IL-2 by T cells. Therefore, PR-957 has immunosuppressive effects [234,235]. Hence, PR-957 may be effective against autoimmune diseases in conjunction with cancer treatment. PR-957 induces an anti-inflammatory response at a low dose as compared to the non-selective inhibitors such as bortezomib and carfilzomib [234,236,237]. Like PR-957, PR-924 is a peptide epoxyketone proteasome inhibitor, and inhibits chymotrypsin-like activity of the immunoproteasome [238]. It impairs the growth of multiple myeloma cell lines and primary tumor cells. It has also been shown to inhibit the tumor growth in animal models without significant toxicities. Unlike PR-957 and PR-924, IPSI-001 is a peptide aldehyde type of inhibitor [239]. It inhibits preferentially the β1i component of the immunoproteasome. It has been shown to inhibit the haematological malignancies in *in vitro* models. It also potently inhibits proliferation in myeloma patient samples [239]. Further, IPSI-001 overcomes conventional and novel drug resistance [239]. Together, these immunoproteasome inhibitors have great potential to be in the clinic in future with more selectivity and less toxicity.

6. Concluding remarks

Here, we have discussed the 26S proteasome complex in different key cellular events and carcinogenesis. It is clear from a large number of studies that the 26S proteasome complex regulates a multitude of cellular processes like cell cycle progression, inflammation, antigen presentation, apoptosis, DNA repair, transcription, and indirectly: cell growth, chemotaxis, angiogenesis, and cell adhesion. Many of these mechanisms are altered to the benefit of cancer cells. For this reason, the 26S proteasome complex has become an attractive target for cancer therapy. In fact, the proteasome inhibition has led to an increased apoptosis and other anti-tumor effects such as cell cycle arrest, and inhibition of angiogenesis and metastasis in various cancer cell lines and xenograft models. The proteasome inhibitor bortezomib is in the clinic to treat multiple myeloma and mantle cell lymphoma patients. Several proteasome inhibitors are now in clinical trials to treat multiple myeloma and solid tumors. Additional proteasome inhibitors with different efficacies are being developed and tested for anti-tumor activities. Several proteasome inhibitors have shown significantly improved anti-tumor activities when combined with other drugs such as HDAC inhibitor, Akt inhibitor, DNA damaging agent, Hsp90 inhibitor, and lenalidomide. In summary, proteasome inhibitors alone or in combination with other therapies have shown very promising results to treat cancer patients in the clinic more effectively.

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