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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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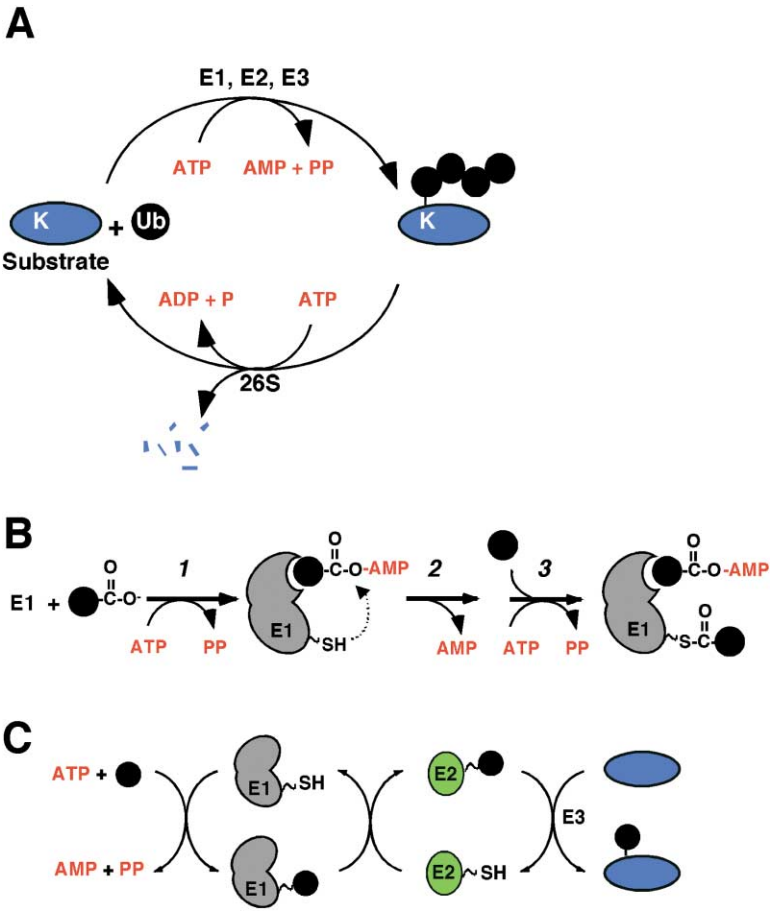

Figure 1. Components and Mechanisms in the Ubiquitin/Proteasome Pathway

(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.

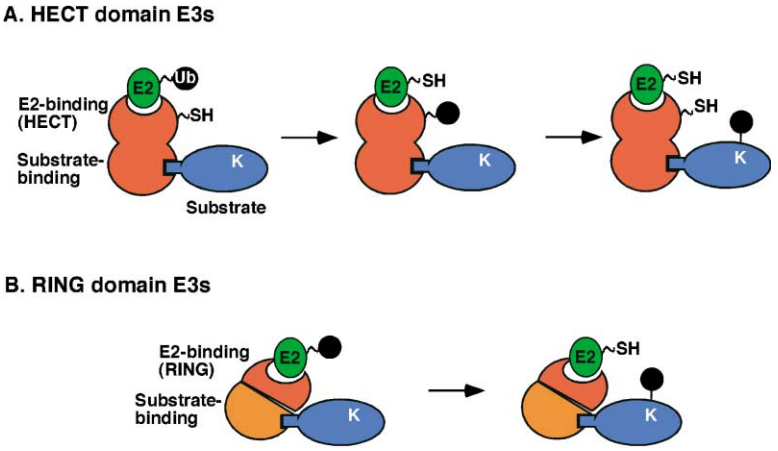

Figure 2. Major E3 Classes

(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

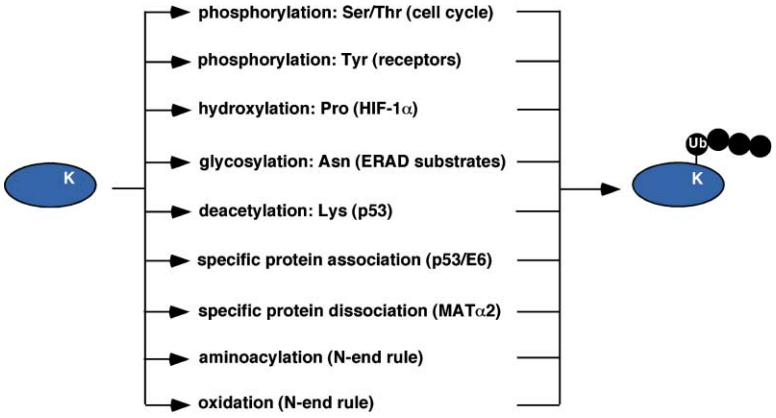

Figure 3. Mechanisms for Modulating Substrate Recognition by E3s

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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