Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets

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Abstract | The ubiquitin–proteasome system (UPS) and ubiquitin-like protein (UBL) conjugation pathways are integral to cellular protein homeostasis. The growing recognition of the fundamental importance of these pathways to normal cell function and in disease has prompted an in-depth search for small-molecule inhibitors that selectively block the function of these pathways. However, our limited understanding of the molecular mechanisms and biological consequences of UBL conjugation is a significant hurdle to identifying drug-like inhibitors of enzyme targets within these pathways. Here, we highlight recent advances in understanding the role of some of these enzymes and how these new insights may be the key to developing novel therapeutics for diseases including immuno-inflammatory disorders, cancer, infectious diseases, cardiovascular disease and neurodegenerative disorders.

Protein homeostasis is essential for most cellular processes. The ubiquitin–proteasome system (UPS) is responsible for much of the regulated proteolysis in the cell, and has non-degradative functions as well. Ubiquitin is a small 76 amino-acid protein that can be reversibly attached to other proteins and lies at the core of an elaborate post-translational modification pathway. Several ubiquitin-like proteins (UBLs) have also been identified, including NEDD8, SUMO and ISG15, which share a characteristic three-dimensional fold with ubiquitin but are otherwise distinct. The UPS and UBL conjugation pathways have multiple essential biological roles and it is not surprising that their function, and often malfunction, are important factors in various human diseases, including numerous cancer types, cardiovascular disease, viral diseases and neurodegenerative disorders. These diseases may feasibly be targeted by modulating components of the UPS and UBL conjugation pathways using small-molecule inhibitors.

Indeed, the therapeutic potential of intervention in the UPS in cancer has been demonstrated by the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals), which was approved by the US Food and Drug Administration in 2003. Recent and ongoing research to elucidate the roles of other components of the UPS and UBL conjugation pathways has identified several enzymes that could be additional targets for therapeutic intervention using small-molecule inhibitors. In this Review, we first provide an overview of the enzyme classes in the UPS and UBL pathways that represent potential therapeutic targets, highlighting considerations that are important for drug discovery and recent progress in the development of small-molecule inhibitors. We then review recent developments in our understanding of the role of the components of the UPS and the UBL pathways in disease and their potential for therapeutic intervention.

Targets in the UPS and UBL enzymatic cascades

Ubiquitin and UBLs typically modulate protein function following covalent attachment to a primary amino group within a substrate protein, usually by forming an isopeptide bond with a lysine side-chain. The ubiquitin pathway is representative of this process and is shown in Fig. 1. The enzymatic cascade that results in protein ubiquitylation and degradation involves several distinct steps, each requiring a different class of enzyme.

In the first step, an E1 activating enzyme (primarily ubiquitin-activating enzyme (UBE; also known as UBA1), although ubiquitin-like modifier activating enzyme 6 (UBA6) can also activate ubiquitin) consumes ATP and forms a high-energy thioester bond with the carboxyl terminus of ubiquitin. Ubiquitin is then passed to one of several E2 conjugating enzymes through a transthiolation

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Figure 1 | Overview of the enzymatic cascade involved in ubiquitin-like protein (UBL) conjugation and the ubiquitin–proteasome system (UPS). a | Ubiquitin-activating enzyme (UAE) binds ATP and ubiquitin (Ub) to form a ternary complex consisting of E1–ubiquitin thioester with ubiquitin–AMP bound (see text for details). The thioester-bound ubiquitin is then passed to one of several E2 conjugating enzymes through a transthiolation reaction. The ubiquitin-charged E2 then forms a complex with an E3 ligase and a protein substrate to transfer ubiquitin to a lysine residue on the substrate. To mark substrates for degradation, multiple ubiquitins are similarly recruited to produce a K48-linked polyubiquitinchain. Following release from the E3, the proteasome recognizes the polyubiquitin chain, and the substrate is deubiquitylated and destroyed. Substrates marked with ubiquitin chains linked through lysines 6, 11, 27, 29 and 33 also seem to be primarily destined for degradation. Alternatively, substrates marked with monoubiquitin, linear ubiquitin chains or K63 ubiquitin chains are involved in signalling functions that are independent of the proteasome. A second E1 (ubiquitin-like modifier activating enzyme 6 (UBA6)) also activates ubiquitin, but the function(s) of this pathway are unknown. b | Nine classes of UBL and eight E1 activating enzymes participate in diverse biological pathways in humans. E1s, E2s and UBLs are structurally and mechanistically related but are unique to each pathway. Ubiquitin and some E2s are exceptions in that they can be used by both the UAE and UBA6 pathways. Ufl1 and C20orf166 were recently identified and reported by Tatsumi et al.139. DUB, deubiquitylating enzyme; NAE, NEDD8-activating enzyme; PP<sup>i</sup>, inorganic pyrophosphate; SAE, SUMO-activating enzyme.
reaction. The ubiquitin-charged E2 then binds an E3 ligase and its protein substrate to transfer ubiquitin to an acceptor lysine residue on the substrate.

To mark substrates for degradation, multiple ubiquitins are similarly attached to produce a K48-linked polyubiquitin chain. On release of the substrate from the E3 ligase, the proteasome recognizes the polyubiquitin chain and the substrate is destroyed. Alternatively, substrates marked by monoubiquitylation or by polyubiquitin chains using different ubiquitin lysine sites are involved in functions such as protein trafficking, signalling and enzyme activation. Deubiquitylating enzymes (DUBs) regulate the function of these various ubiquitin modifications and may, for example, rescue a substrate from degradation by removing a degradative ubiquitin signal or by changing or removing a non-degradative ubiquitin signal.

Although all UBL conjugation pathways are catalysed by structurally and functionally related enzyme cascades, UBLs participate in biologically distinct pathways (Table 1). Ubiquitylation is by far the most thoroughly studied UBL pathway and is ‘information rich’ in that numerous downstream receptors recognize and process differentially ubiquitylated proteins. Substrates that are polyubiquitylated with K48-linked ubiquitin chains are delivered to the 26S proteasome to be degraded into small peptides, with the ubiquitins released to be used again. Proteins that are polyubiquitylated with K63-linked ubiquitin chains are generally not degraded but are essential components of signalling pathways, for example, nuclear factor-κB (NF-κB)-dependent expression of inflammatory and immune genes. The function of ubiquitin chains that are linked through lysines 6, 11, 27, 29 and 33 (Ref. 12) and of chains containing ‘mixed’ linkages is still emerging, although there is increasing evidence that non-K63 chains target proteins for degradation by the 26S proteasome.

With the exception of the proteasome (Box 1; Table 2), the enzymes in the UPS and UBL conjugation pathways represent unprecedented drug targets and target classes. Indeed, significant advances in identifying small-molecule inhibitors of the UBL pathways have recently been achieved by targeting E1s, E2s, E3s, DUBs and the proteasome itself. Each UBL pathway enzyme class is mechanistically distinct. Also, E1s are highly specific for their respective UBLs and match individual UBLs with respective E2s. E3s are mostly associated with the ubiquitin pathway and are primarily responsible for substrate specificity and in some cases directly facilitate UBL transfer. Similarly, DUBs also demonstrate a high degree of substrate specificity. Therefore, there are a number of discrete E1–E2–E3 cascades that could provide opportunities to specifically target the aberrant signalling that is associated with particular diseases, and these are outlined below.

### E1 activating enzymes

There are eight structurally and functionally related E1 activating enzymes that act at the apex of each of the nine classes of UBL conjugation pathways (Fig. 1; Table 1). E1s can be classified on the basis of domain structure. The so-called canonical E1s include UAE, NEDD8-activating enzyme (NAE), SUMO-activating enzyme, UBA6 and UBA7, and the non-canonical E1s include ATG7, UBA4 and UBA5 (see Ref. 15 for a review). All E1s share similar overall structural features and an ATP-dependent, multi-step mechanism for ubiquitin/UBL activation. Because E1s perform the first step of UBL activation, selective inhibition of an individual E1 would be expected to block the activation of each of the substrate UBLs for that E1, and subsequently block the function of affected UBL pathways.

E1s catalyse UBL activation by the multi-step process detailed in Fig. 2. In the first step, ATP and UBL bind together to form a UBL–acyl adenylate intermediate, releasing inorganic pyrophosphate. UBL–AMP then reacts with the E1 active-site thiol to form an E1–UBL thioester (~ denotes a high-energy bond). A second ATP and UBL then bind the enzyme as in the first step to form a ternary complex that contains two UBL.

### Table 1 | E1 activating enzymes, their cognate UBLs and biological roles*

| E1     | Structure   | UBLs       | Roles                                                                 |
|--------|-------------|------------|-----------------------------------------------------------------------|
| UAE (UBA1) | Monomeric   | Ubiquitin  | Multiple, including protein homeostasis, transcriptional regulation and cell cycle progression |
| NAE (APPB1–UBA3) | Heterodimer | NEDD8      | Activation of cullin-based E3s                                        |
| SAE (SAE1–UBA2) | Heterodimer | SUMO1/2/3  | Multiple, including protein stability, transcriptional regulation and cell cycle progression |
| UBA7 | Monomeric   | ISG15      | Antiviral functions; possibly cell growth and differentiation          |
| UBA6 | Monomeric   | FAT10      | Antiviral functions                                                    |
| UBA5 | Homodimeric | URM1       | Antioxidant pathways; tRNA uracyl thioliation                         |
| ATG7 | Homodimeric | ATG12      | ATG5–ATG12 conjugate forms complex with ATG16 that functions as an E3 ligase for autophagic vesicle formation |
| LC3   |             |            | Autophagic vesicle formation                                           |

*For further information see Refs 15, 142, 143. NAE, NEDD8-activating enzyme; SAE, SUMO-activating enzyme; UBA, ubiquitin-like modifier activating enzyme; UBL, ubiquitin-like protein.

**Proteasome**

The 26S proteasome is a protease complex that degrades polyubiquitylated proteins. It is composed of two subcomplexes: a barrel-shaped 20S core particle containing the protease active sites and two 19S regulatory particles that cap the barrel and control access of substrates to the core.

**Nuclear factor-κB (NF-κB)**

A transcription factor with a key role in regulating the immune response. NF-κB is involved in cellular responses to stimuli, including stress, cytokines, free radicals, ultraviolet irradiation and bacterial or viral antigens. Misregulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development.

**REVIEWS**
Box 1 | Targeting the proteasome

In principle, the inhibition of ubiquitin-like protein pathway enzymes would provide specificity for the individual pathways and substrate proteins targeted. By contrast, inhibition of the proteasome blocks the final common step of the degradative pathways and is therefore a relatively nonspecific target. Nevertheless, the first clinically validated drug to target the ubiquitin–proteasome system (UPS) was the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals), which was approved in 2003. Owing to its success, several second-generation proteasome inhibitors are currently in development.

Each of the three types of proteasome active site — the caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) sites — use an amino terminal threonine as the catalytic amino-acid residue. The interactions of these active sites with various types of inhibitor are now known in atomic detail. Bortezomib, as well as the newer inhibitors that are under clinical evaluation, use different chemical ‘warheads’ to covalently modify the N-terminal threonine residue in one or more of the three sites. The properties of these agents, including enzyme inhibition kinetics and pharmacological differences, are summarized in Table 2.

For the most part, these compounds inhibit the β5 sites of both the constitutive proteasome and the immunoproteasome isoforms. Differences in enzyme kinetics may differentiate these proteasome inhibitors with regards to activity and safety; the irreversible nature of carfilzomib, ONX0912 (REF. 100; 101) and NPI-0052 (REF. 102), compared with the slowly reversible binding of bortezomib and CEP18780 (REF. 104), and the more rapidly reversible binding of MLN9708 (REF. 105), might result in differences in tissue distribution, and consequently in levels of UPS inhibition within different tumour types. Similarly, differences in specificity for the three enzymatic sites of the proteasome between these compounds, C1-100-11-101-102 might also result in differences in activity in the different tumour types and in the safety of these proteasome inhibitors. Furthermore, a number of selective inhibitors of the chymotrypsin-like subunit of the immunoproteasome have been reported, including PR957 (REF. 107) and IPSI-001 (REF. 108). Because of their selectivity, these compounds or similar inhibitors might have potential as therapeutic agents for autoimmune disorders.

HECT domain

A domain of ~350 amino acids found at the C terminus of HECT E3s. The HECT domain contains a catalytic cysteine residue that accepts ubiquitin from an E2 to form a thioester intermediate before transferring ubiquitin to a substrate lysine.

U-box

A domain comprising ~70 amino acids that possesses a tertiary structure resembling the RING finger domain. The major difference is that the U-box lacks the characteristic zinc-chelating cysteine and histidine residues of the RING finger. Consequently, U-box E3s use intramolecular interactions other than zinc chelation to maintain the RING finger motif.

RING finger

A domain present in most E3s that is defined by the consensus sequence C-X3-C-H-X3-C-X3-C-H (where X means any amino acid). The RING domain coordinates two zinc ions.

molecules bound to the E1. This form of E1 is competent for transthiolation of the thioester-bound UBL to a pathway-specific E2 and is required for the downstream function of UBL conjugation.

As the enzymatic mechanism of E1s suggests, there are multiple points at which a small molecule could potentially inhibit UBL activation (Box 2). For example, the inhibition of UBL–acyl adenylate formation could be achieved by preventing ATP from binding to E1 using an ATP-competitive small-molecule inhibitor similar to those now commonly used to inhibit kinases and other ATP-dependent enzymes. Alternatively, blocking E1–UBL thioester formation could be achieved by targeting the E1 active-site thiol. Blocking transfer of an E1 thioester-bound UBL to an E2 using molecules that compete with E1–E2 binding is yet another potential mechanism of inhibition. Several strategies for inhibiting E1 function are now available and are beginning to demonstrate the feasibility of selective E1 targeting as a means to further understand UBL pathway biology (Box 2).

E2 conjugating enzymes

E2s are responsible for transferring UBLs to substrate proteins and often function with a single or limited number of E3 ligases, although in some cases no E3 is required. In total, there are approximately 40 E2s, most of which associate with UBE and are involved in ubiquitin conjugation. The other E1s typically associate with one or sometimes two E2s (FIG. 1; TABLE 1). Ubiquitin pathway E2s mediate ubiquitin chain assembly, determine ubiquitin–chain linkage topology and switch between chain initiation and elongation. Together these factors determine the fate of ubiquitylated substrate proteins depending on whether they are mono- or polyubiquitylated and on the site(s) to which ubiquitin is conjugated.

Although several studies have linked E2s from different UBL pathways to cancer and other diseases, a fundamental question remains as to how the limited number of E2s pair with the much greater number of putative E3s. Unfortunately, despite structural and biochemical analyses of E2–E3 interactions, it is not yet possible to predict which E3 functions with a specific E2 and, as potent and selective E2 inhibitors are yet to be reported, our knowledge about their functions in cells is limited. Despite these challenges, there are several emerging strategies that may yield inhibitors of E2s and these are described in Box 3, FIG. 4.

E3 ligases

Substrate selectivity of the UPS relies primarily on the specificity of the hundreds of E3 ubiquitin–protein ligases in the human genome. E3s are represented by four main classes: HECT domain E3s, U-box E3s, monomeric RING finger E3s and multisubunit E3 complexes that contain a RING finger protein (FIG. 3). These classes differ in their protein interaction domains for binding to E2s, as well as in other domains related to substrate binding. The RING finger and U-box domains have an adaptor function in bringing ubiquitin-loaded E2 and the substrate together to promote ubiquitylation, whereas HECT E3s form a thioester intermediate with ubiquitin before transfer to a substrate. Individual E3s within these classes are responsible for the recruitment of specific substrate proteins to be tagged with ubiquitin or, in some cases, another UBL. Selective E3 inhibition may therefore enable direct targeting of an aberrant signalling pathway in cancer or other diseases.

In spite of the tremendous experimental and bioinformatics efforts that have taken place in the past 15 years, the annotation of E3 ligases that are associated with human disease is still in its infancy. The RING finger ligases alone constitute one of the largest enzyme groups in the cell (exceeding the kinases). The molecular mechanisms of E3 function have been elucidated primarily from structural studies. Identifying the substrate-binding surface or related sites mediating ubiquitylation activity, including sites of assembly for components of the E3 complexes, may facilitate the discovery of small-molecule inhibitors of E3s. Indeed, recent reports of inhibitors of cullin–RING ligases (CRLs; of which SCF is an archetype, see REF. 23 for review) represent significant advances. In one example, a chemical genetics screen in yeast for enhancers of rapamycin identified an inhibitor of SCP3 that directly bound to the F-box adaptor Met30 and diminished its binding to the core cullin–RING complex in vivo. Therefore, inhibition was achieved through the apparent failure to assemble a functional SCF complex.
Table 2 | Properties of selected inhibitors of the proteasome and immunoproteasome

| Inhibitor | Development stage | Warhead | Target | Binding kinetics |
|-----------|-------------------|---------|--------|------------------|
| Bortezomib| Approved for multiple myeloma, relapsed mantle cell lymphoma | Peptide boronic acid analogue<sup>103,104</sup> | • 20S proteasome: mainly β5 (IC<sub>50</sub> values: β5 = 2.4–7.9 nM, β2 = 590–4,200 nM, β1 = 24–74 nM)<sup>99,102,103</sup> | Slowly reversible (Half-life: 110 min)<sup>103</sup> |
| Carfilzomib| Phase III (relapsed multiple myeloma) | Peptide epoxyketone<sup>99</sup> | • 20S proteasome: mainly β5 (IC<sub>50</sub> values: β5 = 6 nM, β2 = 3,600 nM, β1 = 2,400 nM)<sup>99</sup> | Irreversible |
| MLN9708 | Phase I | Peptide boronic acid<sup>103</sup> | • 20S proteasome: mainly β5 (IC<sub>50</sub> values: β5 = 3.4 nM, β2 = 3,500 nM, β1 = 31 nM)<sup>101</sup> | Rapidly reversible (Half-life: 18 min)<sup>103</sup> |
| CEP18770 | Phase I | P2 threonine boronic acid<sup>103,106</sup> | • 20S proteasome: mainly β5 (IC<sub>50</sub> values: β5 = 3.8 nM, β2 = >100 nM, β1 = <100 nM)<sup>99,102,103</sup> | Slowly reversible |
| NPI-0052 | Phase I | Non-peptide bicyclic γ-lactam β-lactone<sup>106</sup> | • 20S proteasome: mainly β5, β2 (IC<sub>50</sub> values: β5 = 3.5 nM, β2 = 28 nM, β1 = 430 nM)<sup>106</sup> | Irreversible |
| ONX0912 (formerly PR047)<sup>101</sup> | Preclinical | Peptide epoxyketone<sup>100</sup> | • 20S proteasome: β5 (IC<sub>50</sub> value = 36 nM) | Irreversible |
| PR957 | Preclinical | Peptide epoxyketone<sup>107</sup> | • 20S proteasome: not reported | Irreversible |
| IPSI-001 | Preclinical | Peptidyl aldehyde<sup>108</sup> | • 20S proteasome: β5 (K<sub>i</sub> value = 105 μM), β1 (K<sub>i</sub> value = 239 μM), β2 (K<sub>i</sub> value = 1,455 μM)<sup>108</sup> | Not reported |

In a second example, an inhibitor of SCF<sup>169</sup> was identified in a biochemical screen that disrupted binding of the F-box protein CDC4 and its substrate, phosphorylated SIC1. Structural analysis showed that the compound bound CDC4 at a site 25 Å from the SIC1 binding site, resulting in a distortion of the substrate binding pocket and therefore achieving inhibition through an allosteric mechanism<sup>25</sup>.

A third recent example demonstrates how thalidomide, a compound that was discovered over 50 years ago and was originally prescribed as a sedative until its use was discontinued, exerts its now notorious teratogenic effects. A search for primary targets of thalidomide identified cereblon (CRBN) as a thalidomide-binding protein<sup>109</sup>. CRBN forms a CRL complex with cullin 4 and the adaptor DNA damage-binding protein 1 (DDB1) that has E3 activity and is inhibited by thalidomide. Interestingly, a mutant of CRBN that does not bind thalidomide but retains E3 activity confers resistance to the teratogenic effects of thalidomide in animal models of embryonic development. This suggests that thalidomide exerts its teratogenic effects through CRBN. Together, these results not only shed light on the mechanism of thalidomide teratogenicity, but are also an example of the modulation of an E3 in a clinical setting using a small molecule (although with unintended and tragic consequences). Thalidomide is currently used clinically under strict control to treat multiple myeloma and leprosy, although the molecular target(s) in either of these contexts are unknown.

Collectively, these results indicate the feasibility of obtaining selective inhibitors of at least one important class of E3. However, difficulties remain given the overall protein subunit arrangements combined with the need for small-molecule inhibitors (generally a molecular mass of less than 1,000 Da) to bind and disrupt protein interfaces or otherwise allosterically affect ligase activity. The HECT ligases may ultimately prove to be more amenable to slow-molecule intervention as these enzymes form covalent thioester intermediates with ubiquitin before transfer to lysine residues of target proteins. TABLE 3 provides several examples of E3s with disease implications along with inhibitors or other interventions that have been studied.

**DUBs**

DUBs are proteases that perform three major functions in UBL conjugation (see REF. 12 for a review). First, as UBLs are often translated as pro-proteins or as linear fusion proteins, DUB activity is required to cleave the C termini of UBLs and generate their mature forms. Second, DUBs can remove UBLs from modified substrates, attenuating UBL signalling functions and recycling free UBLs. Third, in the case of ubiquitin, DUBs have a polyubiquitin chain editing or ‘proof reading’ function. In mammals, there are nearly 100 DUBs belonging to five different families, the majority of which are cysteine proteases; the remainder consists of a small number of zinc metalloproteases. Historically, it has been problematic identifying drug-like inhibitors of cysteine proteases, whereas the general class of metalloproteases has been more amenable to inhibition using small-molecule drugs (BOX 4). Recently, a global proteomic analysis identified 774 candidate interacting proteins associated with 75 DUBs, allowing placement of...
Rapamycin
Rapamycin (sirolimus) is a macrocyclic antibiotic produced by a bacterium isolated from soil on Easter Island. Rapamycin binds the cytosolic protein FK-binding protein 12 (FKBP12). The rapamycin–FKBP12 complex inhibits the mTOR (mammalian target of rapamycin) pathway by directly binding mTOR complex1 (mTORC1).

Cysteine proteases
This class of protease uses a cysteine thiol group in its catalytic mechanism. Deprotonation of the cysteine thiol is an intermediate of the catalytic mechanism. Attack of the peptide carbonyl carbon by the oxygen atom of a water molecule at the active site facilitates this reaction by extracting a proton from the attacking water molecule. An active site base follows by nucleophilic attack on the peptide carbonyl carbon. A thioester linking the carbon by the oxygen atom of a water molecule at the active site facilitates this reaction by extracting a proton from the attacking water molecule. An active site base facilitates this reaction by extracting a proton from the attacking water molecule. A thioester linking the carbon by the oxygen atom of a water molecule at the active site facilitates this reaction by extracting a proton from the attacking water molecule. An active site base facilitates this reaction by extracting a proton from the attacking water molecule. A thioester linking the carbon by the oxygen atom of a water molecule at the active site facilitates this reaction by extracting a proton from the attacking water molecule. An active site base facilitates this reaction by extracting a proton from the attacking water molecule. A thioester linking the carbon by the oxygen atom of a water molecule at the active site facilitates this reaction by extracting a proton from the attacking water molecule. An active site base facilitates this reaction by extracting a proton from the attacking water molecule. A thioester linking the carbon by the oxygen atom of a water molecule at the active site facilitates this reaction by extracting a proton from the attacking water molecule. An active site base facilitates this reaction by extracting a proton from the attacking water molecule.

Zinc metalloproteases
A class of protease for which the active sites include two histidine residues that coordinate a zinc ion. During catalysis, the Zn$^{2+}$ promotes attack of the peptide carbonyl carbon by the oxygen atom of a water molecule at the active site. An active site base facilitates this reaction by extracting a proton from the attacking water molecule.

Figure 2 | Mechanisms of E1 inhibitors identified in studies of different ubiquitin-like protein (UBL) pathways. a | In the first step of UBL activation, E1s bind ATP and a cognate UBL and catalyse the formation of a UBL carboxy-terminal acyl adenylate. The E1 catalytic cysteine then attacks the UBL–adenylate to form a thioester with the C terminus of the UBL. The E1 subsequently binds a second ATP and UBL, again forming a UBL–adenylate and resulting in the formation of a ternary complex consisting of an E1–UBL thioester with UBL–adenylate bound to it. This form of the E1 is fully competent to transfer thioester-bound UBL to a cognate E2 enzyme and initiate the downstream effects of UBL signalling. Small molecules, including the pyrazole derivative PYR41, JS–K and MLN4924, use distinct mechanisms to block this process at different stages of the E1 reaction cycle (see Box 2 for further details). b | Substrate-assisted mechanism-based E1 inhibition. E1s use a multistep mechanism to form a ternary complex consisting of an E1–UBL thioester (denotes a high-energy bond) with UBL–adenylate bound to it (steps 1–3). This form of E1 is competent for UBL transfer to an E2 by a transsthioleation reaction (step 4). The NEDD8-activating enzyme (NAE)-selective inhibitor MLN4924 and related adenosine sulphamate analogues are mechanism-based inhibitors of E1s and form covalent UBL–inhibitor adducts. The stability of the UBL–inhibitor adduct within the E1 active site adenylation domain blocks enzyme activity.
pathologies.

The complexity of protein ubiquitylation in signal transduction is illustrated by the fundamental role of ubiquitin in the activation of the transcription factor NF-κB, which has a key role in inflammation and immunity. There are differences in the activation of NF-κB by different ligands, that is, binding to receptors for tumour necrosis factor (TNF), interleukin-1 (IL-1) and T cell receptor, which include the use of linear and K63-linked ubiquitin chains.

The study of the multiple roles of ubiquitin in the enzymatic cascade from receptor–ligand binding to the activation of the expression of NF-κB–dependent genes is complicated by several issues. First, multiple enzymes catalyse ubiquitylation at the same target lysine in a protein. For example, the A20 (also known as TNFα-induced protein 3 or TNFAIP3) and ITCH ligases trigger ubiquitylation and degradation of the RIP kinase, a vital component of the ligand-dependent activation of NF-κB. Second, the lability of ubiquitylated species is a problem due, for example, to regulatory deubiquitylation. Third, there are different types of poly-ubiquitin chain formation at single ubiquitylation sites (lysine residues), such as K63-linked or linear chains, and some proteins also have multiple ubiquitylation sites. Fourth, there are experimental difficulties in studying ubiquitylation reactions, such as the low abundance of ubiquitylated proteins and the need to experimentally use mutated ubiquitins with non-physiological conformational changes.
The recent discovery of the importance of linear ubiquitin chains in NF-kB activation extends the complexity of the regulation of the system. A linear ubiquitin chain-assembly complex (LUBAC) conjugates head-to-tail-linked linear polyubiquitin chains to target proteins. LUBAC is involved in the physiological regulation of the canonical NF-kB pathway by binding to NEMO (NF-kB essential modulator; also known as IKKγ) and in the conjugation of linear polyubiquitin chains on to specific lysine residues of NEMO (26). The recruitment of LUBAC to the TNF receptor 1 (TNF-R1) signalling complex (TNF-RSC) is stimulation-dependent and requires TRADD, TRAF2 and cellular inhibitors of apoptosis (IAPs), but not NEMO or RIP1. It seems that LUBAC is recruited to the TNF-R1 by cellular IAP-generated ubiquitin chains and generates linear ubiquitin chains to increase the efficiency of NF-kB and Jun N-terminal kinase activation. Activation of NF-kB increases TNF-dependent gene expression, whereas activation of Jun N-terminal kinase inhibits TNF-mediated apoptosis. The attraction of NEMO to the TNF-RSC is increased by LUBAC, and the enzymatic activity of LUBAC seems to stabilize the TNF-RSC (27).

This biochemical mechanism is facilitated by the fact that the UBAN (ubiquitin binding in NEMO) motif of NEMO selectively binds linear ubiquitin chains. The specific amino-acid residues of NEMO that are involved in binding linear ubiquitin chains have been reported to be mutated in humans and result in X-linked ectodermal dysplasia and immunodeficiency (28). The binding of polyubiquitin chains to NEMO is interesting in that the C-terminal zinc finger of NEMO can bind ubiquitin as well as UBAN. Although neither UBAN nor the zinc finger show any preference for K63-linked chains, together the domains form a high-affinity K63-specific ubiquitin-binding domain. This suggests that the main function of the C-terminal half of NEMO is to specifically bind K63-linked polyubiquitin chains. The binding of NEMO to linear polyubiquitin chains is dependent on the UBAN alone and does not require the presence of the zinc finger (29). Furthermore, unanchored K63-linked polyubiquitin chains can directly activate TAK1 and IKK kinases. This indicates a new role for free ubiquitin chains in activating kinases. Cleavage of the unanchored K63-linked ubiquitin chains prevents TAK1 and IKK activation (30). Further research may show that unanchored ubiquitin chains are generated as ‘second messages’, as with cAMP.

Whatever the mechanism(s), protein ubiquitylation/deubiquitylation is at the heart of the inflammatory and immune responses, together with protein phosphorylation/dephosphorylation. Therefore, ubiquitylation should be amenable to therapeutic intervention for inflammatory and immune disorders, but at what level? Although specific E3s such as cellular IAPs are obvious targets, the E2s that are involved in K63 ubiquitin chain synthesis (for example, UBC13/UEV1A or UBCH5) or in linear chain assembly (for example, UBC5, E2-25 kDa and UBC7), might be targeted with the provision that multiple signalling pathways may be compromised by inhibitors of these enzymes. It seems that E3s use different E2s to catalyse distinct types of ubiquitin tagging. For example, RING finger IAPs seem to bind to UBC5A, UBC5C, UBC7, UBC8 and UBC13/UEV1A (31). It is also generally thought that, although E3s are the ultimate arbiters of substrate selection for ubiquitylation, the E2s determine what type of polymeric ubiquitin chain will be formed. It remains to be seen how E3s select appropriate E2s for customized polymeric ubiquitin chain assembly on specific target proteins.

In addition, the E3s are key regulators of immune functions. Several E3s, including c-CBL, CBL-b, GRAIL, ITCH and NEDD4, have been shown to negatively regulate T cell activation by contributing to T cell apoptosis. However, the HECT ligase AIP2 positively regulates T cell activation by enhancing T cell proliferation and IL-2 production to suppress apoptosis. AIP2 interacts with and promotes ubiquitin-mediated degradation of EGR2, a zinc finger transcription factor, to suppress EGR2-mediated FasL expression and activation-induced death of T cells (32). Careful selection of the appropriate E3 target for inhibition might ameliorate or block T cell activities.

The taxing biological issue with disease-related consequences is what aspect of NF-kB signalling should be clinically interrupted. For example, normal NF-kB functions are essential for the immune response against microbial infections, but continuous NF-kB activation can lead to inflammation and cancer. For this reason,
tight negative regulation of NF-κB activity occurs through mechanisms involving DUBs. The first DUB that was shown to be involved in the NF-κB system was CYLD, a tumour suppressor involved in a neoplasm called cylindromatosis. The enzyme has a great specificity for K63-linked ubiquitins and the catalytic site involved in the cleavage of K63-linked chains is often mutated in this cancer. Additionally, CYLD (like isopeptidase T) preferentially cleaves unanchored K63-linked polyubiquitin chains and therefore supports the role of these chains in protein kinase activation in the NF-κB pathway. Another DUB with a key role in the negative regulation of the NF-κB response is A20. This enzyme has dual functions in removing K63-linked chains from RIP1 and TRAF6, and uses its ubiquitin ligase activity to attach K48-linked chains to RIP1 for proteasomal degradation.

The inhibition of CYLD and/or A20 could enhance the inflammatory or immune response, but the prolonged consequences are unclear and await assessment in animal models and in early clinical trials. Alternatively, prevention of TNF-dependent NF-κB activation by linear chains through the inhibition of one or more of the E2s (UBCH5, E2-25kDa or UBCH7) may suppress NF-κB activity and inflammatory and immune responses — but again, with what long-term clinical price? For context, the use of steroids for the long-term suppression of various inflammatory conditions is routine, with generally acceptable side effects.

There might be multiple mechanisms to normally ensure the termination of NF-κB activation. However, it is clear that the intracellular ubiquitin-editing protein A20 has a key role in the negative feedback regulation of NF-κB signalling (through TNF and Toll-like receptors (TLRs)) in response to multiple stimuli. The importance of A20 in the negative regulation of NF-κB signalling through TLRs is illustrated by recent findings that A20 disrupts the ubiquitin ligase activities of TRAF6, TRAF2 and CIAP1 by preventing interactions with the E2s UBC13 and UBCH5c and with another regulatory protein, TAX1BP1, to trigger the A20-dependent ubiquitylation of the E2s and their proteasomal degradation. Recent genetic studies demonstrate that several mutations at the human A20 locus relate to immunopathologies such as Crohn’s disease, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes. The gene encoding A20 seems to be a susceptibility gene for many different immune disorders. Finally, and a caveat to any inhibition of A20, is the role of A20 as a tumour suppressor. Acute transient inhibition of A20, by promoting NF-κB activation, may be useful in resolving infections, but chronic inhibition may promote cancer.

During the innate immune response to some microorganisms, the binding of lipopolysaccharide to the TLR receptor complex TLR4–MD2–CD14 leads to the recruitment of the adaptors Mal, MyD88, TRAF6 and several IRAK kinases. TRAF6 is activated by IRAK to cause the attachment of K63 chains to both TRAF6 and IRAK. These chains are recognized by TAB2 and NEMO, leading to TAK1-mediated phosphorylation and activation of the IKK kinase complex. The A20 DUB activity is directed at TRAF6 to downregulate NF-κB activities. It is possible that inhibitors of the DUB activity of A20 may enhance antimicrobial activity in response to intracellular bacterial infections, such as Salmonella, Listeria and Typhimurium.

There are further consequences of manipulating the NF-κB system other than the regulation of inflammatory and immune responses. The system is intricately linked to cell death. For example, internalization of the TNFR1 complex can give rise to further complexes in the cytosol that are dependent on the TRADD component of the TNFR1 complex (to give complex IIA) or RIP1 (to give complex IIB). Complex IIA formation involves the recruitment of the protein Fas-associated death domain (FADD) and caspase 8 to trigger apoptosis, whereas complex IIB is formed when the FADD pathway is blocked by, for example, viral proteins or chemical inhibitors of apoptosis, and mediates cell death by
necroptosis. In this pathway, complex IIB contains both RIP1 and RIP3, which by mutual co-phosphorylation can activate enzymes involved in metabolism. Activated RIP3 increases the activities of enzymes involved in glycolysis and mitochondrial oxidative phosphorylation and, in some cases, can cause the production of reactive oxygen species to mediate necrotic cell death. It is assumed that complex IIB contains deubiquitylated RIP caused by CYLD and A20. Necroptotic cell death may therefore be compromised by inhibition of these DUBs, for example, in virally infected cells, although complex IIA-dependent apoptotic cell death may still operate.32,43. Again, to show the complexity of these cellular responses to various stresses, the necrostatin inhibitors of necroptosis44 can reduce tissue damage in brain ischaemia and myocardial infarction45. Therefore, it is possible that CYLD and A20 inhibitors may prevent tissue necrosis in appropriate clinical situations, such as following stroke and heart attack, for which it is currently difficult to limit tissue damage.

Bone disorders. The roles of NF-κB in inflammation and the immune response, and therefore the usefulness of ubiquitin pathway inhibitors, could be extended to osteolytic disorders. Osteoclasts are responsible for bone resorption and have a pivotal role in the pathogenesis of osteolytic disorders. NF-κB signalling pathways are strictly regulated to maintain bone homeostasis through cytokines, such as RANKL, TNF-α and IL-1, which differentially regulate classical and/or alternative NF-κB pathways in osteoclastic cells. Abnormal activation of NF-κB signalling in osteoclasts has been associated with excessive osteoclastic activity, and is frequently observed in osteolytic conditions, including periprosthetic osteolysis, arthritis, Paget’s disease of bone and periodontitis. NF-κB modulators such as parthenolide and NEMO-binding domain peptide demonstrate therapeutic effects on inflammation-induced bone destruction in mouse models46. Therapeutic intervention in the activities of the UPS may slow disease progression of osteolytic disorders that cause debilitating disease in the ageing population, for example, Paget’s disease of bone.

The UPS and heart disease. The role of the UPS in cardiac function and disease is again complex and sometimes conflicting. Proteasome inhibitors have been used in the experimental manipulation of heart disease. The outcomes so far indicate, as might be expected, that the effects of proteasome inhibitors are concentration dependent, with lower concentrations mediating cytoprotective effects and higher concentrations being toxic. For example, studies on non-toxic proteasome inhibition that cause debilitating disease in the ageing population, for example, Paget’s disease of bone.

Table 3 | Selected E3 ligases, substrates, associations with human disease and mechanistic targeting

| E3 ligase | Substrate | Disease association | Inhibitor/intervention investigated |
|----------|-----------|---------------------|------------------------------------|
| MDM2 (HDM2) | p53, p27 | Multiple cancers, including breast and lung, oesophageal carcinomas, glioblastomas and malignant melanomas | Nutlins14 and RITA15 to promote p53 stabilization; parthenolide to promote MDM2 ubiquitylation16 |
| CRL | p21, p27, cyclin D, β-catenin14, kBa15 | Multiple cancers and other disorders linked to the NF-κB pathway | NAE inhibition results in loss of NEDD8 modification of cullin proteins and inactivation of CRL activity |
| IAP | Various substrates involved in apoptosis and signalling | Various cancers, including oesophageal, liver and lung, ovarian carcinoma and MALT lymphoma | SMAC mimetics neutralize IAP inhibition of caspase-induced apoptosis and promote IAP auto-ubiquitylation and degradation |
| XIAP | AIF, MEKK2, TGFβ-activated kinase | TGFβ stimulation of NF-κB in breast cancer; acute myeloid leukaemia14,15 | Triptolide induction of apoptosis in XIAP-overexpressing AML cells14; antisense compound AEG35156 (REF. 156) |
| E6-AP | p53 | Cervical cancer through HPV16/18 infection; mutations associated with Angelman’s syndrome14,15 | Expression of mRNA decay factor TTP stabilizes p53 through E6–AP targeting in HPV-transformed cervical cancer |
| VHL | HIF1α, HIF2α | VHL syndrome; inactivation associated with renal cell carcinoma14,15 | Bioengineered VHL protein to increase HIF degradation14 |
| Parkin | Synphilin 1, Parkin | Parkinson’s disease | Nitric oxide inhibits E3 activity |
| BM11/RING1A | TOP2A | B and T cell leukaemias, various other cancer cell lines | PRT4165 inhibits BM11/RING1A activity and enhances potency of TOP2 drugs |

AIF, apoptosis-inducing factor; AML, acute myeloid leukaemia; CRL, cullin-RING ligases; HIF, hypoxia-inducible factor; HPV, human papillomavirus; IAP, inhibitor of apoptosis; MALT, mucosa-associated lymphoid tissue; NAE, NEDD8-activating enzyme; NF-κB, nuclear factor-κB; TGF, transforming growth factor; TOP2A, topoisomerase (DNA) II alpha; TTP, tristetraprolin; VHL, von Hippel–Lindau.

Autophagy

Literally means ‘self-eating’, a highly regulated catabolic process in which cellular proteins and organelles are sequestered in a characteristic double-membrane vesicle called an autophagosome and are then degraded following vesicular fusion with a lysosome.

Endosome–lysosome pathway

Endosomes are membrane-bound vesicles that are involved in protein transport between the plasma membrane, Golgi and lysosomes. In the endocytic pathway, internalized molecules are delivered to early endosomes, where efficient sorting occurs. Some molecules, including recycling receptors, are shuttled back to the plasma membrane to be reused. Others, including downregulated receptors, are transported to late endosomes and lysosomes for degradation.
UBL-activating enzymes

A conserved asparagine residue in E2s (exemplified by asparagine 79 in UBC13) has been shown to be crucial for UBL transfer and is thought to form the oxyanion hole required to stabilize the E2 thioester–substrate transition state intermediate. Targeting this site with small-molecule effectors may offer yet another approach for inhibition. Ub, ubiquitin.

Block E2~UBL thioester formation by targeting E3-dependent E2 allosteric activation. In some cases, proteins within the inclusions are ubiquitylated. As in cerebrovascular accidents in the brain, two different processes might be controlled by the proteasome: upregulation of the expression of gene products that ameliorate oxidative damage in surviving cardiomyocytes by proteasome inhibition, and the activation of cardiomyocyte death pathways. The clinical outcome will be dependent on the balance between these proteasome (and general UPS) activities. Caution is needed clinically, as cardiotoxicity has been reported in patients treated with the proteasome inhibitor bortezomib.

More understanding of the UPS in the pathophysiology of heart disease is needed to contemplate new routes for therapy. It seems that pressure overload, ischaemic heart disease or genetic mutations in contractile proteins that cause heart failure are accompanied by elevation in levels of misfolded proteins, which may be removed by the UPS and through autophagy. However, proteasome inhibitors may be useful for the treatment of cardiac hypertrophy and ischaemic heart diseases. A complication is that impaired proteasome function is commonly associated with myocardial ischaemic injury, but recent evidence also supports a cardioprotective role for proteasome inhibitors in myocardial ischaemia. The loss of cardiomyocytes is a key problem in the development of cardiovascular disease. Two main processes mediate cardiomyocyte loss: necrosis and apoptosis. In contrast to necrosis, apoptosis is a relatively well-understood, regulated process that is essential in normal development and tissue homeostasis. Tight regulation of both processes is crucial, especially in post-mitotic cells lacking regenerative capacity, such as cardiomyocytes and neurons.

The UPS is involved in the regulation of cardiomyocyte apoptosis and possibly necrosis.

As in cerebrovascular accidents in the brain, two different processes might be controlled by the proteasome: upregulation of the expression of gene products that ameliorate oxidative damage in surviving cardiomyocytes by proteasome inhibition, and the activation of cardiomyocyte death pathways. The clinical outcome will be dependent on the balance between these proteasome (and general UPS) activities. Caution is needed clinically, as cardiotoxicity has been reported in patients treated with the proteasome inhibitor bortezomib.

In spite of these complexities, consideration should be given to the use of proteasome inhibitors and other modulators of the UPS for viral myocarditis. The primary intracellular protein degradation systems, both the UPS and autophagy, seem to regulate successive stages of viral infectivity. Viral myocarditis, such as that caused by coxsackievirus B3, progresses in three distinct stages: acute viral infection, immune cell infiltration and cardiac remodelling. The UPS has a central role in each of these stages of viral infection and might be modulated to slow viral disease progression and heart disease.

**Chronic neurodegenerative disease.** As expected, ubiquitin-dependent processes, including the degradation of ubiquitylated proteins by the 26S proteasome, by autophagy and in the endosome–lysosome pathway, have central roles in neuronal development, homeostasis and disease. These catabolic systems are essential for neuronal activities, including synaptogenesis, cell–cell interactions (for example, neuromuscular junction formation) and synaptic plasticity. In the adult central and peripheral nervous systems, which are based predominately on non-dividing cells, protein ubiquitylation and deubiquitylation are essential for neuronal survival. Most age-related chronic neurodegenerative diseases involve the accumulation of proteins in aggregates, frequently as paranuclear inclusions (FIG. 6). In every case, proteins within the inclusions are ubiquitylated.
The reasons are incompletely understood but seem to be the result of malfunction or overwhelming of the activities of neuronal 26S proteasomes, autophagy and the endosome–lysosome pathway.

This is shown by experimental genetic findings. PARKIN encodes an E3 that ubiquitylates itself and the α-synuclein interacting protein, Synphilin 1. Familial associated mutations in PARK2/Parkin that are defective for E3 activity are linked to autosomal recessive Parkinson’s disease. Regional ablation of neuronal 26S proteasomes in the brain by deletion of a 26S proteasomal ATPase gene causes the neuropathological hallmarks of Parkinson’s disease and dementia with Lewy bodies. Proteasomal dysfunction may be the primary cause of neurodegenerative disease. Genetic deletion of autophagy genes in the brain causes neurodegeneration with the accumulation of deposits of ubiquitylated proteins, although not with the human hallmark neuropathology. The degradation of ubiquitylated proteins by the 26S proteasome and autophagy is essential for neuronal homeostasis. When either or both protein–catabolic processes are compromised, neurodegeneration ensues.

The normal functioning of the endocytic and multivesicular body (MVB) pathway for receptor internalization and degradation is also essential for neuronal homeostasis, as it seems to contribute to normal autophagic activity. Mutations in the endosomal sorting complex required for transport (ESCRT)-III subunit CHMP2B are associated with frontotemporal dementia and amyotrophic lateral sclerosis. Efficient autophagic protein degradation requires functional MVBs. The ESCRT machinery delivers ubiquitylated proteins into invaginations of endosome membranes. The ESCRT machinery then mediates the breaking off of cargo-containing intraluminal vesicles from the perimeter membrane to form MVBs, which may fuse with lysosomes to cause degradation of their protein content. Defects in this pathway would explain the observed neurodegenerative phenotype seen in patients with CHMP2B mutations.

To extend the cell biological context of neurodegeneration further, it is apparent that the unfolded protein response (UPR) in the endoplasmic reticulum (ER) is connected to autophagy through the key UPR transcription factor XBP1. Genetic XBP1 deficiency causes a significant decrease in the toxicity of mutant SOD1 aggregates (which cause amyotrophic lateral sclerosis) due to an enhanced clearance by autophagy. These data indicate extensive cross-talk between the ER-associated UPR and autophagy to provide protection against neurodegeneration. The malfunction of the UPR in the ER probably triggers autophagy to remove distended ER from the neuron.

Finally, chain-specific protein ubiquitylation has a role in neurodegeneration as K63-linked polyubiquitylation has been detected within pathological lesions of patients with Alzheimer’s, Huntington’s and Parkinson’s disease. Immunoreactivity to K63 chains is also a feature of inclusions in neurons of proteasome-depleted mice, suggesting a proteasomal contribution to the degradation of K63-linked polyubiquitylated proteins in vivo or that K63 polyubiquitylation has a role in inclusion biogenesis.

Chronic neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, dementia with Lewy bodies and amyotrophic lateral sclerosis, are considered to be ‘proteinopathies’ associated with the intraneuronal accumulation of insoluble protein aggregates in surviving neurons (inclusions) and extracellular amyloid deposits. In Alzheimer’s disease, the aggregates contain intraneuronal tau protein and extraneuronal amyloid β-protein (Aβ).

Lewy bodies

Lewy bodies are abnormal protein aggregates that develop inside nerve cells in Parkinson’s disease and Alzheimer’s disease and some other disorders. They are identified when histology is performed on the brain and appear as spherical masses that displace other cell components.
Although it is tempting to represent a general consensus, some enzymological steps are controversial due, for example, to enzymological redundancy. The role of ubiquitin (Ub) in signalling to NF-κB needs considerably more investigation. See the main text for more details. DUB, deubiquitylating enzyme; LUBAC, linear ubiquitin chain–assembly complex.

Figure 5 | Ubiquitin and signalling to nuclear factor-κB (NF-κB). a | Tumour necrosis factor-α (TNF-α) signalling. b | Interleukin-1 (IL-1) receptor 1 (IL1R1) and Toll-like receptor signalling. The diagrams are based on recent reviews. Although it is tempting to represent a general consensus, some enzymological steps are controversial due, for example, to enzymological redundancy. The role of ubiquitin (Ub) in signalling to NF-κB needs considerably more investigation. See the main text for more details. DUB, deubiquitylating enzyme; LUBAC, linear ubiquitin chain–assembly complex.

amyloid-β fragments of the Alzheimer precursor protein. In Parkinson’s disease and dementia with Lewy bodies, there are intraneuronal deposits of α-synuclein. However, the seminal feature of all these diseases is regional extensive neuronal loss in the brain and spinal cord. It has been difficult to recapitulate these features of the diseases using mouse transgenesis to overexpress amyloidogenic proteins in the brain. These approaches have led to intraneuronal aggregates and extraneuronal deposits but not extensive neurodegeneration. By contrast, gene targeting to knock out a proteasomal gene and autophagy genes has resulted in the accumulation of intraneuronal inclusions containing ubiquitylated proteins (some resembling human disease) and extensive regional neuronal loss or neurodegeneration.

Currently, it seems that age-related chronic neurodegenerative disease can be directly attributed to some dysfunction of the UPS and/or autophagy. Any malfunction of these key neuronal processes for regulated removal of proteins results in molecular neuropathological features of neurodegenerative disease. Promotion of the degradation of ubiquitylated proteins by 26S proteasomes (for example, with 26S proteasome activators such as inhibitors of the proteasomal deubiquitylating enzyme USP14 or by autophagy (for example, with mTOR (mammalian target of rapamycin) inhibitors) would facilitate slowing or prevention of chronic neurodegenerative disease.

The regional genetic ablation of a proteasomal ATPase gene causes a reduction in neuronal 26S proteasomes, which is a neuropathological feature of both Parkinson’s disease and dementia with Lewy bodies, and extensive neuronal death accompanied by some features of neuronal apoptosis. The characterization of the neuronal signal transduction pathways that control cell death in these models by approaches such as analyses of gene expression changes by microarray hybridization and proteomics will delineate these pathways and the downstream consequences of changes in these signalling pathways. Therefore, further studies of the consequences of genetic deletion of neuronal 26S proteasomes (and autophagic functions) will identify receptor and enzyme targets for novel therapeutic developments to slow neuronal death and neurodegeneration. An example of a novel signalling target is the discovery that mutations in optineurin cause amyotrophic lateral sclerosis. Optineurin antagonizes the activity of NEMO by similarly binding to ubiquitin.
Many of which develop from glial cells or astrocytes. Tumours. There are nearly 100 types of brain tumour, neuronal 26S proteasomes and extensive neuronal loss, it proteasomal ATPase gene causes a depletion of neuronal UPS and autophagy pathways (which belong to the AAA (ATPases of alternative activities) superfamily) might be useful for the treatment of brain neuronal tumours and, as all cells contain 26S proteasomes, glial tumours and other tumour cell types too. Such an approach offers a new 26S proteasome target that is independent of inhibiting prot

Brain tumours. Brain tumours (including aggressive paediatric tumours) are essentially intractable to current therapies without major side effects (for example, developmental disorders). There is therefore a particular need for novel therapeutic advances in this area. As it is clear that the regional targeted ablation of a neuronal proteasomal ATPase gene causes a depletion of neuronal 26S proteasomes and extensive neuronal loss, it is worth considering intervention in the UPS in brain tumours. There are nearly 100 types of brain tumour, many of which develop from glial cells or astrocytes. The expression of optineurin to inhibit NF-κB activity might be useful in the treatment of this debilitating and essentially untreatable disease.

Viruses and bacteria. Viruses and bacteria use several strategies to block the UPS and to manipulate specific aspects of the system for infection and replication. As exemplified by the classical example of human papillomavirus encoded E6 oncoprotein, which binds and redirects the E3 ligase E6-AP to target p53 to the proteasome, pathogens can be adept at manipulating host-encoded enzymes. Alternatively, pathogen-encoded enzymes that are specific for ubiquitin or UBLs, such as proteases, seem to be a common feature shared by many viruses, bacteria and protozoa. For example, pathogens can code for orthologues of enzymes of the UPS, such as an E2 (REF. 76), or express their own novel analogues of UPS enzymes, such as ubiquitin protein ligases. Some of the proteases share a common origin with mammalian cell-encoded enzymes, but most of them have ancient intrinsic functions, such as processing pathogen protein components, and may have acquired the specificity for ubiquitin or UBLs by interacting with mammalian hosts (and their immune system) throughout evolution. Many of the pathogen-encoded proteases are different from their mammalian counterparts and are therefore attractive targets for drug development to combat infectious diseases. One example of viral manipulation of the UPS is provided by the Ebola Zaire virus VP35 protein. This viral protein interacts with the transcription factor IRF7, which is required for interferon gene expression, and also interacts with the SUMO E2 enzyme UBC9 and the E3 ligase PIAS1. This interaction increases PIAS1-mediated SUMOylation of IRF7, which represses expression of the interferon gene. By contrast, VP35 does not interfere with the activation of NF-κB, which is required for the induction of many pro-inflammatory cytokines that are needed for viral
infection. These SUMO-related events are part of the mechanism that causes rapid overwhelming infection and eventually pathology such as septic shock. A second example is provided by the HIV virus, which uses MVB proteins to egress from the cell and capitalizes on different stages of the autophagic process to mature. Early, non-degradative stages of autophagy increase HIV yield. HIV Gag-derived proteins co-localize and interact with the autophagy factor LC3 to cause autophagy-promoted productive Gag processing. Later, the HIV protein Nef acts as an anti-autophagic maturation factor through interactions with the autophagy regulatory protein Beclin 1, thereby protecting HIV from degradation.

The ISG15 protein was one of the first gene products shown to function as an innate immune protein with broad-spectrum antiviral activity. Protein ubiquitylation might be productive for some RNA viruses but ISGylation is antiviral. A better understanding of the antiviral activities of ISG15 could provide useful insight into the development of novel therapeutic approaches to improve the immune response against such pathogens.

Observations of intracellular bacteria, protein ubiquitylation and autophagy have revealed that autophagy of ubiquitylated proteins requires the p62 protein (also known as SQSTM1), which is an adaptor protein with a C-terminal ubiquitin-associated domain for binding to ubiquitylated proteins and an LC3 interaction region for binding the autophagosome protein LC3. Ubiquitylated proteins on the surface of the intracellular bacterium *Salmonella enterica* serovar Typhimurium recruit p62 and cause the autophagic engulfment of the bacteria. This work demonstrates that the detection of bacteria (and almost certainly of misfolded proteins) occurs by a conserved pathway and that p62 has a role in innate immunity. Additionally, the NDP52 protein, not previously known to contribute to innate immunity, recognizes ubiquitin-coated S. Typhimurium in human cells (in a similar way to p62) and binds the adaptor proteins Nap1 and Sin3bad to recruit Tank-binding kinase 1, a non-canonical member of the IKK kinase family of enzymes. The NDP52 protein also recruits LC3 to activate autophagy against bacteria attempting to colonize the cytoplasm.

Bacteria have evolved mechanisms to evade autophagy. For example, the bacterium *Listeria monocytogenes* efficiently escapes autophagy by recruiting the host Arp2/3 complex and Ena/VASP via the bacterial ActA protein to the bacterial surface to disguise the bacteria from ubiquitylation, p62 binding and autophagic sequestration. Significantly, the ability of ActA to mediate protection from ubiquitylation was elegantly demonstrated by generating a GFP–ActA–Q79C chimera, consisting of GFP (green fluorescent protein), the ActA protein and segments of aggregate-prone polyQ (from the Huntington’s disease protein). GFP–ActA–Q79C forms aggregates in the host cell cytoplasm. However, these ActA-containing aggregates are not targeted for ubiquitylation and p62 recruitment. One or more steps in these pathways may be subjected to therapeutic intervention to slow or prevent viral and bacterial replication in human cells.

Although few bacteria have *bona fide* proteasomes and none has ubiquitin, one very important bacterial pathogen, *Mycobacterium tuberculosis* (Mtb), does contain a proteolytic system that is analogous and orthologous to the UPS. With estimates of 1.3 million deaths per year attributable to this pathogen, there is an urgent unmet medical need for new drugs to target it. Because the prokaryotic ubiquitin-like protein (Pup)–proteasome system of Mtb is essential for pathogenesis, the various components of the system present excellent opportunities for therapeutic intervention. These include the enzymes involved in ‘pupylation’ of substrates, analogous to the ubiquitin E1–E2–E3 cascade, as well as the ‘depupylase’ activity that was recently described. The AAA ATPase complex that regulates the Mtb proteasome could also be targeted. Considerable work has already gone into characterizing active site specificity of the Mtb proteasome to find inhibitors that could selectively block the function of the Mtb proteasome without inhibiting the proteasome of the (human) host.

**Future directions and conclusion**

The fundamental role of the UPS and UBL conjugation pathways in normal cell function and in disease prompts the search for inhibitors that selectively disrupt pathway function. Despite our limited understanding of the molecular mechanisms of pathway targets, the inhibition of pathway enzymes is an attractive, and increasingly tractable, approach to targeting aberrant signalling pathways in multiple cancers and other diseases. Therapeutic intervention in the UPS, the endosome–lysosome system, the autophagic system and in UBL signalling is an emerging area for the treatment of acute and chronic human diseases, including the treatment of viral and bacterial infections. Some cancers and other disease settings in which these pathways are constitutively active have been shown to be more sensitive to inhibition, potentially limiting the side effects of inhibiting all or part of these key cellular systems.

The precedent for UPS inhibition in cancer has been established by the proteasome inhibitor bortezomib, and second-generation inhibitors are now in clinical development with the aim of improving drug pharmacology. Beyond its use in multiple myeloma and mantle cell lymphoma, the most exciting new therapeutic application of bortezomib is as a drug to prevent antibody-mediated rejection of renal allografts in transplant patients by depleting normal antibody-producing plasma cells. In addition, MLN4924 has established the precedent for inhibiting a specific E1 target, with mechanistic studies indicating the possibility of such an approach for other E1s. Understanding the molecular mechanisms involved in the interactions seen at each step of the UPS and UBL conjugation pathways, between E1s, E2s, E3s, substrates, DUBs and the proteasome itself, is increasing through the use of tools such as small-molecule inhibitors and small interfering RNA, improved compound screening strategies and crystal structure studies. Consequently, there are opportunities present and emerging for other novel therapeutics that target numerous specific pathways in the UPS and UBL conjugation pathways.
The ubiquitin–proteasome system (UPS) is a highly conserved protein degradation machinery that plays a central role in various cellular processes, including gene expression, intracellular signaling, and cell survival. The UPS is composed of several components: ubiquitin, a ubiquitin-conjugating enzyme (E2), and an E3 ubiquitin ligase. E3 ligases recruit and activate ubiquitin by transferring it from an E2 to a target protein, which then undergoes degradation by the proteasome.

E3 ubiquitin ligases are classified into different families based on their structures and functions. One such family is the RING finger domain-containing E3 ubiquitin ligases, which are characterized by a RING finger domain. Members of this family, such as the Nedd4 family, play crucial roles in cell survival, proliferation, and response to stress.

Another important family of E3 ubiquitin ligases is the HECT-domain containing E3 ligases, which include the Parkin protein. Parkin is a ubiquitin ligase that is involved in the clearance of aggregating proteins, and its dysfunction is associated with the development of neurodegenerative diseases.

The UPS is highly regulated and can respond to various cellular signals to adjust the rate of protein degradation. This regulation is achieved through the activity of deubiquitinating enzymes (DUBs), which can remove ubiquitin from target proteins, thereby reversing ubiquitin-mediated protein degradation.

Understanding the UPS is crucial for the development of new therapeutic strategies for various diseases, including neurodegenerative diseases. The UPS is a complex system, and further research is needed to fully understand its cellular functions and mechanisms of action.
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