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Review

Therapeutic strategies within the ubiquitin proteasome system

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The ubiquitin-dependent proteolysis system (UPS) is the main driver of regulated protein degradation in all eukaryotic cells, and it is becoming increasingly clear that defects within this pathway drive a large number of human pathologies. Recent success in the use of proteasome inhibitors in the treatment of hematological malignancies validates the UPS as a viable therapeutic pathway, and substantial effort is now focused on the development of both second-generation proteasome inhibitors as well as novel strategies for the inhibition of upstream UPS enzymes. In this review we discuss the potential 'druggability' of key nodes within the UPS and summarize recent advances within the field.
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It is now widely appreciated that the UPS plays a critical role in regulating a wide variety of cellular pathways, including cell growth and proliferation,[1] apoptosis,[2] protein quality control,[3,4] DNA repair,[5] transcription,[6] and immune response.[7,8] Moreover, defects in these pathways have been implicated in a number of human pathologies, most notably in cancer[9] and neurodegenerative disease.[10,11] With few exceptions, destruction of cellular proteins by the proteasome is gated by their ubiquitination; covalent modification by ubiquitin chains acts as a sort of 'tag' signaling their proteolysis. The UPS is conserved throughout eukaryotes (recent work identified a similar covalent modification system in prokaryotes as well[12]) and employs a common, regulated enzyme cascade consisting minimally of E1, E2, and E3 activities (Figure 1). In brief, a ubiquitin-activating enzyme (E1) uses energy from ATP hydrolysis to generate a high-energy thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a catalytic cysteine residue within the E1 itself. This activated ubiquitin is then transferred to an active site cysteine residue within one of several dozen ubiquitin-conjugating enzymes (E2). Finally, the charged E2 enzyme cooperates with one of hundreds of ubiquitin ligases (E3) to transfer the activated ubiquitin to the ε-amino group of a lysine residue within a target protein. In most cases studied thus far, this initial ubiquitin serves as an acceptor for further cycles of ubiquitin modification, generating a polyubiquitin chain. Subsequent ubiquitin addition can occur through isopeptide linkage on all of ubiquitin's seven lysine residues[13] as well as its amino-terminal primary amine,[14] thereby generating a diverse range of chain topologies that can drive a variety of different protein fates. In the canonical degradative pathway, chains linked through ubiquitin Lys48 target the ubiquitinated substrate for destruction at the proteasome, a multi-subunit barrel-shaped cellular protease containing activities that recognize and unfold proteolytic targets as well as mediate their degradation. Chains linked through Lys63 are not generally thought to be targeted for destruction, but rather serve as signals to modulate the activity of diverse pathways such as NF-κB activation[2] and DNA repair.[15] As with Lys63 chain formation, addition of a single ubiquitin monomer (termed monoubiquitination) acts in a signaling capacity, with roles in histone function, endocytosis and membrane trafficking, and DNA replication and repair.[16] Recently, linear polyubiquitin chains, generated by the Hoil-1L/Hoip heterodimeric ligase (also called LUBAC), have been validated as a novel linkage that targets substrates to the proteasome[17] and functions in activation of the NF-κB pathway.[14] Finally, there are nearly 100 deubiquitinating enzymes that counter the activity of E3 ligases in regulatory pathways, as well as functioning in ubiquitin maturation and ubiquitin cleavage and recycling at the 26S proteasome.[18,19]

From a therapeutic standpoint, the surprising efficacy (and rapid clinical approval) of the proteasome inhibitor Bortezomib (PS-341, Velcade) for treatment of multiple myeloma and mantle cell lymphoma has emboldened researchers to explore the possibility of targeting other components of the UPS. The conventional wisdom is that proteasome inhibition, though effective in some tumors, will ultimately be limited by a narrow therapeutic window (poor selectivity in inducing cell death in tumor cells *versus* normal cells). Clearly the 'holy grail' is to develop inhibitors that target specific ubiquitination pathways that are essential for tumor cell growth but not for

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Abbreviations: UPS, ubiquitin proteasome system; E1, ubiquitin activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; DUB, deubiquitinating enzyme; HECT, Homologous to E6AP C-terminus; RING, Really Interesting New Gene; SCF, Skp1-Cullin-Fbox; PHD, plant homeodomain; Ub, ubiquitin; APC/C, Anaphase Promoting Complex/Cyclosome; UBL, ubiquitin-like protein; USP, ubiquitin-specific protease; Protac, proteolysis targeting chimeric molecule
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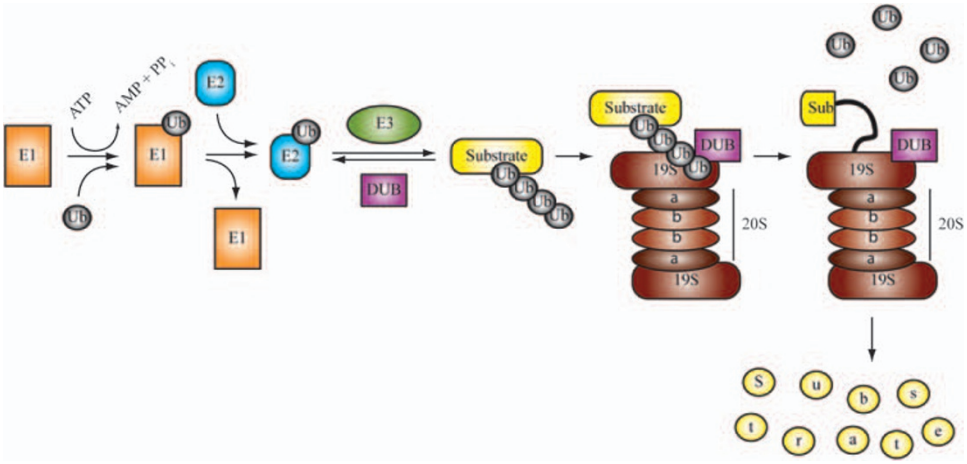

Figure 1 Major enzymatic components of the ubiquitin proteasome pathway (UPS). Ubiquitin is activated and conjugated to target proteins by a conserved series of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) activities. In some cases, an isopeptidase or deubiquitinating enzyme (DUB) may oppose the activity of the E3. Polyubiquitinated proteins are recruited (via ubiquitin receptors) to the 26S proteasome, a multi-subunit, barrel-shaped cellular protease consisting of a 20S core particle bound at one or both ends by 19S cap particles. This 19S cap confers both ATP-and ubiquitin-dependency to proteolysis by the 26S proteasome, and contains isopeptidase activities that remove ubiquitin from the substrate for recycling and ATPase activities that unfold the substrate and feed it into the 20S core for degradation

normal cell growth, which could presumably widen this therapeutic window. As substrate selection in the UPS is driven by the ubiquitin ligases, the majority of research efforts have focused on elucidating the substrates and biology surrounding these enzymes, and a number of ligases have been implicated as therapeutic targets (Table 1). There are several different classes of ubiquitin ligases with very different catalytic mechanisms, which present both unique opportunities and challenges with regard to identifying and developing small molecule inhibitors. In this review, we will summarize the key catalytic steps utilized by the major subtypes of ubiquitin ligases and provide examples of strategies that have or could be used to develop inhibitors. We will also briefly summarize progress in developing inhibitors of non-E3 members of the UPS.

HECT Domain Ligases as Drug Targets

Potentially the most straightforward ubiquitin ligases to inhibit with small molecules are the HECT domain ligases. There are approximately 30 HECT domain ligases in the human genome, all marked by the presence of a conserved HECT (homologous to E6AP C terminus) domain at their extreme carboxyl terminus. The HECT domain contains a catalytic cysteine residue that, much like the E1 and E2 enzymes, accepts the charged ubiquitin from the E2 and directly transfers it to a substrate. Although no HECT domain ligase inhibitors nor high-throughput screens to identify such inhibitors have yet been reported, it is easy to conceive of a small molecule that could occupy the active site and block nucleophilic attack by the HECT catalytic cysteine on the E2-ubiquitin thioester bond. However, currently available structural information reported for the E6AP,[20] WW1/AIP5,[21] Smurf2,[22] and Nedd4L (PDB record 2oni) HECT domains is ambiguous concerning the druggability of this active site pocket. For example, a potentially druggable cleft surrounds the catalytic cysteine in WWP1, lying within a long groove formed by the interface between the amino-terminal and carboxyl-terminal halves of the HECT domain (Figure 2a).[21] The equivalent pocket in E6AP is largely closed off due to a rotation and flexion of the carboxyl-terminal region of the HECT domain within a hinge region.[20] This conformational flexibility (discussed below) suggests that the static images seen in these crystal structures only represent a few of the many potential conformations that are adopted during ubiquitin transfer, and it remains possible that a more chemically attractive cleft may form during the ubiquitination cycle, providing a transient interaction surface that is amenable to targeting with small molecules.

Indeed an alternative strategy for inhibiting HECTs is to exploit these conformational changes to identify noncompetitive, allosteric inhibitors that block transition to one of the catalytic intermediates. Each of the HECT domain structures shows the HECT subdomains (referred to as the N lobe and C lobe) in different orientations with respect to one another, primarily due to a 100° rotation about a flexible hinge region that links the N and C lobes (Figure 2b). Moreover, the structures predict the HECT domain cysteine to be surprisingly distant from the active site of the ubiquitin-charged E2 enzyme (ranging from 16Å in the WWP1 structure to 50Å in the Smurf2 structure), raising doubts that a simple transthiolation reaction could be possible without significant conformational changes within the HECT domain. It was thus proposed that each of these structures may represent intermediate conformations that the HECT domain samples during a catalytic cycle. Flexibility within the hinge would allow the C lobe, which contains the active site cysteine, to first adopt an E2-facing conformation where it accepts the charged ubiquitin from the E2 enzyme via transthiolation, and then swivel to adopt a substrate-facing conformation making the HECT-ubiquitin bond accessible for nucleophilic attack by the target lysine residue. In support of this model, mutation of residues within this primary hinge region to proline, which should generate a more rigid, constrained structure, significantly

Table 1 A sampling of candidate drug targets within the UPS, possible therapeutic indications, and progress in developing small molecule inhibitors (where available)

	Target	Substrate/pathway	Indications	Published inhibitors	References
E3 – HECTs	Nedd4	TM receptor stability (eNaC, IGF-1R, VEGF-R2)	Prostate, bladder cancer	N/A	73
	Smurf2	TGFβ signaling	Esophageal squamous cell carcinoma	N/A	73
	WWP1	KLF transcription factors, TGFβ signaling?	Prostate, breast cancer	N/A	73
	ARF-BP1/ HECTH9	p53, c-Myc	Breast cancer, lymphomas	N/A	74,75
	E6AP	p53 (in conjugation with HPV E6 oncoprotein)	Cervical cancer	N/A	76
E3 – RINGs (single subunit)	Mdm2	p53	AML, multiple myeloma	Nutlin (Roche)	38
	TRAF2		Unknown	N/A	2
	GP78/AMFR	ER-associated degradation (ERAD)	Unknown	N/A	3
	MuRF1	Muscle wasting (Titin)	Cachexia	N/A	77
E3 – RINGs (multi-subunit)	SCF^{Skp2}	$p27^{Kip1}$	Unknown	N/A	1
	$SCF^{\beta TrCP}$	IκBα	Unknown	N/A	2
Other targets	Ubiquitin E1	Ubiquitin-dependent pathways	Unknown	PYR-41	49
	Nedd8 E1	Cullin subunits of multi-subunit E3s (SCF)	Unknown	MLN4924 (Millennium)	54
	UbcH10 (E2)	Mitotic/G_1 targets (with APC/C E3 ligase)	Breast, lung, bladder, ovarian cancers	N/A	78
	USP7/Hausp	Mdm2, p53, FOXO4	$p53^+$ cancers	N/A	79–81
	USP2a	Fatty acid synthase	Prostate cancer	N/A	82,83
	AMSH/STAM	EGFR	Unknown	N/A	84
	USP20	Hif1a	Renal clear cell carcinoma	N/A	85
	USP28	c-Myc	Colon, breast carcinoma	N/A	86
	Proteasome	Degradative ubiquitination	Multiple myeloma, mantle cell lymphoma	Bortezomib (PS-341, Velcade), PR-171 (Proteolix), NPI-0052 (Nereus)	87,88

N/A, none available. Due to space constraints we are unable to discuss each in detail and direct the reader to the cited reviews and primary literature

impaired WWP1 autoubiquitination activity.[21] Although this hypothesis remains tentative at this point, it suggests the intriguing possibility that an allosteric inhibitor that blocks adoption of any of these structural intermediates could efficiently inhibit ubiquitin transfer.

Although targeting protein–protein interactions with small molecules is notoriously difficult, a final route to HECT domain inhibition would be to target the E2–E3 interface. The crystal structure of E6AP bound to the E2 UbcH7 shows a relatively small, focused interaction surface, with main contacts coming from hydrophobic residues in loops L1 and L2 of UbcH7, and secondary interactions from UbcH7 helix H1.[20] Comparison with a structure of the RING ligase (discussed below) c-Cbl bound to UbcH7[23] shows a unique interaction surface on c-Cbl binding to the same L1/L2 loops and H1 helix of UbcH7, suggesting that small molecule inhibitors could theoretically be designed that achieve selectivity through binding to this unique surface on the E3. Although computational chemical modeling failed to identify a region within that interface that would be amenable to small molecule binding (our unpublished results), one cannot exclude the possibility that transient changes in E2 or E3 structure within the cellular environment (for example, on charging of E2 with ubiquitin) could reveal such a druggable surface.

Given the lack of apparent druggability of the HECT domain, the absence of reported inhibitors in the literature is not too surprising. However, it is not clear if attempts to identify inhibitors have been tried and failed, or if groups have instead focused their attention on identifying RING domain or E2 inhibitors. Given the possible flexibility of the HECT ligase domain, it is also possible that inhibitors have been identified, but without a structural model or other way to confirm a binding mode, characterization and optimization of compounds would be very difficult.

RING Finger Ligases as Drug Targets

By far the largest class of ubiquitin ligases (perhaps 500 or more) is the RING (really interesting new gene) finger ligases, defined by the presence of a catalytic zinc finger-like RING domain that uses an octet of cysteines and histidines to chelate two zinc ions in a unique 'cross-brace' structure. Though the catalytic mechanism is less well understood than for the HECT ligases, it is generally accepted that the RING ligases serve a scaffolding rather than direct catalytic role in the ubiquitination reaction. The RING ligases do not covalently accept the charged ubiquitin from the E2; rather the RING finger recruits and orients the E2 for optimal transfer of ubiquitin directly from the E2 to the target protein.[24,25] RING finger ligases can be broadly classified as either single- or multi-subunit ligases. As is the case for the previously described HECT domain ligases, single-subunit RING ligases, such as Mdm2 and Cbl, contain a substrate interaction domain and catalytic RING finger domain within a single

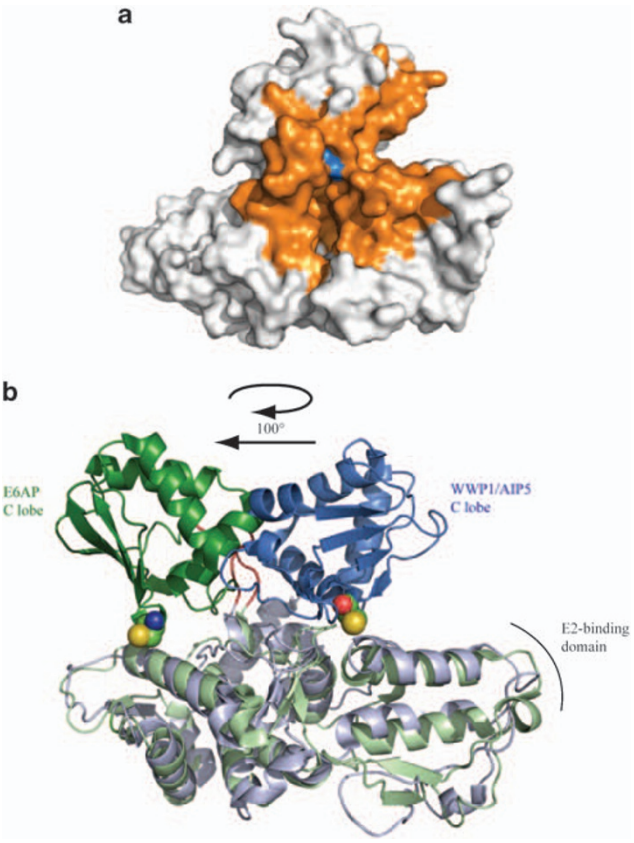

Figure 2 Strategies for HECT domain ligase inhibition. (**a**) The obligate active site cysteine residue within HECT domain ligases lies within a shallow cleft that may be amenable to binding of covalent or noncovalent inhibitors. The structure of the WWP1 HECT domain is shown (1ND7). Residues colored orange lie within potential druggable space based on solvent accessibility and proximity to the catalytic cysteine (blue). (**b**) Remarkable conformational changes within the HECT domain itself that are thought to occur during catalysis may provide opportunities for allosteric inhibition of the E3. The published crystal structures of WWP1/AIP5 (1ND7) and E6AP (1C4Z) are superimposed and aligned based on their respective N-lobe subdomains. The N lobes of WWP1 and E6AP are shown in light blue and light green (respectively), WWP1 C lobe in blue, E6AP C lobe in green, and hinge regions in red. Catalytic cysteines of WWP1 and E6AP are depicted in space-filling representation. Adapted from reference 21

polypeptide. In contrast, the multi-subunit RING finger ligases carry these functions within separate polypeptides (reviewed in detail in reference[26]). A common feature of these more complex E3 ligases is the presence of a catalytic core, containing a Cullin family scaffolding protein and a catalytic RING finger protein, which is then targeted to one of many substrates by binding to a substrate-specific adaptor protein. For example, the best-characterized multi-subunit ligase, the SCF (Skp1, Cullin, F box), is recruited to substrates through binding to one of nearly 70 F-box proteins. Finally, there is substantial evidence for dimerization playing a critical role in catalysis by RING fingers, particularly in the case of Mdm2/MdmX and multi-subunit ligases such as the SCF.[27,28]

Two smaller families of ligases, defined by the presence of either a PHD finger or U box as the catalytic domain, are thought to activate ubiquitination in a manner similar to the RINGs. Unlike the PHD, which is highly similar to the RING domain and differs only in the relative positions of its zinc-chelating cysteines and histidines,[29] the U box contains no chelated zinc ion, lacks the cysteine and histidine residues completely and shows little or no homology to the RINGs in its primary sequence, and yet nevertheless demonstrates remarkable similarity to the RINGs in its tertiary fold.[30,31] Thus, it is likely that these ligases share a similar catalytic mechanism with that of the larger RING finger class.

As RING domain family members are thought to serve as scaffolds in the ubiquitination cascade, inhibiting their activity is likely to be a more difficult undertaking than it would be for the HECT domain ligases. Nevertheless, the clear importance of proto-oncogene RING ligases such as Mdm2 and SCF^{Skp2} in tumorigenesis has driven considerable interest in validating these enzymes as druggable targets. Several ambitious high throughput screens have been developed, using completely reconstituted ubiquitination assays consisting of ubiquitin and recombinant E1, E2, and E3 enzymes, to identify small molecule compounds that inhibit the transfer of ubiquitin either to the E3 ligase itself (termed 'autoubiquitination') or to a physiological substrate. We briefly summarize screening assays as well as progress in developing ligase inhibitors below. Finally, it should be noted that strategies for targeting different classes of RING finger ligases will likely depend on the class of ligase one is looking to inhibit. Inhibitors of enzymatic activity may provide a tractable means of targeting single subunit ligases due to their generally narrow substrate profile, whereas multi-subunit RING finger ligases may necessitate a different approach. The modular design of these ligases greatly expands their substrate repertoire, and also creates significant additional hurdles with regard to their 'druggability'. Inhibitors of enzymatic activity would shut down all versions of the ligase regardless of the substrate-specific adaptor that is bound to the catalytic core; such global inhibition may be too toxic to be viable as a therapeutic. On the other hand, small molecule inhibitors of a specific adaptor protein would almost certainly rely on disrupting the protein–protein interaction between that adaptor and its target protein; targeting such protein–protein interactions can be quite difficult to achieve.

Enzymatic inhibitors. The most straightforward enzymatic screens have tested for inhibitors of E3 ubiquitin ligase activity, most commonly using autoubiquitination of the ligase as the readout for activity. Two groups have reported the identification of inhibitors of Mdm2, which targets the tumor suppressor protein p53 for ubiquitin-dependent proteolysis. Hyperactivation of Mdm2 is a common mechanism for down-regulation of p53 activity in tumors that are genotypically wild type for p53.[32] A group at Bristol-Myers Squibb monitored p53 ubiquitination by preloaded E2-Ub and Mdm2 and identified three compounds with IC_{50}s as low as 3 μM in *in vitro* assays.[33] Biochemical characterization revealed that the compounds did not inhibit the interaction of Mdm2 with p53 and all three compounds appeared to bind in a mutually exclusive fashion, suggesting a common binding pocket. The Vousden lab at the Beatson Institute used an ELISA-based format to screen a 10 000 compound library for inhibition of recombinant Mdm2 immobilized on beads[34,35] and identified a family of closely related 7–nitro-5–deazaflavin compounds

(designated HLI98C, HLI98D, and HLI98E) which inhibit Mdm2 autoubiquitination with an IC_{50} of $\sim 20\,\mu M$ but show relatively poor selectivity (perhaps fivefold selectivity for Mdm2 *versus* the HECT domain ligase Nedd4).[35] The lack of specificity in this study highlights the difficulty in determining mechanism of action in such complicated, multi-enzyme reactions. The failure in specificity is likely due to targeting of the E1 or E2 enzyme in their reaction, because HLI98C inhibits ubiquitin charging by the E2 enzyme UbcH5C with an IC_{50} of $\sim 50\,\mu M$. Encouragingly, cell-based assays show stabilization of p53 after compound treatment, consistent with a mechanism involving inhibition of Mdm2. It should be noted, however, that the mechanism of action of these compounds remains unclear, and this cellular phenotype may be due to general cytotoxicity rather than on-target ligase inhibition.

Several groups have reported high-throughput assay formats for measuring ubiquitination by other ubiquitin ligases, though *bona fide* ligase inhibitors developed using these screening technologies have generally not been published. Rigel has published a method for measuring substrate-independent ubiquitination by immobilizing anaphase-promoting complex/cyclosome catalytic subunits and utilizing an ELISA-based assay to measure incorporation of FLAG-tagged ubiquitin.[36] Examples of two compounds from this screen showed that both compounds inhibited E3 auto-ubiquitination with IC_{50}s of 0.57–1.66 μM. Unfortunately, no evidence was presented that these compounds (or others from the screen) had cell-based activity, displayed significant E3 specificity, or even were direct E3 inhibitors. Celgene reported perhaps the most ambitious screening platform, in which eukaryotically expressed and purified $SCF^{\beta TrCP}$ ligase (containing the proteins Cul1, Roc1, Skp1, and βTrCP) was incubated with recombinant E1 and E2, biotin-labeled ubiquitin, and phosphorylated $I\kappa B\alpha$ substrate; ubiquitination of $I\kappa B\alpha$ was evaluated using a biotin capture antibody followed by detection with europium-labeled streptavidin.[37] It is not clear whether this complex assay format has actually been used to screen for $SCF^{\beta TrCP}$ ligase inhibitors as no resulting compound hits have been published.

Indeed, the disadvantage of all these approaches is in their complexity. Though it may be relatively easy to identify inhibitors (Rigel reported a hit rate of 0.8% in their screen), understanding the precise mechanism of action of inhibitors can be challenging, given the sheer number of components that are necessary for ubiquitination activity. It is not clear if the compounds identified bind directly to the E3 to inhibit its role in ubiquitination, or if they inhibit E1 or E2 activity, or if they inhibit any one of a number of potential protein–protein interactions necessary for ubiquitin chain formation. Until there is a clear understanding of the mechanism of action (likely requiring the development of a number of secondary assays), optimization of hits identified in such enzymatic screens will be extremely challenging.

Alternative strategies could be used that would overcome many of the liabilities of these screening formats. For example, fragment-based approaches have been successfully used to discover small binding fragments that can be readily elaborated into potent small molecule inhibitors. These approaches use techniques such as NMR or crystallography to identify a fragment that binds to a defined region on a protein surface (which in this case would be the E3 catalytic region). As the fragment is discovered using a technique that reveals the precise binding mode, improving the binding affinity and selectivity can be undertaken using a combination of traditional medicinal chemistry optimization in conjunction with structure-guided design. Though there is clearly an opportunity to apply these approaches to E3 small molecule discovery, such techniques have not yet been reported in the literature.

Protein–protein interaction inhibitors. A number of groups have circumvented the questionable druggability of the RING finger ligase catalytic domain and instead have focused on developing small molecules that block binding of the ligase to the substrate. In particular, one of the best examples that exemplify this category is the inhibitors of the p53-Mdm2 interaction. By far, the best characterized of these protein–protein interaction inhibitors are the Nutlins, a series of molecules identified by Roche Holding AG that bind to Mdm2 and prevent its interaction with p53.[38] As the Mdm2 binding domain in p53 is small (ultimately only three residues are responsible for making key interactions with Mdm2; see Figure 3a), perturbation of this interaction surface is thought to stabilize p53 in tumors that retain wild-type p53. The Nutlins block Mdm2-p53 binding (IC_{50} of ~ 140 nM for the most potent member of this class), and the precise mechanism of action of one of these compounds was revealed in a cocrystal structure that showed the Nutlin binding to Mdm2 in a manner almost identical to p53 (Figure 3b). This compound inhibits p53 degradation in cells and reduces tumor growth in an *in vivo* xenograft model. Currently, a compound whose structure has not been disclosed (but is presumably from this series) is undergoing Phase I clinical evaluation.

In addition to the Nutlins, other compounds have been reported that also modulate this interaction. One example is RITA (Figure 3c), a compound that was found in a cell-based screen to identify inhibitors that selectively reduced viability of HCT116 p53 wild-type cells compared to HCT116 p53 null cells.[39] In contrast to the Nutlins, which bind to Mdm2 and prevent binding to p53, RITA binds to p53 and prevents binding to Mdm2. RITA does have the expected activities of an Mdm2-p53 interaction antagonist, as it activates p53 function in tumor cell lines and reduces xenograft tumor growth in a p53-dependent manner. In another example, benzodiazepinedione inhibitors have been reported by Johnson and Johnson Pharmaceuticals that bind Mdm2 and selectively kill p53 wild-type cells *versus* p53 mutant or null cells.[40] Lead compounds from this series (e.g., TDP665759; Figure 3c) were shown not only to stabilize p53 levels in cells but also to increase protein levels of downstream p53 targets such as p21 and PUMAα. Currently Johnson and Johnson has a lead compound (JNJ-26854165) in Phase I clinical trials, but this compound is a novel antagonist that induces a conformational change in Mdm2 but does not prevent binding of p53.[41] Interestingly, binding of JNJ-26854165 to Mdm2-p53 appears to inhibit the interaction of ubiquitinated Mdm2-p53 protein complex to the 26S proteasome. However, the mechanism of action of this compound may not be completely solved as of yet, as it was also found to have p53-independent activity

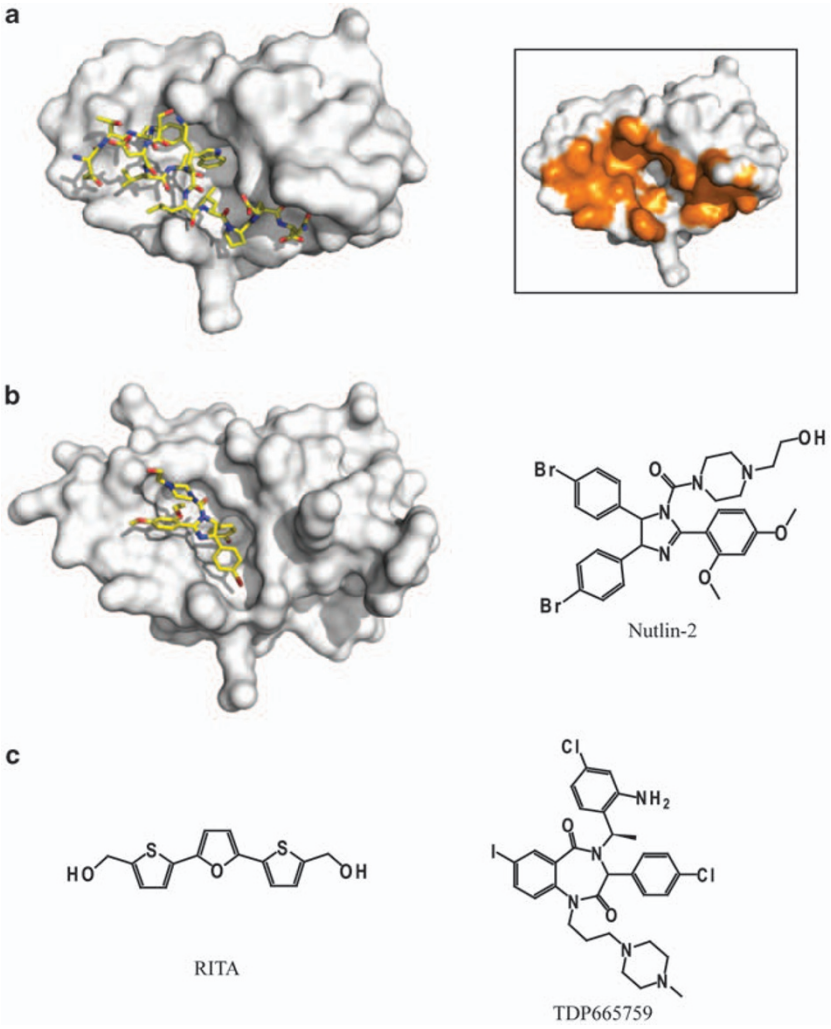

Figure 3 The Mdm2-p53 interaction can be disrupted with a small molecule inhibitor. (**a**) Structure of the p53 interaction domain of Mdm2 (residues 17–125) cocrystallized with a p53 15-mer peptide (residues 15–29) derived from the p53 transactivation domain (1YCR).[72] The surface of the Mdm2 protein is rendered in white and the p53 peptide is shown in yellow. The right panel (inset) illustrates the interface of the Mdm2 protein that is involved in binding the p53 peptide (shown in orange). (**b**) Cocrystal structure of Mdm2 (residues 25–108) and Nutlin-2.[38] Nutlin-2 (compound structure shown on the right) occupies a very similar binding site as the p53 peptide. (**c**) Compound structures of two additional Mdm2-p53 antagonists

across a broad panel of cell lines. Thus, it remains possible that the binding of JNJ-26854165 to Mdm2 disrupts other p53-independent functions, or even inhibits the activity of related E3 ligases or other proteins. Nevertheless, the intriguing mechanism of action appears to increase the number of interaction surfaces within the Mdm2 protein that can be targeted with a small molecule inhibitor.

Clearly, all these data indicate that it is feasible to target a protein–protein interface to inhibit E3 ligase activity. However, small molecule inhibitors that aim to disrupt protein–protein binding have been notoriously difficult to identify, and there may only be a few select cases where this could be applied to effectively inhibit E3 activity *in vivo*.

Ubiquitin E1 and E2 as Molecular Targets

For a number of reasons, relatively little focus has been placed on the development of ubiquitin E1 and E2 inhibitors as therapeutic targets. For many years, a sole E1-activating enzyme was thought to handle the entire ubiquitination load of the cell; this has only recently been expanded to include Ube1-L2/Uba6 in addition to the canonical Uba1.[42–44] The E2 enzymes are significantly more numerous (~40 in human genome), and yet still suffer in diversity compared to the E3 ligases (estimated at more than 500). This suggests that individual E1s and most E2s likely have global functions, and therefore it is not clear if these could be viable therapeutic targets (the anaphase promoting complex/cyclosome-specific E2, UbcH10, is a notable exception; see Table 1). In fact, E1- and E2-directed strategies could elicit unexpected toxicity through inhibition of a variety of nonproteolytic ubiquitination events, such as endocytosis and DNA repair. Moreover, published structural information for Uba1[45] as well as a number of E2 enzymes[20,46] generally show their catalytic cysteines positioned in shallow clefts that are unlikely to allow high affinity-specific interactions with small molecules. An

alternative strategy would be to develop nonubiquitin competitive inhibitors against these enzymes; both of these possible strategies are discussed below.

To date at least two natural product-derived E1 inhibitors have been identified. The mushroom isolate panepophenanthrin[47] and the *Aspergillus* isolate himeic acid A[48] were both demonstrated to inhibit ubiquitin activation by recombinant E1 *in vitro*, though neither study reports cellular activity and their mechanisms of action remain unclear. Weissman and colleagues have isolated a small molecule cell permeable E1 inhibitor, PYR-41, in a larger screen intended to identify inhibitors of Mdm2 ligase activity.[49] PYR-41 was found to inhibit ubiquitination by several different E3 enzymes, and thus was hypothesized to be targeting an upstream component of the ubiquitination cascade. Through a series of *in vitro* experiments, they showed that PYR-41 targets the E1 enzyme, most likely by binding irreversibly to the E1 catalytic cysteine, inactivating it for ubiquitin addition. Initial cell-based studies demonstrated that PYR-41 induced some of the phenotypes expected of an E1 inhibitor, including stabilization of p53 and inhibition of cytokine-induced activation of NF-κB.[49] However, as no data were presented showing inhibition of tumor cell proliferation *in vitro* and the high concentrations of PYR-41 required to inhibit E1 activity (10–50 μM) preclude *in vivo* analysis, the viability of E1 inhibition as a cancer therapeutic remains an open question. A possible alternative strategy for targeting E1 is to target the ATPase activity of E1, as ATP hydrolysis is used to generate a high-energy thioester bond with ubiquitin. Given their wide success as kinase inhibitors, competitive ATP analogs targeting this second active site could present a more chemically tractable means of blocking E1 activity.

Unlike E1s, the E2 enzyme class are likely to be particularly difficult to target with small molecules as the overall structure of a typical E2 suggests no obvious druggable pockets, either at its catalytic cysteine or at the surface which binds the E3 enzyme (references 23, 50; our unpublished analysis). It is important to remember, however, that the available structures represent a static view of one conformation of the E2, and there may be subtle differences in conformation that take place during its catalytic cycle. For example, a mutational analysis of UbcH5B has identified mutations that do not interfere with E3 binding or loading of the E2 active site cysteine residue with ubiquitin, and yet dramatically alter UbcH5B's ability to release ubiquitin.[51] Thus, it was proposed that these residues mediate an allosteric communication between the E3 binding site and the E2 active site. However, this region of the E2 contains no obvious binding pocket either, so the conformational change may have limited significance from a drug discovery viewpoint. Despite the possible technical hurdles in developing small molecule inhibitors of E2s enzymes, there is evidence that these enzymes may be appealing therapeutic targets. The best characterized cancer-related E2 enzyme, UbcH10 (specific for the multi-subunit anaphase promoting complex/cyclosome (APC/C) RING finger ligase), promotes growth of 3T3 cells in soft agar *in vitro* and its expression is elevated in a diverse array of tumor types.[52] Importantly, depletion of UbcH10 by RNA interference combines with agonists of the DR5/TRAIL receptor to drive killing of cancer-derived but not normal cells,[53] providing compelling evidence that UbcH10 inhibition may be useful as a chemosensitizer as well.

Alternative Strategies to Inhibiting E3 Ligase Activity

Targeting Cullin family ligases by Nedd8 inhibition. Nedd8 is a small ubiquitin-like protein (UBL), which is covalently conjugated to target proteins via an ATP-dependent enzyme cascade very similar to that used for ubiquitin conjugation. Modification of proteins with Nedd8 is generally thought to be E3-independent, requiring only the Nedd8-specific E1 (Nedd8-activating enzyme; NAE) and E2 (Ubc12/UBE2M or UBE2F).[54,55] The major cellular 'neddylation' substrates are the Cullins, which are scaffolding components of a family of multi-subunit RING finger ligases.[26] Nedd8 modification of Cullin subunits significantly increases the E3 ligase activity of the holoenzyme, both by blocking association of the Cullin with its negative regulator CAND1[56] and also by directly helping to recruit ubiquitin-charged E2.[57] Thus, specific inhibitors of neddylation are predicted to block SCF activity, which could provide a means of inhibiting the activity of oncogenic SCF ligase complexes (such as SCF^{Skp2} which targets the Cdk inhibitor p27 for ubiquitination) that would otherwise be difficult to target with small molecules. Millennium Pharmaceuticals (part of the Takeda Oncology Company) has developed an NAE inhibitor (MLN4924) that reduces neddylation of Cullins *in vitro* and in cell-based assays.,[58] MLN4924 is reported to stabilize the cellular NF-κB pathway inhibitor IκBα by inhibiting its ligase, $SCF^{\beta TrCP}$, resulting in a loss of NF-κB signaling and induction of apoptosis. The central role of the NF-κB pathway in the cellular response to MLN4924 was further demonstrated *in vivo*; NF-κB-dependent tumor xenografts, using the diffuse large B-cell lymphoma Ly10 cells, were more sensitive to MLN4924 treatment than xenografts that are NF-κB independent (Ly19 cells). MLN4924 is currently undergoing evaluation in Phase I clinical trials for treatment of advanced solid tumors and hematological malignancies.

Another example of a nonubiquitin E1 inhibitor was recently reported. In this case Ginkgolic acid was identified as a compound in *Ginkgo biloba* extract that bound directly to the SUMO E1 enzyme and blocked its ability to become charged with SUMO.[59] Exactly how this compound binds to E1 and prevents SUMO charging are not yet known. Nevertheless, this further demonstrates that E1 enzymes are viable small molecule targets; the challenge, however, is identifying which E1s are therapeutically relevant.

Inhibiting the proteasome. Clearly the most clinically advanced, albeit indirect, method of inhibiting E3 ligase activity is to target the proteasome, which is the cellular protease for the UPS. In 2003, the proteasome inhibitor Bortezomib was approved as a treatment for multiple myeloma; this was later expanded to include mantle cell lymphoma in 2006. Velcade is a dipeptide boronic acid that reversibly inhibits the chymotrypsin-like peptide hydrolyzing activity of the 20S component of the proteasome, which is considered to be the key rate-limiting step in proteasomal degradation.[60] There are a number of pathways that may

explain the somewhat surprising empirical observation that proteasome inhibition shows selectivity in killing tumor *versus* normal cells. In some cells (but not all), induction of apoptosis following Velcade treatment appears to correlate with stabilization of IκBα and inhibition of the NF-κB pathway.[61] Alternatively, proteasome inhibition may be particularly effective in multiple myeloma simply because cells in these tumors are extremely active in protein synthesis (e.g., IgG), and interfering with proteasome activity is expected to overwhelm the cell's protein quality control machinery. Millennium is actively pursuing additional indications for Velcade, and several groups are developing next generation proteasome inhibitors that differ from Velcade in their mechanism of action and selectivity profile (e.g., Carfilzomib, which is a highly specific and irreversible inhibitor of the chymotrypsin-like activity of the proteasome).[62]

Inhibiting recruitment of UPS substrates to the proteasome. Another strategy for inhibiting ubiquitin-dependent proteolysis downstream of E3 ligases is to block recruitment of UPS substrates to the proteasome. A chemical genetics screen using a small (~100 000 compounds) chemical library has been used to identify compounds that inhibit degradation of an unstable luciferase reporter protein in crude *Xenopus* extracts.[63] Elegant biochemical analysis *in vitro* showed that these compounds, christened 'ubistatins', inhibit proteolysis by binding Lys48-linked polyubiquitin chains and blocking their interaction with ubiquitin chain receptors on the proteasome, such as Rad23 and Rpn10.[64–66] The large size and charged nature of these molecules precluded their use in cell-based assays, and it remains unclear how much specificity could be extracted from inhibitors that function at this step in the UPS. Still, optimization of these and similar molecules could provide a means of inhibiting ubiquitin-dependent protein destruction. Theoretically, ubistatin derivatives would be expected to give phenotypes very similar to proteasome inhibition. Some proteins are targeted to the proteasome in a ubiquitin-independent manner, however (e.g., ornithine decarboxylase and the Cdk inhibitor p21), suggesting that the actual cellular phenotype may be different in the two scenarios. It is also possible that different ubiquitin chain receptors resident in the lid of the proteasome may interact with different ubiquitinated substrates, which could afford ubistatin derivatives some selectivity over general proteasome inhibition.

Activating protein degradation. Stabilization of UPS substrates may not always be the appropriate clinical strategy; in a number of cases it could be therapeutically beneficial to stimulate, rather than block, degradation of UPS targets, such as products of oncogenes. The most straightforward means of achieving UPS activation is to inhibit isopeptidase/deubiquitinating (DUB) enzyme activity. The vast majority of these enzymes are cysteine proteases and thus they represent some of the most druggable enzymes within the UPS.[18] However, very little progress has been made in developing inhibitors against these proteases, most likely because our knowledge of the roles of these enzymes and the pathways in which they act has lagged far behind that of the ligases. Though there are a relatively large number of patents describing the identification of DUB inhibitors, very little of this work has been published making it difficult to assess how specific their activity is and whether cellular and *in vivo* data support their proposed mechanism of action. For example, Progenra has screened the NIH library of small molecules (70 000 compounds) and identified a number of compounds that inhibited USP2-dependent cleavage of Ubiquitin-PLA2. In particular, one compound was reported (P5091) that selectively inhibited HAUSP/USP7 activity *versus* a panel of other USPs, and induced p53 and p21 expression (see company web site for details[67]), though no compound structures have been disclosed. In another example, a different group carried out a screen to identify compounds that activated apoptosis in an apoptosome-independent manner, and two molecules (F6 and G5) were identified that appeared to function by inhibition of ubiquitin isopeptidase activities.[68] A subsequent study demonstrated that compound NSC 632839 (compound F6) inhibits purified USP2, USP7, and SENP2 with IC50s of 45 μM, 37 μM, and 9.8 μM, respectively,[69] which suggests that identifying a selective and potent DUB inhibitor may be difficult. Nevertheless, this supports the idea that DUBs are druggable targets, and clearly one of the key issues will be discovering an appropriate selectivity profile for target inhibition.

Finally, there have also been attempts to harness the ubiquitination apparatus to selectively target proteins for rapid destruction that would not otherwise be degraded. The first application of this approach used a chimeric compound (termed 'protac' for proteolysis targeting chimeric molecule) with two binding moieties: a peptide sequence containing the IκBα recognition motif (targeted by $SCF^{\beta TrCP}$) linked to ovalicin, a small molecule that binds methionine aminopeptidase-2 (MetAP-2).[70] This compound efficiently enabled the recruitment of the $SCF^{\beta TrCP}$ ligase to MetAP-2 and promoted its rapid ubiquitination and destruction in *Xenopus* extracts. Further refinement of this strategy has included the development of non-peptide-based protacs that are membrane permeable and thus can be used in cell-based studies.[71] This approach is an extremely elegant and potentially powerful way to target cellular proteins for destruction; however, it is not clear that this could be a viable therapeutic approach *in vivo*, given the inherent complexity and large size of these targeting molecules.

Summary

The UPS represents a major point of regulation for a wide variety of cellular pathways, and the approval of the proteasome inhibitor Bortezomib in 2003 for third-line multiple myeloma, and its more recent expansion to first-line treatment in 2008, has validated UPS enzymes as viable therapeutic targets. The focus of research into the role of the UPS in disease has undoubtedly been the ubiquitin ligases, which drive specificity in the ubiquitination reaction and could provide the selectivity that proteasome inhibitors such as Bortezomib lack. Progress in this area has been frustratingly slow, however. Deciphering druggable opportunities within these families of enzymes has been challenging; our UPS 'parts list' has grown substantially within the past decade, but we still know relatively little about the catalytic mechanisms

used by even the largest and well-studied families of UPS enzymes. The most advanced E3 ligase inhibitor, Nutlin, does not block ligase activity *per se*, but rather targets a protein–protein interaction (Mdm2-p53 binding). Although potentially viable in certain select cases, this strategy almost certainly has limited potential; elucidation of a more general strategy for targeting specific UPS components upstream of the proteasome is clearly required. Thus, tackling the enzymology of ubiquitination will be of paramount importance in the coming years if the initial promise of these enzymes as druggable targets is to be realized.

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