Ligandability of E3 Ligases for Targeted Protein Degradation Applications

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ABSTRACT: Targeted protein degradation (TPD) using proteolysis targeting chimeras (PROTACs) and molecular glue degraders has arisen as a powerful therapeutic modality for eliminating disease-causing proteins from cells. PROTACs and molecular glue degraders employ heterobifunctional or monovalent small molecules, respectively, to chemically induce the proximity of target proteins with E3 ubiquitin ligases to ubiquitinate and degrade specific proteins via the proteasome. Whereas TPD is an attractive therapeutic strategy for expanding the druggable proteome, only a relatively small number of E3 ligases out of the >600 E3 ligases encoded by the human genome have been exploited by small molecules for TPD applications. Here we review the existing E3 ligases that have thus far been successfully exploited for TPD and discuss chemoproteomics-enabled covalent screening strategies for discovering new E3 ligase recruiters. We also provide a chemoproteomic map of reactive cysteines within hundreds of E3 ligases that may represent potential ligandable sites that can be pharmacologically interrogated to uncover additional E3 ligase recruiters.
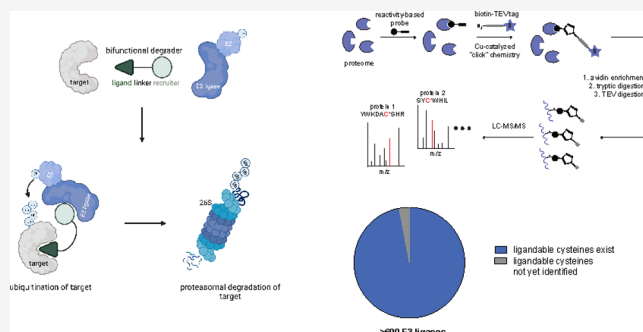
Targeted protein degradation (TPD) has arisen as a powerful chemical biology platform for inducing the degradation of specific proteins in, on, or outside of cells and has made a tremendous impact on drug discovery in both academic and industrial settings.[1−3] TPD uses heterobifunctional or monovalent small molecules or biologics to induce the proximity of a component of the cellular degradation machinery with a neo-substrate protein, inducing the degradation of that specific protein. Heterobifunctional degraders, or proteolysis targeting chimeras (PROTACs), consist of a protein-targeting ligand linked to an E3 ubiquitin ligase recruiter to induce the proximity of an E3 ligase with a neo-substrate protein, causing the ubiquitination and elimination of that protein through proteasomal degradation (Figure 1).[1−3] Molecular glue degraders also bring together an E3 ligase with a neo-substrate protein but employ monovalent small molecules rather than heterobifunctional small molecules.[4] Whereas the genesis of TPD as a potential therapeutic paradigm arose from heterobifunctional or monovalent small molecules that induce the proximity of an E3 ubiquitin ligase with a neo-substrate protein to ubiquitinate and degrade the target protein via the proteasome, next-generation TPD strategies have arisen that exploit (1) the targeted degradation of the cell surface and extracellular proteins through the lysosome with lysosome-targeting chimeras (LYTACs), (2) the targeted degradation of larger intracellular proteins and cell compartments through autophagy with autophagy-targeting chimeras (AUTACs), (3) the targeted degradation of transcription factors with tran-

scription-factor-targeting chimeras (TRAFTACs) and transcription factor PROTACs (TF-PROTACs), (4) the targeted degradation of bacterial proteins through the recruitment of bacterial proteases with BacPROTACs, and even (5) the targeted degradation of RNA with ribonuclease-targeting chimeras (RIBOTACs).[5−9] Induced proximity paradigms are even being extended beyond degradation to include the targeted manipulation of other protein post-translational modifications beyond ubiquitination, including (1) targeted phosphorylation with phosphorylation-inducing chimeric small molecules (PHICs), (2) targeted dephosphorylation with phosphatase-recruiting chimeras (PHORCs), and (3) targeted deubiquitination with deubiquitinase-targeting chimeras (DUB-TACs).[10−12] These induced proximity paradigms have garnered significant interest within pharmaceutical companies because of the potential of TPD and next-generation induced proximity paradigms to tackle the "undruggable" proteome, representing most of the proteome that still cannot be therapeutically exploited using classical drug discovery approaches. All of these potential therapeutic paradigms are enabled by the discovery of
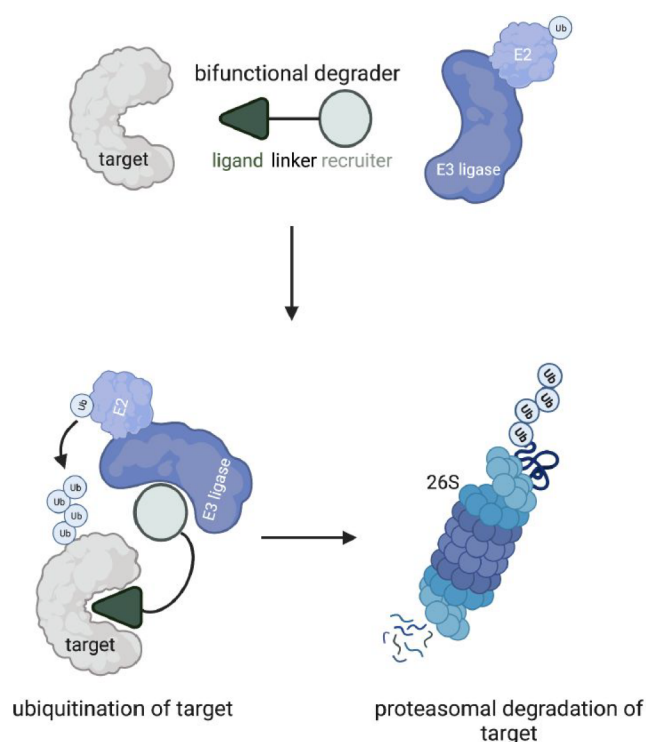
Figure 1. Targeted protein degradation with heterobifunctional PROTACs. PROTACs consist of a protein-targeting ligand linked to an E3 ubiquitin ligase recruiter via a linker to induce the proximity of an E3 ligase with a neo-substrate target protein to induce ubiquitination and proteasome-mediated degradation of the target protein.

small-molecule, peptide, or biologic recruitment handles that, either by themselves or through linkage to protein-targeting ligands, enable the induced proximity of a protein modulator with a neo-substrate protein of interest.

Despite the emergence of many PROTAC and molecular glue degrader drug candidates in the clinic, in clinical development, or entering clinical trials and the development of new induced proximity paradigms beyond TPD, a major bottleneck in using classical PROTACs and molecular glue degraders for TPD is still the paucity of pharmacological tools that exist for the ubiquitin-proteasome system, in particular for E3 ubiquitin ligases. E3 ubiquitin ligases are of particular interest because they are responsible for substrate recognition and specificity in the ubiquitin-proteasome system. E3 ligases are also the most diverse component of the ubiquitin-proteasome system, with over 600 estimated members.[13,14] Yet only a handful of E3 ligase recruiters currently exist, with most attention placed on two E3 ligases—cereblon (CRBN) and von Hippel−Lindau (VHL).[15−17] These two E3 ligase recruiters are likely insufficient to degrade any and every protein target of interest with the desired selectivity. Evidence of this includes the lack of degradation observed against KRAS G12C with CRBN-based recruiters but successful degradation achieved with VHL recruiters.[18,19] In a recent beautiful study by Donovan, Ferguson, Sim, Gray, and Fischer et al., where they developed >90 CRBN- and VHL-based kinase PROTACs with various linkers and protein-targeting ligands, the authors impressively showed the degradation of >200 kinases, but there was still a significant number of kinases that were not degraded out of the >500 kinases that were detected.[20]

The discovery of new E3 ligase ligands will undoubtedly help in expanding the scope of TPD and the targets that can be degraded by PROTACs and molecular glue degraders. Furthermore, the development of tissue-type-, cell-type-, or cell-compartment-restricted E3 ligase recruiters may enable the development of degraders that achieve the location-specific degradation of targets. Here we review current E3 ligase recruiters, ligands, and molecular glues that have been successfully used in TPD and discuss chemoproteomic and covalent ligand screening approaches that have been used to discover new E3 ligase recruiters. We also present an aggregated chemoproteomic atlas of reactive cysteines that can potentially be targeted by cysteine-reactive covalent ligands across a large swath of the human E3 ligase family.

UBIQUITIN-PROTEASOME SYSTEM AND E3 LIGASES

The ubiquitin-proteasome system is a major regulator of protein homeostasis and is responsible for the degradation of a wide range of cellular proteins. Degradation occurs through an enzyme cascade that results in ubiquitination, a covalent post-translational modification. Hershko, Ciechanover, and Rose discovered this cascade in the 1970s while studying the degradation of denatured globin. They showed that degradation was ATP-dependent and proteins were marked for degradation by the covalent addition of multiple molecules of a small protein later identified as ubiquitin.[21,22] Ubiquitin is activated via the consumption of ATP by an E1 ubiquitin-activating enzyme. The activated ubiquitin is transferred from the E1 enzyme to the E2 ubiquitin conjugation enzyme via a transthiolation reaction. Ubiquitin is then transferred to the substrate by an E3 ubiquitin ligase. The transfer is accomplished via an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine side chain on the substrate. The cycle is repeated to poly ubiquitinate the substrate, marking it for proteasomal degradation (Figure 1).[23] The system, although complex and regulated, is generally applicable to the diverse proteome.

E3 ligases are classified based on their mechanisms of action. There are two main classes: RING E3 ligases, which recruit E2 ubiquitin conjugates via the RING domain and catalyze the transfer of ubiquitin directly to the substrate, and HECT (Homologous to the E6-AP Carboxyl Terminus) E3 ligases, which transfer ubiquitin to a substrate only after forming a thioester bond with ubiquitin. Within RING E3 ligases, there are three subcategories. Cullin-Really Interesting New Gene (RING) ligases (CRLs) are multisubunit complexes where a Cullin protein links an E2-binding subunit and a target-binding subunit.[24] Anaphase-promoting complex (APC) ligases are also multisubunit complexes that bring E2 proteins and the target protein together without a linker protein. RING-between-RING (RBR) ligases are considered RING E3 ligases because they bind to E2 proteins via their RING domain, but they form a thioester bond with ubiquitin before transferring it to the target protein.

E3 LIGASE RECRUITERS

In 2001, the first example of artificial E3 ligase recruitment was shown with the E3 ligase ß-TrCP. A PROTAC linked a ß-TrCP peptide ligand to a covalent methionine aminopeptidase 2 (MetAP2) ligand, resulting in the degradation of MetAP2.[25] A peptide ligand was also used as the recognition sequence for the VHL E3 ligase in the first cell-permeable PROTAC in 2004.[26] As interest in E3 ligase recruitment grew, the field shifted away from

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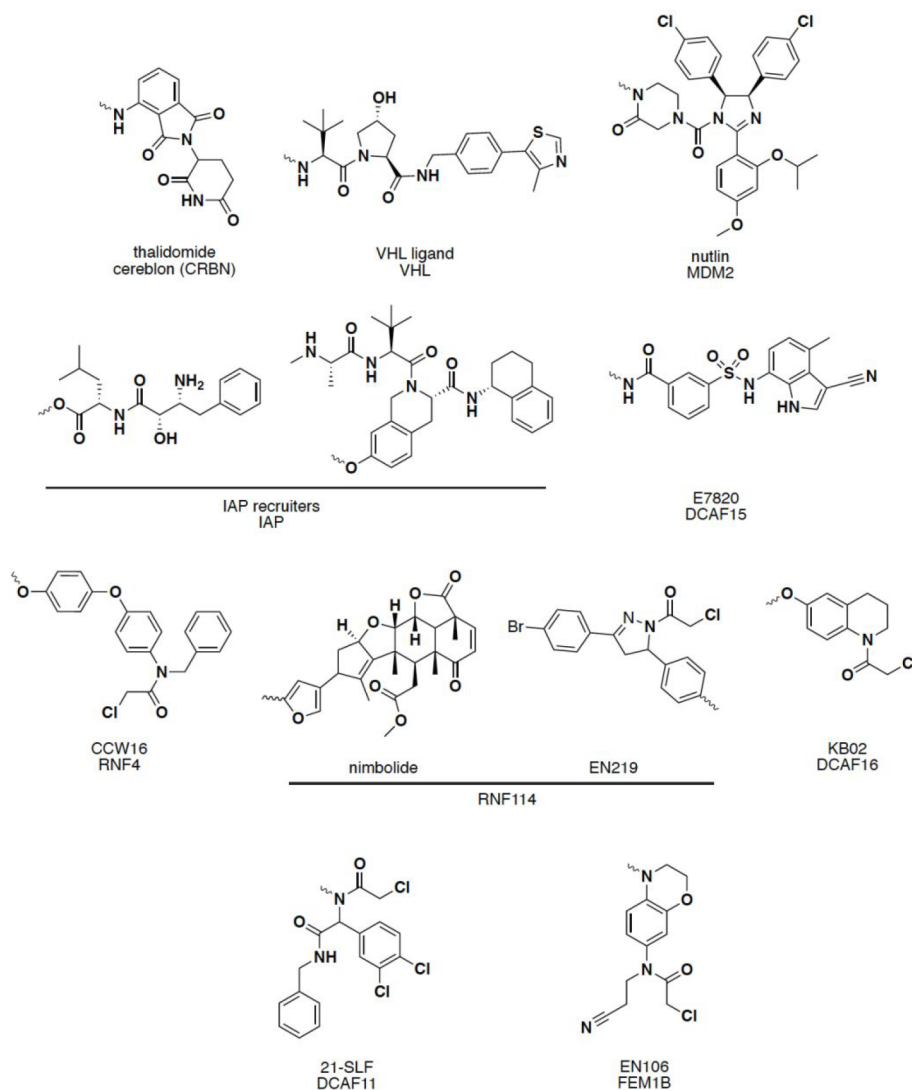
Figure 2. Existing E3 ligase recruiters for targeted protein degradation applications.

peptide ligands and toward reversible small-molecule E3 ligase recruiters, with the recruitment of the MDM2 E3 ligase by the ligand nutlin in 2008.[27] Reversible small-molecule ligands continued to gain recognition with the recruitment of the E3 ligase cIAP in 2010.[28] Thalidomide, an immunomodulatory drug (IMiD), was shown to bind to the E3 ligase CRBN in 2010 by Ito, Ando, and Handa.[29] In recent years, more covalent small-molecule E3 ligase recruiters have been identified.

Von Hippel−Lindau. The VHL complex E3 ligase consists of VHL, elognins B and C, cullin 2, and RING box protein 1. VHL was one of the first E3 ligases targeted artificially by mimicking the recognition sequence of hypoxia-inducible factor-1 (HIF-1α), the primary target of VHL. In the early 2000s, the central hydroxyproline residue in the ALAPYIP recognition sequence of HIF-1α was determined to be essential for the formation of the HIF-1α/VHL complex via hydrogen bonding between the hydroxyproline of HIF-1α and a hydroxyproline pocket on VHL.[30,31] In 2004, Schneekloth and Crews et al. used the ALAPYIP recognition sequence as a VHL recruiter for the first cell-permeable PROTAC, targeting and degrading FK506 binding protein (FKBP12).[26] This set the stage for interfering with proteins on a post-translational level, presenting the opportunity to create chemical knockdowns to

study protein function without genetic modification. In 2005, a PROTAC targeting estrogen receptor-α (ERα) was developed that used a shorter peptide recognition sequence to recruit VHL. The shorter, pentapeptide sequence used for VHL recruitment improved the cell permeability of the PROTAC. This E2-penta PROTAC successfully degraded ERα, and angiogenesis was inhibited as a result of ERα degradation.[32] VHL continued to be recruited with peptide recognition sequences resembling the HIF-1α sequence, but as more proteins were targeted, issues with cell permeability and drug-like properties became more apparent.[33]

Following the discovery of small-molecule recruiters for mouse double minute 2 homologue (MDM2) and cellular inhibitor of apoptosis protein (cIAP), discussed later in this Perspective, a more drug-like small-molecule ligand for VHL was identified. In 2012, Buckley, Crews, and Ciulli et al. identified a small-molecule ligand that interrupted the interaction between HIF-1α and VHL. By analyzing the crystal structure of the HIF-1α/VHL complex, they were able to identify the key residues for binding and design small-molecule analogues to the key binding motif.[34] Between 2012 and 2015, the original small-molecule VHL recruiter was optimized to yield a VHL ligand with nanomolar potency.[35,36] In 2015,

Bondeson and Crews et al. used this optimized VHL recruiter in several PROTACs to specifically degrade ERα and receptor interacting serine/threonine kinase 2 (RIPK2) in cells and *in vivo* in mice (Figure 2).[36] Also, in 2015, Zengerle, Chan, and Ciulli et al. designed a PROTAC linking JQ1, a bromo- and extra-terminal (BET) protein inhibitor, to the small-molecule VHL ligand. JQ1 is a nonselective BET inhibitor, but they observed selectivity of the PROTAC for BRD4. At the time, chemical knockdown had only been achieved for all BET family members, but none selectively. A different downstream pharmacological response was observed for BRD4 degradation than for nonspecific BET inhibition. The PROTAC was successful at low concentrations, and the endogenous expression of HIF-1α was not impacted.[37] The VHL recruiter has since been used in many PROTACs to degrade a wide range of targets.[2,20,38,39]

More recently, Bond, Chu, and Crews et al. targeted the oncogenic G12C mutant form of Kirsten rat sarcoma viral oncogene homologue (KRAS) with a small-molecule PRO-TAC-recruiting VHL. The Crews lab designed a PROTAC linking MRTX849, a covalent KRAS^{G12C} ligand, to a VHL ligand. The degradation of KRAS^{G12C} was observed along with the downstream suppression of MAPK signaling. This was the first successful PROTAC to target KRAS^{G12C} in cancer cells.[40] This work follows the unsuccessful degradation of KRAS^{G12C} by the Gray lab using CRBN recruiters.[41]

Cereblon. CRBN is one of the substrate receptors for the Cullin-RING E3 ubiquitin ligase 4 (CRL4) containing DDB1, CUL4, and RBX1. Whereas the endogenous substrates of CRBN are still under investigation, CRBN recognizes neo-substrates upon binding to thalidomide and other IMiD analogs by engaging in ternary complexes, subsequently leading to the ubiquitination and proteasome-mediated degradation of these neo-substrates. Thalidomide, a drug originally developed for morning sickness in pregnant women in the 1950s, was found to exert profound birth defects called phocomelia, hallmarked by short limbs.[42] CRBN was identified as the primary target of thalidomide responsible for these birth defects by Ito and Handa et al. in 2010.[43] The neo-substrate protein responsible for the birth defects associated with thalidomide, Sal-like protein 4 (SALL4), was not uncovered until more recently through concurrent discoveries by Matyskiela and Chamberlain et al. and Donovan and Fischer et al. in 2018.[44,45] Once CRBN was identified as the target of thalidomide responsible for its immunomodulatory effects, less toxic IMiDs such as lenalido-mide and pomalidomide were developed as cancer drugs to target CRBN as treatments for multiple myeloma.[46] A series of studies in 2014 and 2015 provided both structural and biological insights, showing that different thalidomide analogs altered the E3 ligase neo-substrate specificity of CRBN and that the anticancer effects of lenalidomide and pomalidomide were through the CRBN-mediated degradation of neo-substrate transcription factors Ikaros (IKZF1) and Aiolos (IZKF3).[47−51] Subsequent studies have continued to showcase the diversity of neo-substrate degradation with an expanding scope of IMiD analogs.[48,52−55]

In 2015, Winter and Bradner et al. demonstrated that IMiD CRBN recruiters could be linked to protein-targeting ligands to selectively degrade specific proteins in cells (Figure 2). They the showed selective degradation of the transcriptional coactivator BRD4 and FKBP12 by linking IMiDs to their previously developed BET family protein ligand JQ1 and FKBP12 ligand SLF, respectively, in a CRBN-dependent manner.[56] Alongside

this paper in 2015, Lu and Crews et al. also published on IMiD-based PROTACs for BRD4.[57] These results opened up the possibility for a fully synthetic drug-like heterobifunctional degraders that could be used to specifically degrade target proteins in cells and spurred the development of many IMiD-based PROTACs that have now been exploited against countless protein targets and PROTACs that have entered clinical development.[2,20,38,39]

MDM2. In 2008, the Crews lab demonstrated that nutlin, a known MDM2 E3 ligase ligand that displaces the interaction between MDM2 and the tumor suppressor p53, could be used in a PROTAC to recruit MDM2 to degrade the androgen receptor (AR) (Figure 2). AR was targeted by a selective androgen receptor modulator (SARM) linked to nutlin by a polyethylene glycol (PEG) linker.[27] Using a small-molecule E3 ligase ligand instead of the peptidic E3 ligase ligands that existed at the time helped to increase both the cell permeability and stability of PROTACs while decreasing the molecular weight. The Crews lab found success in recruiting MDM2 with nutlin again in 2019 with the successful degradation of BRD4. Because nutlin inhibits the interactions between MDM2 and p53, MDM2-based PROTACs have the potential to both degrade their target and stabilize p53. The Crews lab demonstrated that their MDM2 PROTAC worked synergistically to exert anticancer activity by both degrading BRD4 and stabilizing p53.[58]

cIAP1. Cellular inhibitor of apoptosis protein 1 (cIAP1) degrades caspase proteins and is overexpressed in many cancer cells. In 2008, the Naito lab demonstrated that methyl bestatin (MeBS) bound to cIAP1 and promoted autoubiquitylation, facilitating its proteasomal degradation (Figure 2).[59] This study indicated that cIAP1's intrinsic ubiquitin ligase activity had the potential to be manipulated for the degradation of other proteins. In 2010, Itoh and Hashimoto designed specific and nongenetic inhibitor-of-apoptosis protein-dependent protein erasers (SNIPERs) that utilized MeBS to recruit cIAP. The original SNIPER contained an ester, which was readily hydrolyzed in the cell and caused off-target degradation.[28] Amide-linked SNIPERs were designed with an all-trans retinoic acid (ATRA) ligand to bind the cellular retinoic acid binding protein II (CRABP-II). The selectivity of the SNIPER was improved, and degradation of CRABP-II was observed.[60] In 2011, the group demonstrated that the SNIPER technology could be applied to different protein targets by degrading a variety of nuclear receptors (retinoic acid receptor, ERα, and AR).[61]

Following the facile expansion of SNIPER technology, the Naito lab designed a SNIPER(TACC3) in 2014 to target the spindle regulatory protein transforming acidic coiled-coil-3 (TACC3). Although degradation of TACC3 was observed, it was determined that cIAP1 was not the E3 ligase responsible for the degradation. Through mechanistic studies, it was determined that the E3 ligase anaphase-promoting complex/cyclosome (APC/C^{CDH1}) was responsible for mediating TACC3 degradation.[62]

In more recent years, cIAP ligands have been improved over the original compounds, as demonstrated by GlaxoSmithKline (GSK) in 2020 with the PROTAC-mediated degradation of receptor-interacting serine/threonine protein kinase 2 (RIPK2) (Figure 2).[63] This study demonstrated that RIPK2 could be selectively degraded *in vivo* at nanomolar potency. It is important to note that the RIPK2 PROTAC is more potent than the RIPK2 inhibitor alone due to the catalytic pharmacological benefits offered by PROTACs.[63] These

findings demonstrate that PROTACs recruiting a variety of E3 ligases have therapeutic potential.

In 2021, Genentech demonstrated that E3 ubiquitin ligases could degrade themselves in a proteasome-dependent manner using modified X-linked inhibitor of apoptosis protein (XIAP) recruitment ligands.[64] This novel degradation modality is composed of an XIAP recruitment ligand linked to a nucleophilic primary amine. The degradation depends on the isopeptide bond formation between the nucleophilic amine and the C-terminus of ubiquitin, leading to the XIAP-promoted ubiquitination of the small-molecule recruitment ligand. This complex is then recruited to the proteasome, leading to the degradation of XIAP. Interestingly, the ubiquitylation state of XIAP is not changed, and the degradation depends only on the binding to and E3 ubiquitin ligase function of XIAP. This K-tag approach demonstrates not only the successful recruitment of XIAP, but also the expansion of the field of TPD to include the self-degradation of E3 ubiquitin ligases.[64]

RNF4. In 2019, Ward and Nomura discovered a small-molecule cysteine-reactive covalent RING finger protein 4 (RNF4) recruiter using activity-based protein profiling (ABPP)-based covalent ligand screens, to be described in more depth later. RNF4 is an E3 ligase that ubiquitinates SUMOylated proteins for proteasomal degradation.[65] Cysteine-reactive covalent ligands were screened against the fluorescent cysteine-reactive iodoacetamide probe (IA-rhodamine) labeling of pure human RNF4 in a gel-based ABPP screen, described later in this Perspective. The initial covalent ligand hit TRH 1-23 was found to react with the zinc-coordinating cysteines C132 and C135 within RNF4 without inhibiting the RNF4 ubiquitination activity.[65] Upon hit optimization, CCW 16 was identified as the most potent compound against RNF4 and was subsequently linked to JQ1 to demonstrate the RNF4-dependent degradation of BRD4 (Figure 2).[65] This study demonstrated the utility of target-based covalent ligand screening to discover new E3 ligase recruiters.

RNF114. Concurrent with the discovery of covalent RNF4 recruiters, the neem tree-derived anticancer natural product nimbolide was discovered as a covalent RNF114 recruiter.[66] In 2019, Spradlin, Maimone, and Nomura et al. discovered that nimbolide reacts preferentially with the intrinsically disordered N-terminal cysteine 8, involved in the substrate recognition of the E3 ubiquitin ligase RNF114 using the ABPP-based chemoproteomic approaches discussed later in this Perspective.[66] The authors showed that nimbolide exerts its anticancer effects by inhibiting RNF114 substrate interactions with tumor suppressors p21 and p57, leading to their accumulation and eventual cell death. More importantly, the authors demonstrated that nimbolide could be used as a covalent RNF114 recruiter for PROTACs in TPD, where they linked nimbolide to JQ1 to show both the selective degradation of BRD4 and the stabilization of endogenous RNF114 tumor suppressor substrates (Figure 2).[66] Subsequent studies showed that nimbolide could also be used to degrade the fusion oncogene BCR-ABL in leukemia cancer cells. In this study, the authors demonstrated that RNF114-based degraders showed the preferential degradation of BCR-ABL over c-ABL compared with CRBN or VHL-based degraders that showed the preferential degradation of c-ABL.[67]

To develop a more synthetically tractable RNF114 recruiter, Luo, Spradlin, and Nomura et al. performed a target-based covalent ligand screen using gel-based ABPP approaches against RNF114 to identify a fully synthetic recruiter, EN219, that mimicked the action of nimbolide in preferentially targeting C8

of RNF114 in cells.[68] The authors subsequently linked EN219 to JQ1 to demonstrate the RNF114-dependent degradation of BRD4 (Figure 2).[68] Whereas nimbolide was discovered as an RNF114 recruiter through target identification studies using ABPP-based chemoproteomic approaches, this later study showcased how target-based covalent ligand screening can be used to discover new E3 ligase recruiters against specific E3 ligases of interest.[68,69]

DCAF16. Also concurrent with the discovery of covalent RNF4 and RNF114 recruiters, Zhang and Cravatt et al. discovered a novel covalent DDB1 and CUL4 associated factor 16 (DCAF16) E3 ligase recruiter that could be deployed in TPD applications in 2019 using a cellular covalent PROTAC screening strategy coupled to downstream chemoproteomic target identification.[70] The authors linked covalent and promiscuous scout ligands to the FKBP12 ligand SLF and screened for the selective degradation of nuclear FKBP12 that possessed a C-terminal nuclear localization signal. Through this effort, they identified the covalent scout ligand KB02 as a recruiter for TPD applications (Figure 2). To identify the E3 ligase target of KB02, they performed FKBP12 pulldown proteomic studies to identify ubiquitin-proteasome system proteins that were specifically enriched upon the treatment of cells with their KB02-based PROTAC. Through this effort, they identified and subsequently validated DCAF16, a substrate recognition component of the CUL4-DDB1 E3 ligase complex, as the E3 ligase target of KB02 responsible for the degradation activity of their PROTAC. The authors demonstrated that the KB02-based PROTAC was able to degrade its target proteins with the low fractional engagement of DCAF16. With a KB02-based PROTAC linked to JQ1, they also demonstrated the degradation of BRD4.[70]

DCAF15. Since the mid-2000s, sulfonamides have been utilized in the clinic for cancer treatment due to their antitumor activity.[71−73] However, the mechanisms of action of sulfonamide derivatives indisulam, E7820, and chloroquinoxaline sulfonamide were uncharacterized. In 2017, Uehara et al. and Han et al. independently reported that the sulfonamide derivatives form a complex between the coactivator of activating protein-1 and estrogen receptors like RNA-binding protein 39 (RBM39) (also known as CAPERα) and the DCAF15-DDB1-CUL4 complex. The formation of the complex led to the induced ubiquitination and proteasomal degradation of RBM39.[74,75] In 2019, three crystal structures of the RBM39-sulfonamide-DCAF15-DDB1-CUL4 complex were reported.[76−78] In 2020, Li and Chen et al. reported the first DCAF15-targeting PROTAC based on E7820 (Figure 2).[79]

KEAP1. In 2020, Tong, Luo, Maimone, and Nomura et al. demonstrated that the targeted degradation of BRD4 could be enabled by exploiting the triterpene derivative bardoxolone methyl (CDDO-Me), a ligand reported to activate the antioxidant NRF2 pathway by targeting the E3 ligase Kelch-like ECH-associated protein 1 (KEAP1).[80] Whereas DCAF16, RNF4, and RNF114 recruiters acted irreversibly toward their respective E3 ligases, bardoxolone was unique as an E3 ligase recruiter due to its presumed covalent reversible interactions with cysteines on KEAP1. Bardoxolone may also interact with targets beyond KEAP1, and it will be of future interest to better understand the mechanisms through which bardoxolone may act as a recruiter for TPD applications.

DCAF11. In 2021, Zhang and Cravatt et al. discovered another covalent E3 ligase recruiter against the Cullin-RING E3 ligase substrate receptor DCAF11 using a similar approach to
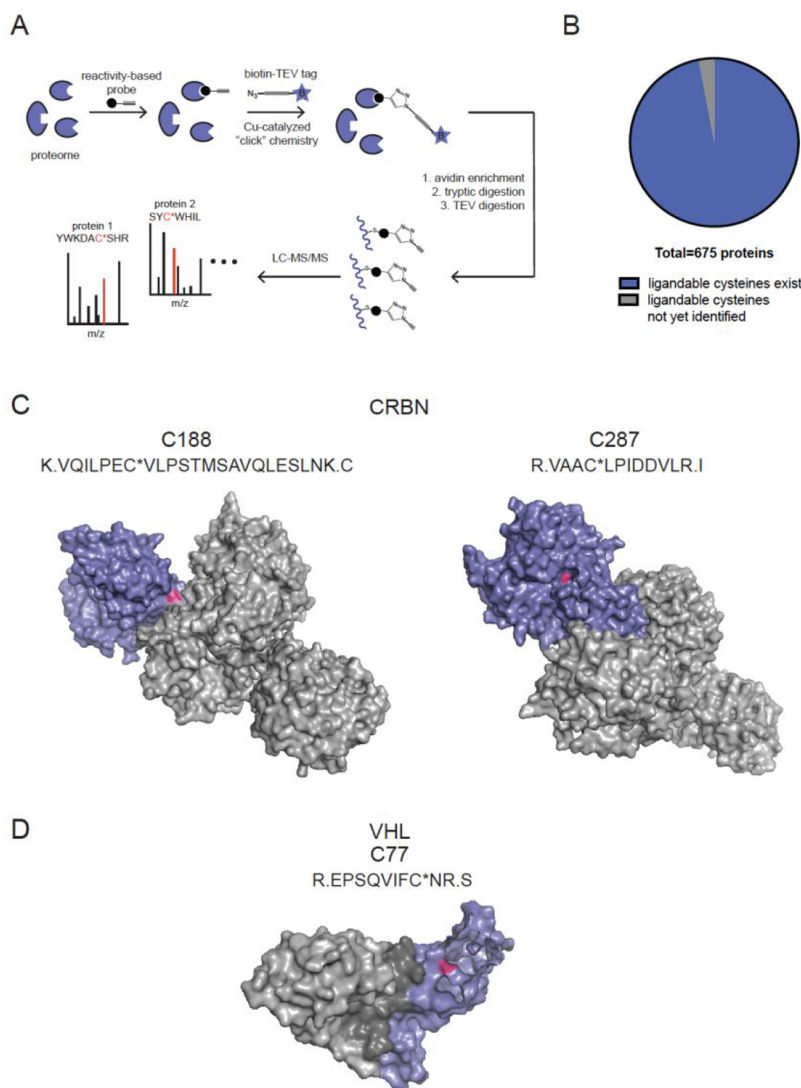
Figure 3. Using chemoproteomic platforms to identify potential reactive and ligandable cysteines across the human E3 ligase family. (A) ABPP-based chemoproteomic approaches using reactivity-based probes. Reactivity-based alkyne-functionalized probes can be used to label reactive cysteines in complex proteomes, after which a TEV protease-cleavable biotin-azide tag can be appended to probe-labeled proteins using copper-catalyzed azide—alkyne cycloaddition (CuAAC). Probe-modified proteins can be avidin-enriched and digested with trypsin, and probe-modified tryptic peptides can be eluted by TEV protease for quantitative proteomic analysis. (B) Aggregating our lab's chemoproteomic data sets of all probe-modified cysteines from ABPP experiments, we have identified probe-modified cysteines across 97% of human E3 ligases. Data can be found in Table S1. (C) Probe-modified tryptic peptides and structure of the reactive cysteines C188 and C287 in CRBN. The structure of CRBN-DDB1 is shown with CRBN in blue, DDB1 in gray, and C188/C287 in magenta (PDB 4TZ4). (D) Probe-modified tryptic peptides and structure of the reactive cysteine C77 in VHL. The structure of VHL-elongin B/C is shown with VHL in blue, elongin B/C in gray, and C77 in magenta (PDB 1VCB).

their strategy for discovering their DCAF16 recruiter.[81] In this study, the authors screened a library of PROTACs consisting of the FKBP12 ligand SLF linked to a wider panel of electrophilic ligands to identify compounds that degraded luciferase-conjugated FKBP12 in a luciferase reporter assay. Through this effort, they identified 21-SLF as a covalent PROTAC that degraded luciferase-FKBP12 in 22Rv1 cancer cells but not in other cancer cell lines tested (Figure 2). Upon pulldown proteomic studies to identify E3 ligase components that were enriched with FKBP12 and PROTAC treatment, they identified and validated DCAF11 as the E3 ligase responsible for the degradation of 21-SLF. Interestingly, the authors showed that whereas this PROTAC caused degradation through targeting C460 on DCAF11, in the absence of this cysteine, C443 and C485 also serve as additional engagement sites that support 21-SLF-induced target degradation. Using this DCAF11 recruiter,

the authors also demonstrated the degradation of the AR in 22Rv1 cells.[81]

FEM1B. Also, in 2021, Henning, Manford, Rape, and Nomura et al. discovered a cysteine-reactive covalent E3 ligase recruiter against the CUL2 E3 ligase FEM1B through a targeted functional screen.[82] FEM1B was recently discovered as a critical regulator of the cellular reductive stress response, a cellular environment where there is a persistent depletion of reaction oxygen stress.[83] Manford and Rape et al. discovered that FEM1B, under reductive stress, recognizes its substrate FNIP1, leading to the FEM1B-dependent ubiquitination and protea-some-mediated degradation of FNIP1 and the restoration of mitochondrial activity and redox homeostasis.[83] In this study, the authors identified a key cysteine residue, C186, that was critical for substrate recognition. To target this substrate recognition cysteine in FEM1B as a possible FEM1B recruit-

ment site, Henning, Manford, Rape, and Nomura et al. performed a fluorescence polarization screen with a cysteine-reactive covalent ligand library to identify compounds that would disrupt FEM1B interactions with a fluorescently tagged FNIP1 degron peptide sequence. Through this screen, the authors identified a covalent ligand EN106 that bound to C186 of FEM1B to displace FEM1B−FNIP1 degron interactions (Figure 2). They subsequently linked this EN106 recruiter to JQ1 to demonstrate selective FEM1B-dependent BRD4 degradation in cells.[82] This study highlighted the utility of covalent ligand libraries coupled to functional E3 ligase-substrate degron displacement screens to discover new recruiters against specific E3 ligases of interest.

DISCOVERY STRATEGIES FOR NEW E3 LIGASE RECRUITERS

Despite the significant advances made in recent years, only a handful of E3 ligase recruiters exist for the >600 E3 ligases that can potentially be exploited for targeted protein degradation or other induced proximity paradigms. As has already been described in the above examples, chemoproteomic platforms and covalent ligand discovery approaches have arisen as powerful strategies for discovering new E3 ligase recruiters and more broadly for discovering covalent ligands and ligandable sites across the wider proteome. One particularly useful chemoproteomic strategy has been the ABPP approach. ABPP uses activity-based or reactivity-based chemical probes to profile proteome-wide reactive, functional, and ligandable sites directly in complex biological systems.[84−87]

Weerapana and Cravatt et al. demonstrated in 2010 that broadly promiscuous cysteine-reactive probes could be used in complex proteomes using a quantitative proteomic platform, termed isotopic tandem orthogonal proteoslysis-ABPP (iso-TOP-ABPP), to identify solvent-accessible cysteines and hyper-reactive cysteines that were enriched in functional sites or binding pockets due to the local protein microenvironment.[88] Since this discovery over 10 years ago, quantitative mass-spectrometry-based ABPP platforms have been coupled to covalent ligand screening paradigm targeting to radically expand the scope of proteome-wide ligandability. Starting with discoveries from Wang and Cravatt et al. showing that isoTOP-ABPP could be used in a competitive format with competing lipid electrophiles to enable target identification[89] and Backus and Cravatt et al. demonstrating that this platform could be used to identify more drug-like covalent ligands against a broad swatch of ligandable cysteines in the proteome,[90] there have been an increasing number of studies using chemo-proteomics-enabled covalent ligand discovery approaches against cysteines, lysines, and other amino acids to enable ligand discovery against the proteome.[69,91−99]

As previously described in this Perspective, chemoproteo-mics-enabled covalent ligand discovery platforms, whether through target-based or target discovery approaches, have greatly enabled the expansion of E3 ligase recruiters for targeted protein degradation and PROTAC applications, including covalent recruiters against RNF4, DCAF16, RNF114, DCAF11, and FEM1B.[65,69,81,82,100] Recently, Henning and Nomura et al. extended this approach to develop a deubiquitinase recruiter against OTUB1 and showed proof-of-concept for DUBTACs for targeted protein stabilization by stabilizing cystic fibrosis transmembrane conductance regulator (CFTR).[12] As such, isoTOP-ABPP and other chemoproteo-mics-enabled covalent ligand discovery platforms are powerful

approaches for uncovering unique ligandable sites and corresponding ligands to enable TPD, PROTACs, and other induced proximity-based therapeutic modalities.

Our lab, like many others in the TPD field, has been performing many ABPP-based chemoproteomic experiments using reactivity-based chemical probes for various target identification, target engagement, and selectivity profiling studies of covalently acting small molecules, accumulating large data sets that include a large amount of probe-modified peptide data from human proteomes. To better enable the mining of potential reactive cysteines that may exist across the human E3 ligase family of proteins, we have aggregated all of our chemoproteomic data from 455 distinct experiments using the alkyne-functionalized iodoacetamide probe and the isoTOP-ABPP method first reported by Weerapana and Cravatt et al.[88] across various human cell line proteomes. We have quantified the total number of spectral counts for each tryptic peptide identified within each E3 ligase family member across all experiments (Figure 3A; Table S1). These include data from our research group's published papers and our currently unpublished studies.[12,65,68,69,96,97,99,101−109] We show reactive cysteines identified across a representative set of E3 ligases in Table 1.

Across 675 E3 ubiquitin ligases, substrate receptors, and related components mined, we identified probe-modified cysteines across 653 of these proteins. These sites of probe labeling represent potential solvent-accessible reactive cysteines that exist across 97% of E3 ligases (Figure 3B). We have included Table S1 that includes all probe-modified sites within these 653 proteins identified from our aggregate chemo-proteomic data with the individual peptides and sites of modification identified for each E3 ligase, the aggregate spectral counts identified for each site, and the number of experiments in which the particular probe-modified tryptic peptide has been observed. There are several caveats to this list of probe-modified sites within human E3 ligases that we point out here before the further interpretation of these data below. First, these 675 proteins mined do not represent the total list of all human E3 ligases. There are likely many more E3 ligases that we failed to mine here. Second, whereas we believe that within these data there exist many potential ligandable cysteines within E3 ligases, there is also likely a large fraction of these sites that do not represent true ligandable sites and may just represent surface cysteines that are not part of binding pockets. Third, there are very likely many reactive and ligandable cysteines within E3 ligases that are not represented in this list. This may be for many reasons, including E3 ligases that may (1) be of low abundance, (2) not be expressed in the cell lines profiled to date, (3) show poor ionization of tryptic peptides, (4) show poor reactivity with the iodoacetamide probe, or (5) show destabilization, unfolding, or aggregation upon cell lysis. Fourth, this list includes only probe-modified cysteines and does not include other amino acids that can potentially be interrogated by other reactivity-based probes. These data merely represent an aggregate list of probe-modified cysteines wherein we have quantified the number of times each particular probe-modified tryptic peptide has appeared across our group's internal chemoproteomic data sets.

To identify potentially interesting cysteines that could be targeted by covalent ligands, we postulated that probe-modified cysteines within E3 ligases that showed higher aggregate spectral counts for a particular cysteine compared with other sites within the same E3 ligase may represent more reactive and potentially

Table 1. Representative List of Reactive Cysteines within E3 Ligases[a]

E3 ligase	UniProt ID	reactive cysteine(s)
RNF114	Q9Y4L5	C8,[b] C110
RNF4	P78317	C132/C135[b]
DCAF16	Q9NXF7	C177 or C179[b]
DCAF11	Q8TEB1	C460[b]
FEM1B	Q9UK73	C186[b]
CRBN	Q96SW2	C188,[c] C287
VHL	P40337	C77[c]
RNF2	C99496	C72[c]
RNF14	Q9UBS8	C262,[c] C417
RNF20	Q5VTR2	C383, C905, C924
RNF25	Q96BH1	C316[c]
RNF31	Q96EP0	C504,[c] C551
RNF111	Q6ZNA4	C968[c]
RNF113A	O15541	C15[c]
RNF123	Q5XPI4	C461[c]
RNF126	Q9BV68	C32
RNF128	Q8TEB7	C15[c]
RNF130	Q86XS8	C320[c]
RNF149	Q8NC42	C295[c]
RNF167	Q9H6Y7	C271[c]
RNF180	Q86T96	C28[c]
RNF213	Q63HN8	C614[c]
UBR1	Q8IWV7	C180, C1603[c]
UBR2	Q8IWV8	C1360, C1619,[c] C576
UBR4	Q5T4S7	C1358, C2554,[c] C4049, C2222, C1274
ZNF598	Q86UK7	C456[c]
KCTD21	Q4G0X4	C166[c]
KBTBD7	Q8WVZ9	C527/C529[c]
KLHL20	Q9Y2M5	C356[c]
KLHL24	Q6TFL4	C359[c]
KEAP1	Q14145	C23, C288, C196, C319
FBXL18	Q96ME1	C468[c]
FBXL7	Q9UJT9	C386/C401[c]
FBXW8	Q8N3Y1	C579[c]
FBXO10	Q9UK96	C430[c]
FBXO22	Q8NEZ5	C228[c]
FBXO30	Q8TB52	C592,[c] C433, C723/C725
FBXO9	Q9UK97	C68[c]
SKP1	P63208	C120, C160
SKP2	Q13309	C113,[c] C240, C205, C345
ASB6	Q9NWX5	C153[c]
ASB9	Q96DX5	C216[c]
RAB40C	Q96S21	C159[c]
NOSIP	Q9Y314	C8[c]
PRPF19	Q9UMS4	C298,[c] C230, C351
STUB1	Q9UNE7	C199,[c] C48
UBE4A	Q14139	C79,[c] C989, C1002
DCAF5	Q96JK2	C503[c]
DCAF7	P61962	C61,[c] C120
DCAF8	Q5TAQ9	C297, C272
DCAF13	Q9NV06	C190, C215, C120

[a]Reactive cysteines annotated are derived from the aggregate chemoproteomic data in Table S1. Listed cysteines show at least seven aggregate spectral counts for the particular site. [b]From experimental data from Zhang, Cravatt et al. 2019; Spradlin, Nomura et al. 2019; Ward, Nomura et al. 2019; Zhang, Cravatt et al. 2021; or Henning, Nomura et al. 2021.[65,66,81,82,100] [c]Represents the dominant probe-modified site over other sites identified in the same E3 ligase from the aggregate chemoproteomic data in Table S1.

more ligandable cysteines. Caveats to this premise include cysteines located in regions within a protein sequence that do not yield suitable tryptic peptides with respect to the ionization and compatibility with mass spectrometry-based sequencing and the labeling of surface-exposed cysteines that may not be part of binding pockets. However, we conjectured that the aggregate chemoproteomic data would still yield potential ligandable sites within E3 ligases that may be of interest to the TPD community.

Consistent with this premise, if we assess RNF114, a RING E3 ligase for which we have previously identified nimbolide and EN219 as covalent recruiters that can be used in PROTAC applications,[68,69] we observe nine probe-modified cysteines— C8, C49, C32/C52, C64/C67, C110, C173, and C176. However, among these nine sites, C8 collectively shows 1641 aggregate spectral counts over the next most recognized site C110 with 40 spectral counts (Table S1). Both nimbolide and EN219 react specifically with C8 on RNF114. However, this does not always correlate. For example, in DCAF16, we observe three probe-modified cysteines, C119, C173, and C179, where C119 shows the highest number of aggregate spectral counts. However, Zhang and Cravatt et al. found that their KB02 likely engages with C177 or C179 and not C119. However, C119 may represent another additional site that could be liganded for DCAF16 recruitment. FEM1B and DCAF11 either have not yet appeared in our chemoproteomic profiling experiments or were captured with low aggregate spectral counts.

Nonetheless, we still believe that this data set likely has many potential entry points into ligand discovery against E3 ligases. For example, in assessing CRBN, an E3 ligase for which IMiDs exist as recruiters but for which a covalent recruiter has not yet been disclosed, we observe four probe-modified cysteines— C188, C287, C326, and C441—in which C188 and C287 show the highest aggregate spectral counts with 422 and 100, respectively, compared with the other sites (Table S1). Interestingly, C188 sits at the interface between CRBN and DDB1, indicating that targeting this site might either disrupt or stabilize CRBN/DDB1 interactions (Figure 3C). In contrast, C287 appears to be in an allosteric site within CRBN that sits at the bottom of defined pocket (Figure 3C). C287 may be a potential ligandable cysteine that can be targeted within CRBN. VHL also possesses a probe-modified cysteine C77 that also appears to be part of a potential binding pocket (Figure 3D).

An important consideration for using this database of potential ligandable cysteines within E3 ligases to prioritize the development of covalent E3 ligase recruiters is the location of the cysteine in the protein. Many E3 ligase recruiters that have been discovered thus far, including those for CRBN, VHL, RNF114, DCAF15, cIAP, and FEM1B, target substrate recognition domains within the E3 ligases. Whether an E3 ligase-targeting ligand needs to bind to a substrate recognition site to be successfully recruited for TPD remains unclear. Targeting allosteric sites within the E3 ligase that are not directly involved in substrate recognition may still yield successful recruitment. However, if the cysteine rests within a known interaction domain between the E3 ligase and an important functional adapter protein necessary for the function of the E3 ligase complex, then these sites would likely not be ideal for recruiter development.

Other important considerations for prioritizing E3 ligases within the database include the cell type, tissue, or cell compartment expression of the E3 ligase in relation to the target of interest if one aims to achieve the location-specific degradation of a protein. Another critical consideration is the

functional role of the individual E3 ligases. Many E3 ligases remain poorly characterized as to their endogenous biochemical, cellular, and physiological functions, and the E3 ligases listed in our Table S1 may cause toxicity upon recruitment, may not be involved in ubiquitin conjugation, and may not confer specific ubiquitin linkages that destine a protein for degradation versus the many other roles of ubiquitin or ubiquitin-like proteins.[110] Some classes of E3 ligases, such as the RBR proteins, are highly regulated and are autoinhibited and may not be ideal or more complicated for recruitment and TPD.[111] The ligandability and the ability to develop potent and selective chemical matter for a particular E3 ligase must be balanced with these aforementioned aspects associated with the biology of each E3 ligase.

Hundreds of additional probe-modified cysteines exist across the >600 human E3 ligases mined here against our lab's aggregate chemoproteomic data. Many of these sites may represent unique ligandable sites that can be accessed with covalent ligand discovery approaches, and we encourage the ubiquitin proteasome and targeted protein degradation fields to mine this database and deploy covalent ligand screening platforms to expand the arsenal of E3 ligase recruiters that can be exploited in PROTACs and molecular glue degraders.

CONCLUSIONS AND FUTURE DIRECTIONS

In this Perspective, we have summarized the E3 ubiquitin ligases that have been successfully recruited and manipulated to date and present our aggregate chemoproteomic data of reactive cysteines that have been identified across the human E3 ligase family of over 600 members.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.1c00464.

Description of Table S1 and supplementary references (PDF)

Table S1. Aggregate quantified chemoproteomic data of probe-modified peptides for E3 ligases and other components of the ubiquitin proteasome machinery (XLSX)

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