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Expanding the landscape of E3 ligases for targeted protein degradation

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ABSTRACT

Targeted protein degradation (TPD) is a rapidly developing field in chemical biology and drug discovery. Various TPD modalities have emerged, with proteolysis-targeting chimeras (PROTACs) being the most well-developed at present. In PROTACs, a heterobifunctional molecule is used to recruit an E3 ligase to degrade a protein of therapeutic interest. Most of the PROTAC candidates that have been developed thus far use either CRBN or VHL as the hijacked E3 ligase, which poses several limitations. In order to overcome these limitations and furthermore realize the full potential of TPD as a therapeutic modality, the field will need to unlock additional E3 ligases. This review will therefore present 11 alternative E3 ligases for TPD. It will also describe some of the ongoing platform development that is facilitating the discovery of additional E3 ligases for TPD.

Targeted protein degradation (TPD) has emerged as a new therapeutic strategy to expand the druggable proteome (Schapira et al., 2019). In TPD, a compound induces proximity between a target protein and an effector protein to initiate either proteasome- or lysosome-mediated protein degradation (Ding et al., 2020). As a technology that opens up this avenue, proteolysis targeting chimeras (PROTACs) consist of two moieties connected by a linker and induce ternary complex formation by simultaneously binding the E3 ligase and the target protein, leading to ubiquitination and degradation of the target protein (Fig. 1). This modality presents several advantages over traditional small molecule drugs. For instance, PROTACs require only target engagement rather than functional activation or inhibition and can therefore bind at silent binding pockets on proteins that would be 'undruggable' through conventional means. PROTACs also have the potential to degrade proteins possessing multiple functional domains, which are challenging to target with traditional small molecule drugs.

Much of the research in TPD thus far has focused on identifying new ligands for proteins of therapeutic interest. PROTACs have been used to degrade over 60 unique proteins, and some candidates, such as B-cell lymphoma (BCL)-XL degraders and androgen receptor (AR) degraders, have begun clinical trials (Mullard, 2021). Most of these reported PROTACs recruit E3 ligase CRBN or VHL, with 30+ proteins being degraded through CRBN and 20+ through VHL (Bricelj et al., 2021).

One potential disadvantage of relying solely on CRBN or VHL for PROTAC development is the emergence of drug resistance. In a 2019 work by Zhang et al., treatment of tumor cells with BET-based PROTACs that engage CRBN or VHL failed to completely eliminate tumor cells. In subsequent genomic analysis, surviving tumor cells carried gene alterations on CRBN or VHL complexes, indicating that PROTAC resistance may arise from mutations in the degradation machinery itself rather than the protein target (Zhang et al., 2019a). Another challenge in PROTAC development is inducing ternary complex formation. The chimeric small molecule typically needs to form a ternary complex between the E3 ligase and its neo-substrate protein which persists long enough for the E3 ligase to ubiquitinate its substrate (Zaidman et al., 2020). Although CRBN and VHL have been shown to ubiquitinate a wide variety of substrate proteins, some protein-ligase pairs do not persist long enough for ubiquitination to occur (Gadd et al., 2017). In order to ensure that these proteins can be degraded by PROTACs, it is important to identify alternative hijackable E3 ligases. Alternative E3 ligases also offer several distinct advantages. E3 ligases are expressed heterogeneously and their expression can vary with tissue type, tumor type, cellular compartment, or cell state. Consequently, recruiting E3 ligases with a specific expression profile could lead to the development of exquisitely specific PROTACs for precision medicine. In addition, recruiting essential E3 ligases for TPD may avoid gene alteration-mediated drug resistance. In light of these potential advantages, this review will therefore focus on 11 alternative E3 ligases for TPD (Fig. 2) as well as technologies that will facilitate the ongoing search for additional E3 ligases.

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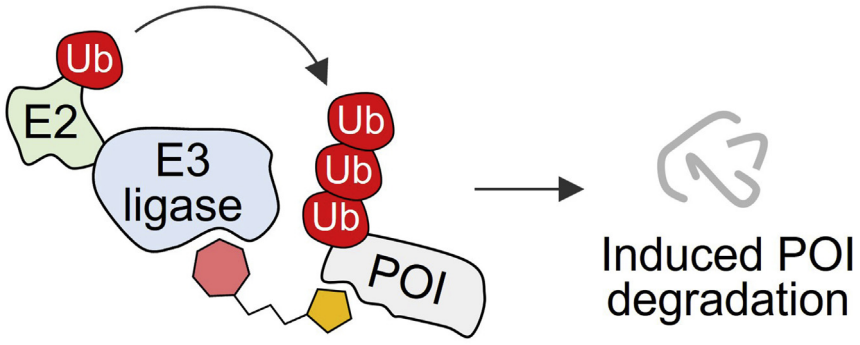

Fig. 1. Schematic representation of PROTAC-mediated ubiquitination and subsequent degradation of protein of interest (POI).

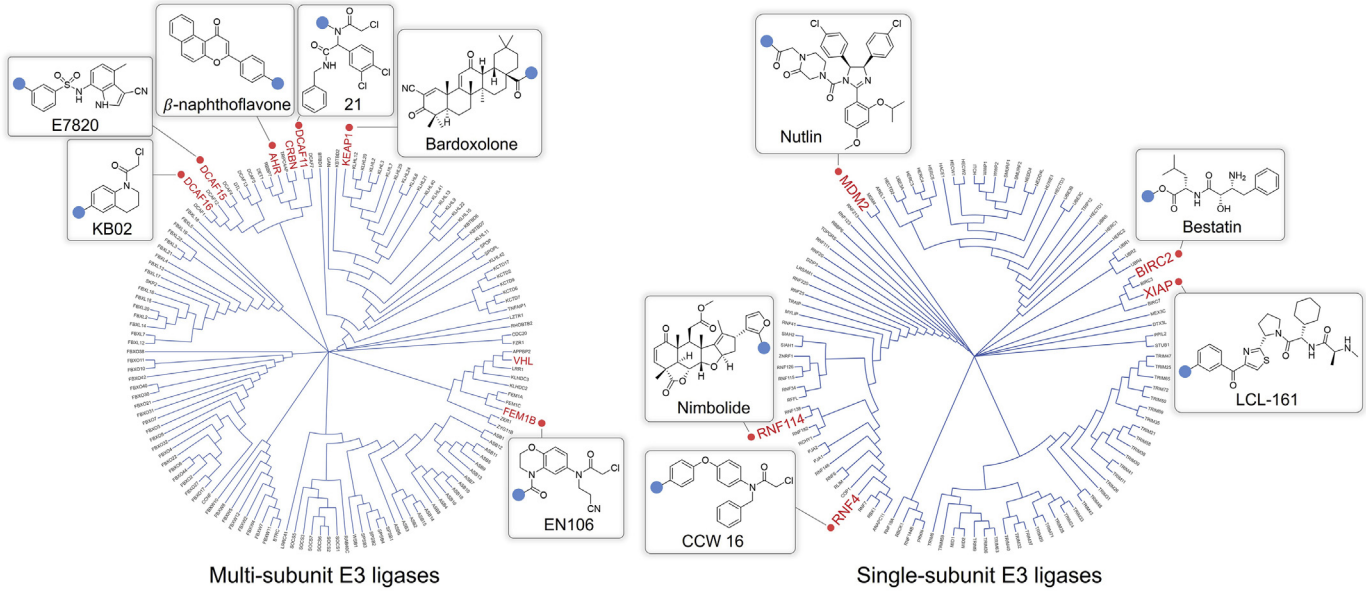

Fig. 2. E3 ligases that have been demonstrated to support PROTAC-induced target degradation. Multi-subunit and single-subunit E3 ligases involved in the ubiquitin-proteasome system (UPS) were extracted in UbiHub (confidence ≥3) (Liu et al., 2019). Red-highlighted E3 ligases have been validated to support PROTAC-induced target degradation. Their representative ligands are shown in the corresponding box (the blue dot indicates the linker attached position).

1. PROTACs discovered through previously reported ligands

1.1. MDM2

MDM2 is an E3 ligase that recognizes and degrades the tumor suppressor p53. The synthetic small molecule class nutlins were developed to disrupt this interaction, thereby stabilizing p53 and promoting p53-mediated apoptosis. In 2008 Schneekloth et al. reported a PROTAC conjugating nutlin to the non-steroidal AR ligand for TPD, making it the first all-small molecule PROTAC reported (Schneekloth et al., 2008). Recently, MDM2 has re-emerged as an E3 ligase of interest in the context of the search for alternative E3 ligases. In 2019 Hines et al. reported that the nutlin-based PROTAC A1874 is capable of recruiting MDM2 to degrade BRD4 and also retains nutlin's function of stabilizing p53 (Hines et al., 2019), leading to synergy between BRD4 degradation and p53 stabilization.

1.2. cIAP1 and XIAP

The E3 ligase cIAP1 (also known as BIRC2) inhibits apoptosis by ubiquitinating its substrate RIP1, and its overexpression has been linked to several cancer types. The ligand methyl bestatin (MeBS) prevents cIAP1 from associating with RIP1 and also induces cIAP1 auto-ubiquitination and degradation (Sekine et al., 2008). A PROTAC conjugating MeBS and the CRABP2 ligand all-*trans*-retinoic acid (ATRA) was developed to recruit cIAP1 for ubiquitination and subsequent degradation of CRABP1 and CRABP2 (the authors dubbed this class of PROTACs specific and non-genetic inhibitor of apoptosis protein-dependent protein erasers, or SNIPERs) (Itoh et al., 2010). Notably, because MeBS-based PROTACs also promote apoptosis by blocking cIAP1-induced RIP1 ubiquitination, it is thought that these PROTACs exert their antitumor efficacy through two distinct mechanisms. XIAP, another IAP family protein, has analogously been demonstrated to support LCL-161-based PROTAC-mediated degradation of estrogen receptor α (ERα) (Ohoka et al., 2017).

1.3. AhR

The aryl hydrocarbon receptor (AhR) has dual functionality as both an E3 ligase and a transcription factor controlling diverse cellular responses (Ohtake et al., 2007). In 2019 Ohoka et al. linked the previously reported AhR ligand β-naphthoflavone (NF) to the CRABP2 ligand ATRA to induce degradation of CRABP2 (Ohoka et al., 2019). A β-NF-JQ1 conjugate was also developed in the same study to induce degradation of the BET bromodomain proteins BRD2, BRD3, and BRD4. In addition, a

PROTAC substituting an alternative AhR ligand ITE induced degradation of CRABPs, indicating the versatility of AhR as an E3 ligase for TPD.

1.4. KEAP1

KEAP1 is an E3 ligase that participates in the oxidative stress response by regulating the ubiquitination and subsequent degradation of transcription factor NRF2, a master regulator of anti-oxidative responses, and has also been shown to support TPD by a peptide-based PROTAC (Lu et al., 2018). Tong et al. used the terpenoid derivative methyl bardoxolone, a reversible covalent binder of KEAP1, to degrade BRD4 (Tong et al., 2020). In another work (Wei et al., 2021), Wei et al. harnessed a noncovalent KEAP1 binder MS83 and showed that MS83-based PROTAC induced BRD4 degradation.

1.5. DCAF15

DCAF15 is a poorly understood E3 ligase and was recently found to play a critical role in suppressing natural killer cell-mediated cancer cell clearance. In 2017 Han et al. found that anticancer sulfonamides dimerize DCAF15 and the RNA splicing factor protein RBM39, leading to the ubiquitination and degradation of RBM39 (Han et al., 2017). Inspired by this finding, Li et al. leveraged sulfonamide E7820 and generated a PROTAC that induces DCAF15-mediated degradation of BET proteins BRD2, BRD3 and BRD4 (Li et al., 2020).

2. PROTACs discovered through novel ligand identification

Each of these six E3 ligases (MDM2, cIAP1, XIAP, AhR, KEAP1 and DCAF15) has previously identified ligands which make it possible to harness them for PROTAC development. Of the 600+ human E3 ligases, the majority do not have reported ligands. Thus, new technologies have been developed to discover novel E3 ligands and subsequently incorporate them into functional PROTACs.

One strategy for identifying E3 ligase ligands is activity-based protein profiling (ABPP), a competition-based chemoproteomic approach that enables quantitative and site-specific ligand-target interaction profiling on a proteome-wide scale (Fig. 3) (Vinogradova et al., 2020). The identified E3-ligand pairs can be further tested for bifunctional PROTAC-mediated TPD. In addition to ABPP, a 'function-first' strategy has also been developed to simultaneously discover E3 ligases and their paired ligands for TPD. In this strategy, a focused bifunctional compound library is generated by conjugating structurally diverse moieties to a selective ligand for a protein of interest (such as SLF for FKBP12). Cells are then treated with these compounds and analyzed via a degradation assay to identify hits that induce target degradation. Subsequent target deconvolution approaches such as Affinity Purification Mass Spectrometry (AP-MS) are used to identify the E3 ligases that support the hit compound-induced target degradation (Fig. 3). Several hijackable E3 ligases have been identified using these strategies.

2.1. RNF4

RNF4 is a poorly understood E3 ligase and may be involved in spermatogenesis. Ward et al. used an ABPP approach to search for covalent ligands against RNF4 and identified the chloroacetamide-bearing TRH 1-23, which engages RNF4 through two cysteine residues (Cys132 and Cys135) (Ward et al., 2019). This compound was subsequently optimized and coupled to JQ1 to give CCW 28-3, which recruits RNF4 to degrade BRD4. CCW 28-3 was evaluated for off-target degradation and found to substantially degrade four additional non-BRD4 proteins.

2.2. RNF114

The terpene natural product nimbolide is known to exhibit anticancer activity. In a 2019 study, by using ABPP, Spradlin et al. identified the E3 ligase RNF114 as the target of nimbolide (Spradlin et al., 2019). They then harnessed nimbolide to recruit RNF114 for BRD4 degradation. In another study, Luo et al. screened a library of electrophiles and identified EN219 as a non-natural product-based ligand for RNF114 that covalently engages Cys8, the same cysteine on RNF114 targeted by nimbolide (Luo et al., 2021). EN219-based PROTACs were further shown to degrade BRD4 and the oncogenic fusion protein BCR-ABL.

2.3. FEM1B

The E3 ligase FEM1B was previously shown to degrade its substrate FNIP1 as part of the reductive stress response. An ABPP approach was used to identify covalent ligands for FEM1B. The chloroacetamide-bearing EN106 was shown to covalently engage C186 on FEM1B and also disrupt FEM1B binding to FNIP1 (Henning et al., 2021). A PROTAC that conjugates EN106 to JQ1 was further shown to degrade BRD4. A proteome-wide analysis showed that one additional protein, PNMAL1, was substantially degraded, indicating that the EN106-JQ1 PROTAC induces minimal off-target degradation.

2.4. DCAF16

DCAF16 is an uncharacterized E3 ligase, although a recent GWAS study showed that it is associated with lung cancer risk. In a 2019 study, we reported the use of heterobifunctional small molecules to recruit DCAF16 for TPD (Zhang et al., 2019b). In this study the FKBP12 ligand SLF was coupled to electrophilic 'scouts' and a functional assay was used to identify compounds capable of inducing FKBP12 degradation. An affinity purification approach was then used to identify the E3 ligase that associates with FKBP12 in a ligand-dependent manner, which led to the identification of DCAF16. Notably, DCAF16 appears to be a nuclear E3 ligase that supports ligand-induced degradation of nuclear proteins. The DCAF16 ligand KB02 was then coupled to JQ1 to induce degradation of the nuclear protein BRD4.

2.5. DCAF11

DCAF11 is an E3 ligase that is known to degrade p21, a cell cycle checkpoint protein dysregulated in osteosarcoma. Using a similar strategy to the 2019 work that discovered DCAF16, we generated a focused FKBP12-directed bifunctional compound library. These compounds were screened in four cancer cell lines expressing luciferase-fused FKBP12 and one compound, 21-SLF, was found to induce FKBP12 degradation. A similar affinity purification-mass spectrometry strategy identified the E3 ligase DCAF11 that supports 21-SLF-mediated FKBP12 degradation. Another PROTAC that conjugates DCAF11 ligand to androgen receptor ligand (ARL) was subsequently synthesized and shown to degrade AR in prostate cancer cell line 22Rv1 (Zhang, 2021).

3. Outlook and perspectives

Although PROTAC-induced target degradation through CRBN and VHL is widely applicable and effective, the potential for drug resistance to these two E3 ligases in tumors has already been shown, indicating the need to discover alternative E3 ligases for TPD. Alternative E3 ligases also pose several potential benefits, including specificity for tissue, tumor, cell type, or cell state; as well as producing synergistic antitumor effects secondarily through disinhibition of pro-apoptotic cell cycle regulatory proteins.

In the search for alternative E3 ligases for TPD, two primary strategies have been pursued: "ligand first" and "ligase first." In the first strategy, an E3 ligase is recruited via its known ligand, which is conjugated to a second ligand for a protein of interest. While this approach has resulted in the discovery of several additional E3 ligases for TPD, it relies on previously reported ligand-ligase associations and therefore does not propose a schedule for deliberately targeting the remaining 600+ human E3 ligases.

In the second strategy, an E3 ligase of interest is queried using chemoproteomic, rational design or ligand discovery approaches to identify a new ligand (Fig. 3), which is then paired with the known protein ligand for targeted degradation. Thus far, ABPP has chiefly been used for ligand-cysteine interaction studies. Consequently, many of the ligands discovered via this strategy are electrophiles that recruit E3 ligases through cysteines (CCW16-RNF4, nimbolide-RNF114 and EN106-FEM1B). One caveat is that as a competition-based method, ABPP may not capture low stoichiometric ligand-ligase interaction. A 'function-first' approach has been developed to identify ligand-induced target degradation that proceeds with substoichiometric E3 engagement and results in the identification of two E3 ligases for TPD (DCAF16 and DCAF11), suggesting that this approach could potentially be more sensitive than ABPP at detecting weaker ligand-ligase interactions. ABPP and function-first screening could therefore be complementary approaches in the search for additional E3 ligases.

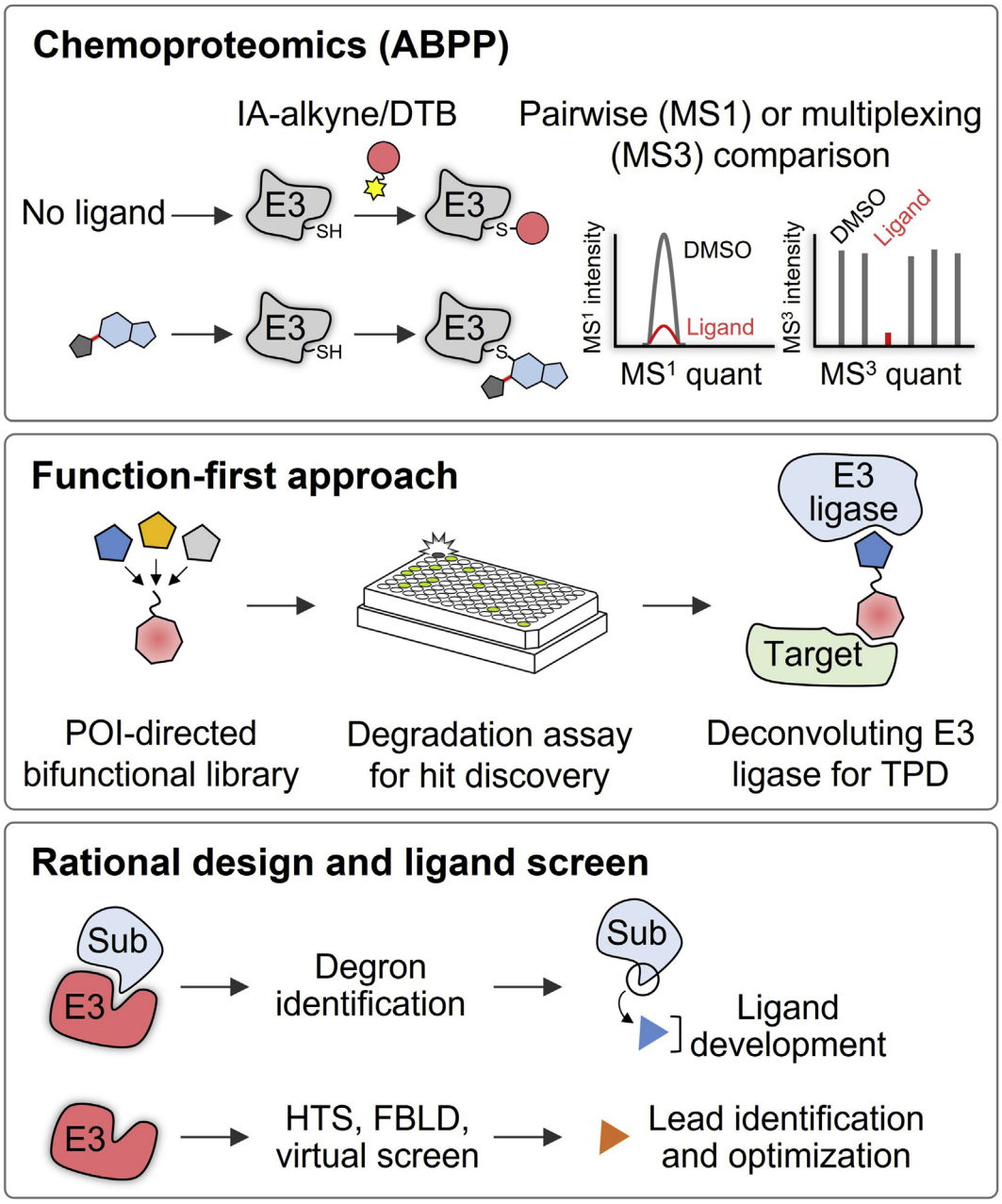

Fig. 3. Strategies for identifying alternative E3 ligases for TPD. In the ABPP approach (top panel), a broad-spectrum cysteine reactive probe with a clickable (IA-alkyne) or affinity (IA-DTB) handle is used to capture proteins bearing reactive cysteines. Affinity purification combined with trypsin digestion will generate probe-modified cysteine containing peptides with signature MS1 or MS3 spectra, which will allow the assessment of cysteine reactive electrophiles in a site-specific manner. In a function-first strategy (middle panel), a focused heterobifunctional compound library is generated by conjugating structurally diverse moieties to a ligand that selectively binds to POI. A degradation assay (for example, Western blotting analysis or luciferase assay) is performed to identify hit compounds that degrade the POI. Proteomics (affinity purification mass spectrometry) or genetic (CRISRP knockout screen) approaches will then be employed to deconvolute the E3 ligases that support ligand-induced degradation of POI. In rational design strategy (bottom panel), the degron sequence from the endogenous protein substrate of an E3 ligase is identified, followed by a medicinal chemistry campaign that imparts the drug-like property to the E3 ligand. In the ligand screen strategy (bottom panel), HTS, FBLD or a virtual screen is performed to identify lead compounds that bind to the E3 of interest. Further medicinal chemistry will be carried out in order to improve the selectivity and potency of the lead compounds. Sub, substrate. HTS, high-throughput screening. FBLD, fragment-based lead discovery. ABPP, Activity-Based Protein Profiling. IA, iodoacetamide. DTB, desthiobiotin.

Electrophilic PROTACs could potentially pose several advantages: they induce the formation of a pseudo-binary complex rather than a ternary complex as in traditional PROTACs, which could improve kinetic stability and enhance degradation. Moreover, in the case of DCAF16 and DCAF11, target degradation was shown to proceed with low fractional E3 engagement, indicating that those PROTACs may support the degradation of target proteins while minimally perturbing endogenous substrates of the recruited E3. However, there are also potential disadvantages of leveraging covalent ligand-ligase interaction for TPD. Electrophilic PROTACs could be metabolically labile, and also show general toxicity at higher concentration, which could limit their therapeutic window.

As the field continues to explore alternative E3 ligases, several considerations will become important. For instance, for E3 ligases that have solely been shown to degrade BRD4, more work is needed to demonstrate their capability of degrading additional proteins of therapeutic interest. Although BRD4 is a protein of therapeutic interest, it also may be easier to destabilize than other proteins even within the same bromodomain (BRD) family. Thus, one important consideration preceding clinical applications is to confirm the versatility of each E3 system degrading non-BRD4 proteins.

Another important consideration is the need for additional screening platforms to discover more E3 ligases for TPD. Covalent functionalization followed by E3 electroporation ('COFFEE') was recently elaborated to identify alternative E3 ligases with ligandable cysteines for TPD (Pinch, 2022). Another strategy for future work could involve expanding the scope of ABPP to screen novel ligands for binding to other nucleophilic residues, including serine, lysine, and tyrosine. This approach could probe underexplored, cysteine-poor regions on the surfaces of E3 ligases and dramatically expand the landscape of E3 ligases which might be inaccessible through the current cysteine-based methodologies. Collectively, these approaches are anticipated to continue to yield new alternative E3 ligases for TPD.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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