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Review

Harnessing the Power of Proteolysis for Targeted Protein Inactivation

Rati Verma,[1,*] Dane Mohl,[1] and Raymond J. Deshaies[1]
[1]AMGEN Research, One Amgen Center Drive, Thousand Oaks, CA 91320, USA
*Correspondence: rverma03@amgen.com
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Two decades into the twenty-first century, a confluence of breakthrough technologies wielded at the molecular level is presenting biologists with unique opportunities to unravel the complexities of the cellular world. CRISPR/Cas9 allows gene knock-outs, knock-ins, and single-base editing at chromosomal loci. RNA-based tools such as siRNA, antisense oligos, and morpholinos can be used to silence expression of specific genes. Meanwhile, protein knockdown tools that draw inspiration from natural regulatory mechanisms and facilitate elimination of native or degron-tagged proteins from cells are rapidly emerging. The acute and reversible reduction in protein levels enabled by these methods allows for precise determination of loss-of-function phenotypes free from secondary effects or compensatory adaptation that can confound nucleic-acid-based methods that involve slow depletion or permanent loss of a protein. In this Review, we summarize the ingenious ways biologists have exploited natural mechanisms for protein degradation to direct the elimination of specific proteins at will. This has led to advancements not only in basic research but also in the therapeutic space with the introduction of PROTACs into clinical trials for cancer patients.

Natural Examples of Targeted Protein Degradation (TPD)

The Ubiquitin-Proteasome and Autophagy Systems

The eukaryotic cellular milieu is in dynamic flux with biomolecules synthesized and degraded continuously. Proteins that are destined to be degraded are targeted to the proteasome by ubiquitin (Ub), which is covalently attached to acceptor lysines on the substrate by a sequential cascade of Ub-activating (E1), Ub-conjugating (E2), and Ub-ligase (E3) enzymes (Figure 1). Ub conjugation can also serve as a signal for the autophagic pathway whereby cytosolic contents destined for degradation are first sequestered in a compartment bounded by a double membrane, after which the outer membrane fuses with the lysosome to deliver the encapsulated vesicle into the lysosome for destruction. Recent reviews have been written on the Ub-proteasome system (UPS) (Bard et al., 2018) and autophagy (Dikic and Elazar, 2018) and thus we will not describe them in further detail here other than to point out that the UPS operates in both the nuclear and cytosolic compartments whereas autophagy operates in the cytosol and can degrade individual proteins, protein complexes, macromolecular aggregates, and even entire organelles. In addition, both systems involve recognition of specific signals on targets and thus can potentially be repurposed to effect the specific elimination of individual proteins (via the UPS) or assemblages (via autophagy) from cells.

Viruses Hijack the UPS to Circumvent Cellular Defenses

A wealth of early molecular studies on DNA tumor viruses indicated that they subvert cellular regulatory pathways by coding for proteins that bind to and inactivate key cellular factors like the tumor suppressor proteins p53 and Rb (Dybas et al., 2018; Mahon et al., 2014). In a landmark study from Peter Howley's group, investigation of the E6 protein of human papillomaviruses HPV-16 and HPV-18 illuminated an unexpected twist on this theme: the E6 protein of these viruses deploys the cellular E6AP (E6-associated protein) Ub ligase to ubiquitinate p53, resulting in rapid p53 degradation by the proteasome (Scheffner et al., 1993).

Another early "lesson from nature" that illustrated the power of hijacking the UPS was courtesy of HIV-1 (human immunodeficiency virus type 1). The viral protein VPU is an integral membrane phosphoprotein that binds to newly synthesized host membrane proteins such as the HIV receptor CD4 and MHC class I molecules. Two conserved phosphoserine residues in the cytoplasmic domain of VPU recruit the F-box protein β-TRCP1 resulting in the formation of a ternary complex comprising CD4, VPU, and Ub ligase $SCF^{\beta\text{-}TRCP}$, which triggers ubiquitination and proteasomal degradation of CD4 (Margottin et al., 1998). It is now appreciated that inducing degradation of cellular proteins is a common strategy employed by a broad range of viruses to subvert cellular regulatory mechanisms and antiviral defenses (Mahon et al., 2014). It is estimated that no fewer than two dozen viruses deploy one or more proteins that serve as a molecular bridge to link a cellular protein target to a Ub ligase. HIV-1 is perhaps the most opportunistic, employing four different proteins (VPU, VPR, VPX, and VIF) to target host proteins for elimination. Apart from $SCF^{\beta\text{-}TRCP}$ (a member of the CRL1 cullin-RING ligase family), VPR and VPX hijack CRL4 and VIF repurposes CRL5 to degrade antiviral proteins (Mahon et al., 2014; Yu et al., 2003). It is particularly instructive that viruses, which are under intense evolutionary pressure and must make do with compact genomes, have repeatedly selected for targeted degradation as a means to circumvent cellular defenses.

SmallMolecule-Induced Degradation of Proteins via the UPS Is a Common Theme in Signaling by Plant Hormones

Auxin (indole-3-acetic acid or IAA) is a plant hormone that regulates plant development by promoting degradation of the

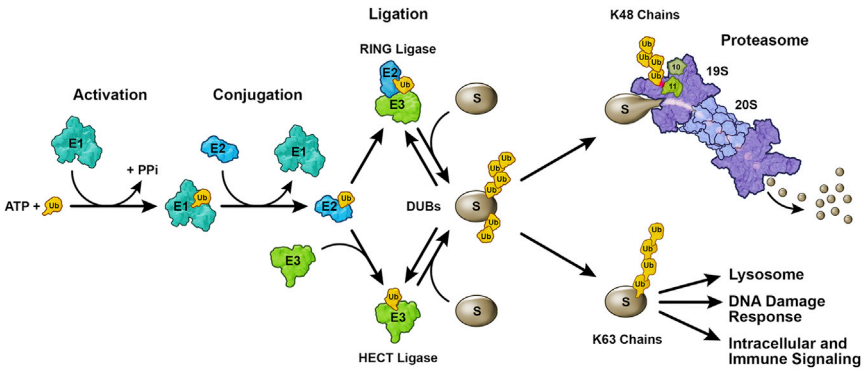

Figure 1. Main Enzymatic Steps in the Ubiquitin-Proteasome System (UPS)

The E1 enzyme activates ubiquitin (Ub) in a two-step ATP-dependent reaction to form a high-energy thioester bond with its active site cysteine and the carboxy terminus of Ub. The thioesterified Ub is then transferred to the active site cysteine of a Ub-conjugating enzyme E2. There are currently 2 E1s and 40 E2s known in mammalian cells. E3 Ub ligases bind to both substrates (S) and E2~Ub thioesters, which results in the transfer of Ub to substrate. Ligases can be classified very broadly into two classes: HECT (homologous to E6AP carboxyl terminus) ligases, which undergo an additional trans-thioesterification, and RING (really interesting new gene) ligases, which don't. About 30 HECT ligases and >500 RING ligases aid in substrate ubiquitination at acceptor lysines by catalyzing formation of an iso-peptide bond between the ε amino group of acceptor lysine and c-terminal Gly 76 of Ub. Ub itself has 7 lysine residues as well as a free amino end. PolyUb chains that are formed via lysine 63 usually confer signaling functions on substrates. Chains formed via lysines 11, 29, or 48 (or branches thereof) generally result in substrate being targeted to the 26S proteasome for degradation. Most RING ligases contain the canonical RING domain although some variants such as those containing the U-box, the B-box, and the RBR (RING-between-RING) are known. 95 deubiquitinase enzymes (DUBs), with varied linkage specificities, can stabilize substrates by removal or trimming of Ub chains. Ubiquitinated substrates are escorted to the 26S proteasome by shuttling receptors or bound directly by intrinsic receptors such as Rpn10 and Rpn13 present in the 19S regulatory cap. An additional region of disorder in the substrate that is translocated into the 20S peptidase core of the proteasome by the 19S ATPases commits the substrate to degradation. The substrate-engaged proteasome undergoes a conformational change that results in removal of the Ub chain en bloc by the intrinsic deubiquitinating enzyme Rpn11. The core peptidase subunits degrade the substrate to generate peptides that are processed before presentation by MHC class I molecules.

Aux/IAA family of transcriptional repressors via the Ub ligase SCF^{TIR1}. Auxin binds directly to the F-box substrate receptor TIR1 (Dharmasiri et al., 2005; Kepinski and Leyser, 2005) and stabilizes association of Aux/IAA substrates—which share a homologous "auxin-inducible degron" (AID)—with SCF^{TIR1}, resulting in ubiquitination and degradation of the Aux/IAA proteins (Figure 2). X-ray crystallography of a TIR1-auxin-AID ternary complex revealed that auxin occupies a cavity in the Aux/IAA binding site of TIR1, and thereby creates a platform that makes contact with and stabilizes binding of the AID (Tan et al., 2007). Even more remarkable is that the plant hormone jasmonate works by an identical mechanism through the F-box substrate receptor COI1 (Thines et al., 2007), and strigolactones, salicylic acid, and cytokinins also manipulate protein degradation in different ways (Larrieu and Vernoux, 2015). Together with the data on viruses summarized above, these examples illustrate the extraordinary potential of using small molecules and engineered proteins to induce targeted degradation of proteins in either the therapeutic or research space.

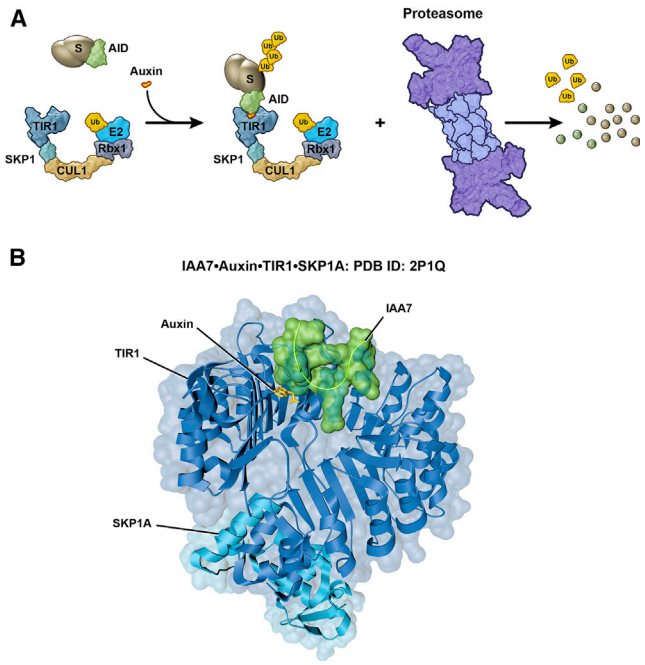

Figure 2. The Auxin Inducible Degron (AID) Promotes Degradation of Intracellular Proteins

(A) Treatment of cells with auxin promotes formation of a ternary complex that contains auxin, the TIR1 substrate receptor subunit of an SCF Ub ligase complex, and an endogenous or engineered substrate (S) that contains an auxin-inducible degron (AID). This results in SCF^{TIR1}-dependent ubiquitination of the AID-containing protein marking the substrate protein for proteasomal degradation. SKP1, CUL1, and RBX1 are other subunits of the Ub ligase SCF^{TIR1}.
(B) PDB structure 2P1Q showing ternary complex formation.

Targeted Degradation of Native Proteins with Engineered Small Molecules

PROTACs: Chimeric Small Molecules that Recruit a Ub Ligase to a Cellular Protein to Trigger Its Ubiquitination and Degradation

The examples provided by viral hijacking of the UPS inspired an effort to develop bi-specific small molecules that could mimic the action of E6 and VPU and induce proximity of a specific cellular "protein of interest" (POI) with a Ub ligase. At the time, the limiting factor was the paucity of portable, well-defined ligands for Ub ligases. As proof of concept, a ten amino acid phosphopeptide degron from IkBα that binds the β-TRCP substrate receptor subunit of Ub ligase $SCF^{β\text{-}TRCP}$ (Winston et al., 1999; Yaron et al., 1998) was covalently linked to ovalicin, a small molecule inhibitor of methionine aminopeptidase-2, to yield PROTAC-1, for PROteolysis Targeting Chimeric molecule #1 (Figure 3). Strikingly, PROTAC-1 induced ubiquitination and degradation of methionine aminopeptidase-2 in frog egg extracts, thus demonstrating for the first time the feasibility of exploiting Ub ligases to deliberately program elimination of a

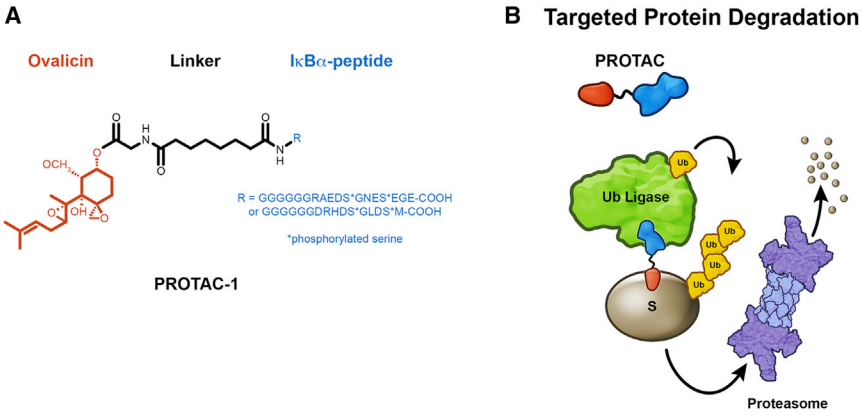

Figure 3. Chimeric Small Molecules Termed PROTACs Recruit Intracellular Proteins to Ub Ligases

(A) The first PROTAC, PROTAC-1, consisted of the MetAP2 covalent inhibitor ovalicin linked via an aminohexanoic acid linker and six glycine residues to the phosphoSer-containing decapeptide of IkBα that is recognized by $SCF^{\beta\text{-}TRCP}$.

(B) Schematic of PROTACs that are heterobifunctional molecules comprising an E3 ligand linked to a substrate protein ligand. The induced proximity between E3 and substrate (S) results in ubiquitination and proteasomal degradation of the latter.

specific cellular substrate with an engineered compound (Sakamoto et al., 2001). The same approach was employed to target the estrogen (ER) and androgen (AR) receptors for degradation (Sakamoto et al., 2003). Shortly after the original Sakamoto et al. (2001) report was published a patent was issued that proposes the use of peptides that bind the Ub ligase UBR1 linked to different ligands to trigger ubiquitination and degradation of target proteins. However, no data were shown to illustrate the effectiveness of this approach (US Patent No. US6306663B1).

Because the first PROTACs employed a phosphopeptide ligand for β-TRCP, they did not penetrate cells. An opportunity to engineer cell-permeable PROTACs presented itself with the discovery of a seven residue hydroxyproline-containing peptide from HIF-1α that specifically binds the substrate receptor subunit VHL (Von Hippel-Lindau) of ubiquitin ligase $CRL2^{VHL}$ (Hon et al., 2002; Min et al., 2002). This peptide was used to assemble PROTACs that induce degradation of AR and FKBP12 (FK506-binding protein) in cells (Schneekloth et al., 2004).

Although PROTACs based on the VHL peptide ligand work in cells, they suffer from relatively low potency, most likely due to poor bioavailability. This spurred engineering of the first "all small molecule" PROTAC based on Nutlin-3a, a ligand of the Ub ligase MDM2 that competes for binding of the MDM2 substrate p53. Nutlin-3 conjugated to the AR antagonist hydroxyflutamide specified AR degradation in a proteasome-dependent manner (Schneekloth et al., 2008).

Another approach to the development of small molecule degraders is based on a ligand for the Ub ligase IAP (Inhibitor of Apoptosis) (Fulda and Vucic, 2012). Bestatin methyl ester is linked to ligands that bind a POI to create bifunctional SNIPERS (specific and nongenetic IAP-dependent protein erasers) that direct degradation of targets such as CRABPI and CRABPII (Itoh et al., 2012; Itoh et al., 2010). A limitation of these early SNIPERs is that bestatin induces cIAP autoubiquitination and degradation. Nonetheless, SNIPER(ER) was used successfully to suppress the growth of ERα-positive breast tumors in mice (Ohoka et al., 2017). Further derivatization of the IAP ligand module yielded SNIPER(ER)-110, which recruits XIAP and prompts degradation of both cIAP and ERα (Ohoka et al., 2018).

IMiD Drugs Work through a PROTAC-like Mechanism

By 2010 PROTACs able to induce degradation of multiple substrates had been described, yet the approach remained challenged by the lack of high-affinity Ub ligase ligands as well as a compelling proof of concept that PROTACs had the potential to become actual drugs. This began to change with the important discovery that cereblon (CRBN), a substrate receptor of Ub ligase CRL4, is the primary target of the multiple myeloma drug thalidomide (Ito et al., 2010). A subsequent study reported the unusual finding that for thalidomide and its analogs lenalidomide and pomalidomide (collectively known as immunomodulatory or "IMiD" drugs) to be cytotoxic toward multiple myeloma cells, it is essential that the cells express CRBN (Lopez-Girona et al., 2012; Zhu et al., 2011). This curious result was explained by the observation that IMiDs promote recruitment of the transcription factors IKZF1 and IKZF3 to CRBN, after which the IKZF proteins are ubiquitinated and degraded (Gandhi et al., 2014; Krönke et al., 2014; Lu et al., 2014). A subsequent study found that lenalidomide promotes recruitment of casein kinase-1α (CK1α) to CRBN (Krönke et al., 2015).

The finding that lenalidomide recruits IKZF proteins and CK1α to CRBN (Figure 4) was a puzzle because no obvious degron sequences are shared between these proteins. Crystallographic studies of CRBN bound to CK1α in the presence of lenalidomide shed considerable light on how IMiDs mediate ternary complex formation (Petzold et al., 2016). As was shown previously for the mechanism of signaling by auxin, IMiDs sit at the Ub ligase-neosubstrate interface and stabilize the complex. The glutarimide ring of lenalidomide nestles in the "tri-Trp" pocket of CRBN whereas the phthalimide ring remains solvent exposed, allowing contact with a β-hairpin loop containing a critical glycine (amino acids 35–41) in CK1α. Residues within the same CK1α loop also contact CRBN. The complex is thus formed by a balance of lenalidomide-protein and protein-protein contacts at the interface. Importantly, the atoms in the β-hairpin that contact CRBN are in the main chain and not the side chains. This explains how lenalidomide stabilizes complex formation with diverse neosubstrates, because similar β-hairpin loops are present in zinc finger proteins including IKZF1/3 and other IMiD-dependent $CRL4^{CRBN}$ neosubstrates including ZFP91 and SALL4 (An et al., 2017; Donovan et al., 2018; Fink et al., 2018; Matyskiela et al., 2018). This realization spurred a proteomic screen of zinc finger proteins, which revealed that at least 11 and perhaps as many as 150 family members are IMiD-induced $CRL4^{CRBN}$ neosubstrates (Sievers et al., 2018). Notably, although the contact between CRBN and the β-hairpins is sustained by main chain interactions, there are nevertheless

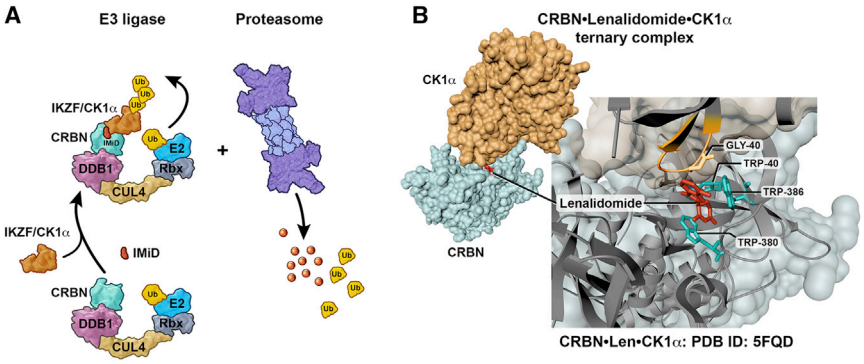

Figure 4. IMiD-Induced CRBN-Dependent Degradation of Neosubstrates

(A) Ub ligase substrate receptor subunit CRBN bound to IMiD promotes Ub and proteasome-dependent degradation of neosubstrates CK1α and IKZF1. DDB1, CUL4, and RBX1 are other subunits of the Ub ligase $CRL4^{CRBN}$.

(B) Lenalidomide buried in the "tri-Trp" pocket of CRBN with the solvent-exposed phthalimide ring contacting CK1α via a β-hairpin loop.

sequence preferences that explain the selectivity of IMiDs for particular zinc finger proteins. The insights derived from the structural biology are now being used to tune the selectivity of IMiDs for particular β-hairpin-containing proteins. Interestingly, a β-hairpin with a key glycine residue promotes CRBN binding and degradation of the translation termination factor GSPT1 by the glutarimide analog CC-885 (Matyskiela et al., 2016). Since CRBN is widely expressed and GSPT1 is essential, its degradation represents a potential liability for CRBN-based degraders. However, structure-activity analyses aided by chemoproteomics are allowing for the design of more specific IMiD analogs that no longer degrade GSPT1 (Hansen et al., 2018; Ishoey et al., 2018).

Following the landmark studies on IMiDs, it was shown that the aryl sulfonamide antineoplastic drug indisulam works by stabilizing the interaction between the Ub ligase $CRL4^{DCAF15}$ and the neosubstrate RBM39 (Han et al., 2017; Uehara et al., 2017) as well as the closely related splicing factor RBM23 (Du et al., 2019; Faust et al., 2020; Ting et al., 2019). It will be interesting to see how many "orphan" drugs with unknown mechanism-of-action work by inducing degradation of a cellular protein.

Prospective Development of Potent and Selective PROTACs

As the IMiD-CRBN story was being unraveled, a parallel effort aimed to optimize the hydroxyproline-containing peptide that binds VHL. A combination of fragment-based screening and structure-based computational simulations yielded VHL-1 and its derivatives, which bind VHL with sub-micromolar affinity (Buckley et al., 2012a, 2012b; Van Molle et al., 2012). The availability of these ligands and IMiDs as non-peptidic, cell-permeable molecules with good affinity for their cognate Ub ligases enabled a flurry of activity in 2015 that opened the door to developing PROTACs as therapeutics. In a series of four papers from the Bradner, Ciulli, and Crews laboratories (Bondeson et al., 2015; Lu et al., 2015; Winter et al., 2015; Zengerle et al., 2015), a set of potent PROTACs based on VHL-1 and IMiDs was described. These papers established that PROTACs:

(1) Work on multiple different proteins. Targets as varied as FKBP12, the BET family of epigenetic readers BRD2-4, the steroid receptor ERRα, and protein kinase RIPK2 could be efficiently degraded (see Table 1 for a more complete list of proteins targeted by PROTACs thus far).
(2) Are exceptionally potent—more so than the POI ligands employed, which contributes to their catalytic mode of action.
(3) Induce rapid degradation of substrate.
(4) Are specific as revealed by proteome-wide mass spectrometry.
(5) Exhibit a more sustained effect than the corresponding POI ligand.
(6) Can induce target degradation *in vivo*.

In addition to those enumerated above, three additional findings emerged from these and subsequent studies that have important implications for the design and use of PROTACs. First, PROTACs can have unexpected (and, as yet, unpredictable) specificity. Although JQ1 is a pan-BET inhibitor, tethering JQ1 to VHL-1 with different linkers yielded one PROTAC that induced degradation of all three BRD proteins versus another (MZ1) that showed selectivity for BRD4 (Zengerle et al., 2015). The crystal structure of the VHL-MZ1-BRD4 ternary complex revealed that the PROTAC induced *de novo* contacts between VHL and BRD4 resulting in highly positive cooperative formation of the ternary complex. This enabled rational design of an even more selective BRD4 PROTAC (Gadd et al., 2017).

To further understand the substrate specificity of PROTACs, a promiscuous compound (foretinib) that inhibits fifty-four protein kinases was linked to either CRBN or VHL ligands (Bondeson et al., 2018). Of the 54 protein kinases, 9 were depleted by the VHL-based PROTAC, and 14 by the CRBN-based PROTAC with six being degraded by both. Ternary complex formation was the best predictor of the efficacy of the VHL PROTAC. Interestingly, changing the length and attachment point of the linker joining foretinib to the VHL ligand enhanced selectivity toward the MAP kinase p38α. To date, there are no conventional inhibitors that selectively target p38α over the other three family members. Similarly, clinically approved CDK4/6 inhibitors were used to design paralog-selective PROTACs, allowing for differentiation of function of these two important kinases for the first time (Brand et al., 2019; Jiang et al., 2019; Rana et al., 2019). It is now clear that selectivity between closely-related family members can be achieved with PROTACs, but the path to engineer selectivity prospectively is not understood yet.

The second unexpected finding to emerge from the initial PROTAC studies was the "hook effect" (Bondeson et al., 2015). Because PROTACs contain two ligands that can bind their

Table 1. Compilation of Cellular Proteins Targeted by Non-peptidic PROTACs

Target(s)	Ligase(s)	Reference(s)
ABC50	CRBN	(Cieślak et al., 2019)
ALK	CRBN	(Powell et al., 2018; Zhang et al., 2018)
AR	MDM2	(Schneekloth et al., 2008)
AURKA/B	CRBN	(Huang et al., 2018)
BCL2	CRBN	(Wang et al., 2019)
BCL6	CRBN	(McCoull et al., 2018)
BCR-ABL	IAP; CRBN; VHL	(Demizu et al., 2016; Lai et al., 2016)
BRAF	CRBN	(Chen et al., 2019a)
BRD2, BRD3, BRD4	CRBN; VHL	(Lu et al., 2015; Winter et al., 2015; Zengerle et al., 2015)
BRD7, BRD9	VHL	(Zoppi et al., 2019)
BTK	CRBN	(Buhimschi et al., 2018; Sun et al., 2018; Zorba et al., 2018)
c-ABL	CRBN; VHL	(Lai et al., 2016)
CDK4/6	CRBN	(Jiang et al., 2019; Zhao and Burgess, 2019a)
CDK9	CRBN	(Olson et al., 2018; Robb et al., 2017)
cIAP1	IAP	(Itoh et al., 2012)
CRABP I/II	IAP	(Itoh et al., 2012; Itoh et al., 2010)
CRBN	VHL; CRBN	(Steinebach et al., 2019; Steinebach et al., 2018)
EGFR	VHL	(Burslem et al., 2018a)
ER	IAP	(Demizu et al., 2012; Itoh et al., 2011)
ERK1/2	CRBN	(Lebraud et al., 2016)
ERRα	VHL	(Bondeson et al., 2015)
FAK	VHL	(Cromm et al., 2018)
FKBP12	CRBN	(Winter et al., 2015)
FLT3	VHL; CRBN	(Burslem et al., 2018b; Huang et al., 2018)
GSPT1	CRBN	(Matyskiela et al., 2016)
HCV NS3/4A	CRBN	(de Wispelaere et al., 2019)
HDAC6	CRBN	(Yang et al., 2018)
HER2	VHL	(Burslem et al., 2018a)
IRAK4	VHL	(Nunes et al., 2019)
ITK	CRBN	(Huang et al., 2018)
Mcl1	CRBN	(Wang et al., 2019)
MDM2	CRBN	(Li et al., 2019b)
p38a/delta	VHL; CRBN	(Bondeson et al., 2018; Smith et al., 2019)
PARP1	MDM2	(Zhao et al., 2019)
PCAF/GCN5	CRBN	(Bassi et al., 2018)
PIRIN	CRBN	(Chessum et al., 2018)
PTK2/B	VHL; CRBN	(Popow et al., 2019)
RAR	IAP	(Itoh et al., 2011)
RIPK2	VHL	(Bondeson et al., 2015)
Rpn13	CRBN	(Song et al., 2019)
SGK3	VHL	(Tovell et al., 2019b)
sirtuin-2	CRBN	(Schiedel et al., 2018)
SMAD3	VHL	(Wang et al., 2016)
SMARCA2/4	VHL	(Farnaby et al., 2019)
TACC3	IAP	(Ohoka et al., 2014)
tau	CRBN	(Silva et al., 2019)
TBK1	VHL	(Crew et al., 2018)

(*Continued on next page*)

Table 1. *Continued*

Target(s)	Ligase(s)	Reference(s)
TEC	CRBN; VHL; IAP	(Zorba et al., 2018; Huang et al., 2018)
TRIM24	VHL	(Gechijian et al., 2018)
TrkC	CRBN	(Zhao and Burgess, 2019b)
ULK1	CRBN	(Huang et al., 2018)
VHL	VHL	(Maniaci et al., 2017)

For proteins that have been targeted by multiple PROTACs, only the chronologically first degrader is cited due to space limitations

targets independently, one expects that there might be a sharp optimum concentration corresponding to maximal ternary complex formation and that at higher concentrations the formation of the binary complexes (PROTAC-POI and Ub ligase-PROTAC) would squelch ternary complex assembly (i.e., the hook effect). The positive cooperative assembly of ternary complex exhibited by MZ1 described above would mitigate the hook effect, and indeed fortuitous contacts between the ubiquitin ligase and neosubstrate have been observed in ternary complexes induced by both IMiDs and PROTACs (Gadd et al., 2017; Petzold et al., 2016; Sievers et al., 2018). However, these "neocontacts" do not always cooperatively promote ternary complex assembly. For example, the structure of CRBN-BRD4 complexes nucleated by different IMiD-based PROTACs revealed a surprising plasticity in the docking of BRD4 to CRBN (Nowak et al., 2018). The inter-protein contacts contributed little to binding affinity and no positive cooperativity was observed. Likewise, another study utilizing CRBN to target Bruton's tyrosine kinase concluded that degradation was dependent on ternary complex formation, but thermodynamic cooperativity was unnecessary (Zorba et al., 2018).

The third unanticipated observation is that although both CRBN and VHL ligands can be used to assemble highly efficacious PROTACs, those based on IMiDs may be active toward a broader range of substrates (Huang et al., 2018). While this is not fully explored yet, it may be related to either the structure or mobility of the substrate receptor-adaptor-cullin complex. Of the CRLs, $CRL2^{VHL}$ is predicted to have one of the largest gaps between the substrate receptor and docked E2, whereas $CRL4^{CRBN}$ has one of the smallest (Cardote et al., 2017; Cavadini et al., 2016). Moreover, the CRL4 adaptor DDB1 consists of 21 WD40-like repeats that fold into a unique triple β-propeller, whereas all the other cullin adaptors are based on a more rigid BTB fold. The greater rotational flexibility afforded by DDB1 may thus allow its bound substrate receptors, including CRBN, to sample a larger volume (Fischer et al., 2011). CRBN itself is unique in that the IMiD-binding domain defines the CULT domain family that is conserved across species including prokaryotes but is not found in other members of the DCAF family of substrate receptors of CRL4 (Lupas et al., 2015). The domain adopts a β-tent fold and contains a cradle-shaped groove lined by aromatic residues that can accommodate different ligands across organisms (Hartmann et al., 2015).

Detailed mapping of sites of ubiquitination following PROTAC addition would aid in understanding differences between recruited ligases and selectivity achieved among target paralogs as exemplified by two earlier studies that painstakingly mapped sites of ubiquitination by mass spectrometry (Gadd et al., 2017; Petzold et al., 2016).

Principles of PROTAC Design

As earlier comprehensive reviews have emphasized (Churcher, 2018; Cromm and Crews, 2017; Maniaci and Ciulli, 2019; Mayor-Ruiz and Winter, 2019) PROTACs offer the potential to target any POI as long as a ligand is available. Because PROTACs can have a DC_{50} (concentration of PROTAC required to reduce the level of target protein by 50%) that is substantially lower than the K_D of the POI ligand, these ligands need not be high-affinity binders. Ability of a PROTAC to induce ternary complex formation (Ub ligase-PROTAC-target) is critical, and biochemical and cell-based methods for assessing such complexes are available (Riching et al., 2018; Roy et al., 2019). However, as noted above the role of cooperativity remains unclear (Bondeson et al., 2018; Roy et al., 2019; Zorba et al., 2018). Although ternary complex formation is necessary, it may still be insufficient because in some cases ubiquitination of the target does not occur (Smith et al., 2019). Given these observations, efforts to develop and optimize PROTACs should monitor ternary complex formation and target ubiquitination.

In developing PROTACs it is important to consider that events downstream of ubiquitination may also determine whether the target is degraded. The rate-limiting step for degradation by the 26S proteasome is unfolding (Bard et al., 2019). Initiation of degradation occurs at regions of disorder in the substrate, which the ATPases of the proteasome 19S regulatory particle grasp and translocate into the proteolytic core of the 20S proteasome. The sequence composition and length of the disordered region can be as important for substrate degradation as the Ub chain itself (Yu and Matouschek, 2017). For PROTAC targets for which multiple ligands are available, the choice of ligand could be dictated more by the position of acceptor lysines relative to intrinsic regions of disorder than by ligand affinity. For tightly folded, compact substrates that cannot be engaged by the proteasomal ATPases, the conserved ATPase Cdc48/p97/VCP may come to the rescue (Olszewski et al., 2019). This ATPase typically functions downstream of ubiquitination and upstream of the proteasome and is required for IMiD-induced degradation of IKZF1/3 and CK1α (Nguyen et al., 2017). Small molecule p97/VCP inhibitors can trap ubiquitinated targets, which may facilitate visualization of PROTAC-induced ubiquitination (Zhong et al., 2019).

Whither PROTACs?

What does the future hold for PROTACs? A flood of papers over the past 4 years has established that PROTACs represent a versatile modality that can potentially be used to inactivate the

80% of the proteome that is undruggable, provided that a ligand can be obtained for the POI. We will now turn to addressing the major challenges that remain to be overcome and promising directions that might yield significant improvements to the technology.

One challenge is that it remains to be shown that a prospectively engineered PROTAC can be used safely and efficaciously to treat human disease. We may soon have an answer to this question, now that oral PROTACs that target AR (ARV-110) and ER (ARV-471) are being dosed in patients suffering from metastatic castration-resistant prostate cancer (https://clinicaltrials.gov/ trial number NCT03888612) and metastatic breast cancer (https://clinicaltrials.gov/ trial number NCT04072952), respectively. Based on presentations at scientific meetings, we can expect to see a few additional clinical trials initiated over the next year.

A second challenge will be to make PROTACs that have suitable pharmaceutical properties (e.g., molecular weight [MW], metabolic stability, rate of clearance, and oral bioavailability) that enable convenient, efficient, and highly specific target depletion in the relevant tissue *in vivo*. We propose that success in these endeavors will be enhanced by developing PROTACs that mimic the IMiDs—i.e., low MW, compact molecules that bring neosubstrate into close proximity with the Ub ligase, enabling formation of inter-protein contacts that confer positive cooperativity to ternary complex formation with the caveat that the consequent reduction in flexibility may compromise ubiquitination. An example of this approach was reported recently (Simonetta et al., 2019). β-catenin is normally ubiquitinated by $SCF^{\beta\text{-}TRCP}$ but this is blunted by the naturally occurring β-catenin S37A mutation found in a subset of colorectal cancers. Simonetta et al. describe a "molecular glue" that has no appreciable affinity for either β-catenin or β-TRCP alone but stabilizes the binding of mutant β-catenin to β-TRCP by binding at the interface to restore contacts disrupted by the S37A mutation. We define a molecular glue as a molecule that stabilizes a native or nucleates a non-native protein-protein interaction but binds with only weak or undetectable affinity to the individual proteins. According to this definition, IMiDs, indisulam, and auxin are molecular glues but PROTACs are not, because with PROTACs, the E3 and target are typically each individually bound with sub- or low micromolar affinity. Molecular glues that display positive cooperatively should not only have higher potency but should increase both the therapeutic window (by reducing the hook effect) and target specificity. Because the inter-protein contacts that drive positive cooperativity are fortuitous, the odds of capturing such contacts may be enhanced by generating a suite of PROTACs that bring a POI into proximity with multiple different Ub ligases, to find the optimal pairing. In addition, this approach should minimize the risk of forming ternary complexes that do not yield productive ubiquitination.

As our understanding of PROTACs becomes more sophisticated, it may be possible to model *in silico* the structure of ternary complexes and predict the likelihood of positive cooperativity and neosubstrate ubiquitination to identify optimal Ub ligase-target pairs. There is potentially a lot we can learn from such efforts. A recent quantitative study highlights the dramatic range in efficiency for TPD. On a molecule of substrate per molecule of Ub ligase per minute basis, indisulam-promoted degradation of RBM39 is 1,250-fold more efficient than lenalidomide-induced turnover of CK1α and 420-fold more efficient than dBET-induced elimination of BRD4 (Reichermeier et al., 2019). Thus, even though $CRL4^{CRBN}$ is the most abundant CRL4 and $CRL4^{DCAF15}$ is one of the least (in HEK293 cells), targeted degradation of RBM39 is more rapid than either CK1α or BRD4. In addition, methods that enable efficient identification of more compact, molecular glue-like molecules—including computational approaches or powerful screening tools like DNA-encoded libraries—would help drive the field forward and are urgently needed.

A third challenge will be to extend use of PROTACs beyond cancer cells to normal tissues. Neurodegenerative diseases like FTD (frontotemporal dementia) and other tauopathies are associated with accumulation of aggregated forms of mutant tau protein leading to neuronal loss. PET (positron emission tomography) tracers that bind to aberrant tau but not wild-type protein are used in diagnoses of tauopathies. In a clever adaptation, a mutant tau PET probe was linked to a CRBN ligand to generate a PROTAC that promotes clearance of tau in FTD patient-derived neuronal cell models (Silva et al., 2019). Because many of the >500 ubiquitin ligases encoded in the human genome are expressed or activated in a spatially or temporally restricted manner, it may be possible to develop PROTACs that only work in a particular tissue, at a particular time, or in response to a particular physiological state.

One potentially interesting future direction for PROTACs is as enhancers of immuno-oncology drugs. PROTACs induce presentation of specific MHC class I peptides from the proteins whose degradation they induce (Jensen et al., 2018). Because PROTACs are likely to induce novel sites of ubiquitination in the target, they may result in MHC presentation of "neopeptides" that could enhance the immune attack on tumors.

Another possibility is to develop PROTACs that recruit an oncogenic Ub ligase with a ligand that disrupts binding to its tumor suppressor substrate. In a recent example, a PROTAC comprising the MDM2 ligand Idasanutlin conjugated to the BRD2-4 ligand JQ1 was designed. This PROTAC was more effective than its component ligands in inhibiting proliferation since both p53 was stabilized and BRD4 was degraded (Hines et al., 2019). Additionally, the repertoire of ligases recruited for PROTAC deployment is expanding via the use of electrophilic warheads that modify cysteine residues on E3s (Spradlin et al., 2019; Ward et al., 2019; Zhang et al., 2019).

The idea underlying PROTACs can be expanded to include degradation in other compartments (such as the lysosome) and of other classes of biomolecules (such as secreted and membrane proteins). Ligands that bind the cell surface lysosome targeting receptors when conjugated to ligands binding extracellular domains were able to direct endocytosis and degradation of extracellular and membrane-embedded proteins (Nalawansha et al., 2019; Banik et al., 2019). These ENDTACs and LYTACs echo the principle underlying "sweeping" antibodies (Igawa et al., 2013) and could be generalized to encompass the degradation of any extracellular protein for which a ligand can be generated. Meanwhile, formation of a chimera between a small

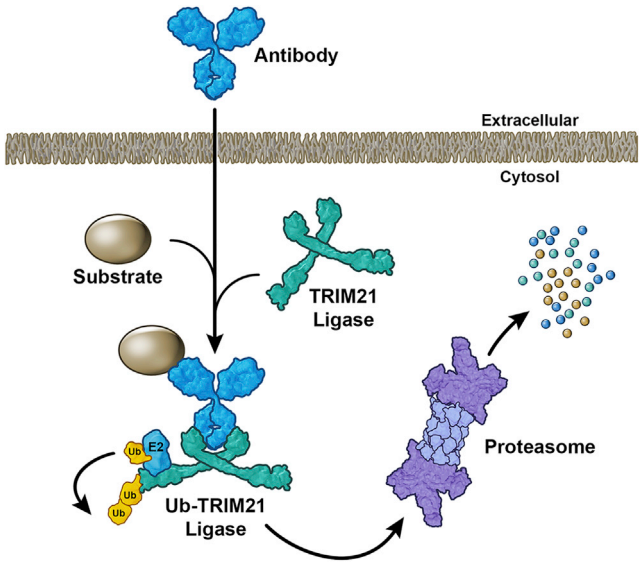

Figure 5. Antibody-Mediated Degradation of Endogenous Proteins via Trim-Away

Cytosolic antibody receptor Trim21 promotes Ub-mediated degradation of antibody-protein complexes. Trim21 recognizes the Fc region of IgG and is auto-ubiquitinated. Ub marked Trim21 and its antibody/protein cargo are targeted for proteasomal degradation.

molecule that binds a specific RNA and another that binds RNase L led to specific elimination of the RNA target from cells (Costales et al., 2019) (RIBOTACs). Most recently, it was shown that guanylation of cysteine, which naturally triggers autophagy of bacteria that invade the cytosol (Ito et al., 2013), can be exploited to develop guanine-based AUTACs that activate autophagy of a target protein (Takahashi et al., 2019). Expansion of these lines of investigation holds tremendous promise.

Targeted Degradation of Native Intracellular Proteins with Biologics

Trim-Away: Antibody-Mediated Degradation of Endogenous Proteins

Trim21 is a ubiquitously expressed Ub ligase that functions as a cytosolic antibody receptor by binding the Fc region of IgG molecules (James et al., 2007). Trim21 recruits the UPS to attack antibody-coated pathogens such as bacteria and viruses that gain access to the cytosol (Figure 5). The observation that Trim21 is a cytosolic antibody receptor has been cleverly exploited to repurpose it to degrade endogenous proteins (Clift et al., 2017). Antibodies introduced by microinjection or electroporation are sufficient to induce degradation of their target antigen within a few hours. In all, nine different endogenous proteins in ten different cell types were shown to be degraded in a specific, antibody-dependent manner. In addition, the Trim-Away approach was recently used to create null-like phenotypes in zebrafish embryos (Chen et al., 2019b). Trim-Away depleted proteins faster than morpholinos, allowing loss-of-function analysis of regulatory proteins during early embryogenesis. Disadvantages of Trim-Away include the following: (i) Trim21 is degraded together with the antibody:antigen complex, so the method is therefore limited by the endogenous amount of Trim21 unless additional Trim21 is co-introduced with the antibody; (ii) it requires electroporation of antibodies into the cytosol and because of their high MW it is not suitable for nuclear proteins; and (iii) it is only as effective and specific as the antibody being used. For a discussion of protocols and pitfalls to be avoided, consult Clift et al. (2018).

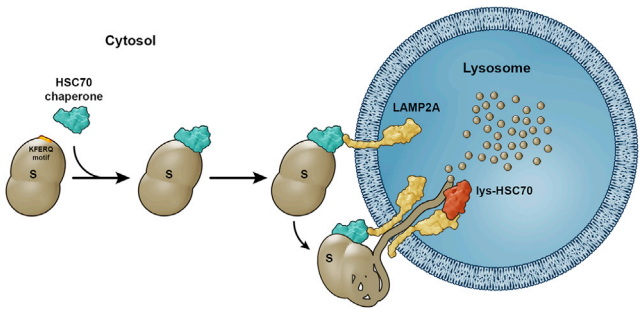

Figure 6. Chaperone-Mediated Autophagy Targets Proteins for Lysosomal Degradation

Substrate proteins tagged with KFERQ-like peptide sequences are recognized by the cytosolic chaperone HSC70. The complex, aided by co-chaperones, binds the transmembrane lysosomal receptor LAMP2A and drives its multimerization, which is required for substrate translocation. Translocation and dissociation of substrate are further aided by the low-pH-dependent lysosomal form of HSC70 (lys-HSC70), resulting in substrate degradation.

Targeting Proteins to the Lysosome via Chaperone-Mediated Autophagy

Chaperone-mediated autophagy (CMA) is a specialized form of autophagy in which soluble cytosolic proteins bearing a KFERQ motif or close matches to it are recognized by HSC70 (heat shock cognate 71 kDa protein). Aided by other co-chaperones, the complex binds to the cytosolic tail of lysosomal transmembrane protein LAMP2A, which drives oligomerization of LAMP2A and transport of the KFERQ-bearing protein cargo across the membrane (Kaushik and Cuervo, 2012). Natural substrates for CMA include tau and α-synuclein (Figure 6). However, mutant Huntingtin (Htt) and its fragments are not efficiently cleared by CMA. Expression of an engineered fusion protein containing two copies of HSC70-binding motifs and two copies of QBP1 (polyglutamine-binding peptide 1 that binds to expanded polyQ tracts in mutant Htt but not wild-type Htt) resulted in specific degradation of mutant Htt via CMA in mammalian cells (Bauer et al., 2010).

In another adaptation, tripartite fusions were generated that contained the cell membrane-penetrating sequence TAT followed by a target protein binding domain (PBD) and tandem repeats of the CMA-targeting motif (Fan et al., 2014). These chimeric proteins were applied to primary neuronal cultures and shown to degrade endogenous proteins such as DAPK1 (death associated protein kinase 1) and the scaffolding protein PSD-95. Degradation was sensitive to lysosome inhibitors and insensitive to inhibitors of the proteasome and macroautophagy. Disadvantages of this approach include its dependence on (i) a domain that specifically binds the POI, (ii) transduction of proteins or DNA constructs into cells, and (iii) uptake of sufficient amounts of the targeting construct to degrade the target, on the presumption that it does not act catalytically.

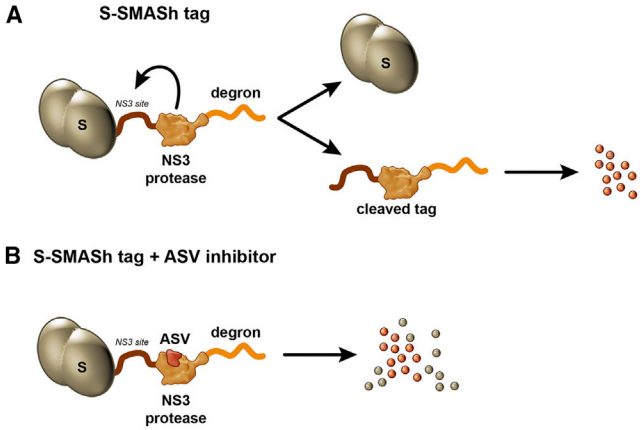

Figure 7. SMASh-Mediated Control of Protein Expression
(A) SMASh, composed of a hydrophobic degron sequence and NS3 protease cleavage site flanking the NS3 protease, auto-cleaves to release native (untagged) substrate protein (S).
(B) In the presence of NS3 protease inhibitor asunavir (ASV), the NS3 protease is inactivated and the hydrophobic degron of SMASh marks the tagged substrate protein for degradation.

Targeted Degradation of Tagged Proteins with Small Molecules

Smallmolecule-based PROTACs and molecular glues have enabled the application of TPD as a therapeutic strategy. However, TPD is also a powerful way for inducing protein depletion in engineered cells and organisms to study the effect of acute loss of protein function in normal and diseased states. In this section, we will describe various strategies that have been developed to enable rapid, controlled, and selective protein depletion in model systems. All of the strategies described here are based on the concept that many proteins contain natural "degrons," which are short peptide sequences that confer metabolic instability by serving as binding motifs for Ub ligases (Varshavsky, 1991). Importantly, degrons are transferable in that they can be attached to any other protein to induce its degradation via the Ub ligase that recognizes the degron.

The Auxin-Inducible Degron (AID)

Discovery of the mechanism underlying auxin signaling enabled development of a powerful and efficient method to deplete any protein (Figure 5). In this approach, the AID from IAA17 is fused to a POI (Nishimura et al., 2009). Since all components of the SCF machinery (except the plant F-box receptor TIR1, which is introduced ectopically) are conserved in eukaryotes, the technique works in yeast, flies, *C. elegans*, and human cell lines (Nora et al., 2017; Rao et al., 2017; Trost et al., 2016; Zhang et al., 2015). The method works for both nuclear and cytoplasmic proteins, which are degraded with half-lives as short as 9 min (Holland et al., 2012). More recently, a number of modifications have been made to the system to expand its utility (García-Rodríguez and Ulrich, 2019; Natsume et al., 2016) including the development of the inhibitor auxinole for tighter control of the degron (Yesbolatova et al., 2019). The size of the original AID has been reduced from 25 kDa to 7 kDa (mini-AID) (Morawska and Ulrich, 2013; Natsume et al., 2016). An advantage of this approach is that it is completely bio-orthogonal since neither TIR1 nor auxin is produced in animals. A disadvantage is that it requires transgenic expression of TIR1 and the target protein must function as an AID fusion. To our knowledge proteomic studies have not been performed to determine whether TIR1 expression causes depletion of endogenous protein but it does result in auxin-independent degradation of tagged protein in some instances. A more recent iteration utilizing the F-box protein AtAFB2 and the short degron miniIAA7 (67 aa) reportedly overcomes the latter limitation (Li et al., 2019a). The technique has not been used *in vivo* partly because of the high concentrations of auxin required (100–500 μM). Development of a higher-affinity derivative would circumvent this limitation.

The dTAG (Degradation Tag) System and FKBP-Directed PROTACs

Engineered variants of FKBP12 such as $FKBP12^{F36V}$ create a "cavity" in the protein that allows for specific recognition by a "bumped" synthetic ligand that does not bind endogenous FKBP12 (Clackson et al., 1998). The bumped ligand was conjugated to lenalidomide to yield a heterobifunctional degrader (dTAG) that recruits $CRL4^{CRBN}$ to a POI (Brand and Winter, 2019; Nabet et al., 2018). Addition of the dTAG-13 degrader molecule leads to acute loss of proteins tagged with $FKBP12^{F36V}$ including BRD4 and $KRAS^{G12V}$. Additionally, administration of dTAG-13 to mice engrafted with leukemia cells that express luciferase–$FKBP12^{F36V}$ results in loss of bioluminescence, demonstrating applicability of the technique *in vivo*. A disadvantage that this method shares with other approaches described in this section is that the target protein must function as a fusion.

In a related approach, PROTACs directed against FKBP12 were evaluated in larger mammals such as pigs and rhesus monkeys (Sun et al., 2019). Oral administration or intraperitoneal injections resulted in PROTAC-dependent depletion of FKBP12 in most organs and tissues except the brain where intra-cerebroventricular injections were required. Cardiac dysfunction was noted, mirroring *Fkbp12* conditional knockout mice. FKBP12 expression recovered within days to a week depending on the tissue. While this approach allows for inducible degradation of FKBP12 fusion proteins, a disadvantage is that it does not spare endogenous FKBP12.

The IKZF3 Degron

Another useful conditional approach employs a minimal IMiD-responsive IKZF3 degron fused to a POI. Administration of pomalidomide induces degradation of the POI–IKZF3 chimera, enabling loss-of-function studies *in vivo* (Koduri et al., 2019). Interestingly, the IKZF3 degron functions best when fused to the C terminus of a POI. The authors estimate that approximately 30%–40% of the CRISPR/Cas9 tagged proteome is amenable to this approach. An advantage is that pomalidomide crosses the blood-brain barrier and can induce depletion of brain targets. Additionally, IMiDs do not induce binding of IKZF3 to mouse CRBN.

SmallMolecule-Assisted SMASh

In this strategy, a tetrapartite fusion is constructed that contains the POI fused to a cassette that includes the hepatitis C virus NS3 protease, a hydrophobic degron, and a cleavage site for NS3, such that intramolecular cleavage by NS3 severs the degron-containing cassette from the POI (Chung et al., 2015). Under normal conditions, NS3 rapidly excises the cassette as

Table 2. Summary of the Advantages and Disadvantages of Described Methods of Targeted Protein Degradation

Method	Advantages	Disadvantages
PROTACs	Catalytic; reversible; endogenous wild-type and mutant targets; paralog-selective; *in vivo* applications.	Lengthy SAR analyses of linker and target ligands; high MW and not "rule-of-5" compliant.
Molecular glues	Catalytic; reversible; endogenous wild-type and mutant targets; paralog-selective; *in vivo* efficacy in humans with some BBB-permeable.	Challenging to identify/synthesize prospectively; specificity issues.
Trim-Away	Endogenous targets.	Limited by endogenous levels of Trim21, Ab access to cytosolic but not nuclear targets; Ab specificity to target.
CMA	No dependency on ubiquitination machinery for targeting to lysosome.	Ectopic expression of engineered constructs; non-catalytic; only cytosolic targets.
AID	Reversible; versatile.	Ectopic expression of F-box receptor; 7 kDa target modification; no *in vivo* application; leaky degradation in absence of auxin.
dTAG	Catalytic; reversible; *in vivo* applications.	12 kDa target modification.
IKZF3 degron	Catalytic; reversible; *in vivo* applications including CNS; rodent IKZF3 not targeted.	3 kDa target modification best at C terminus; not all targets degraded.
SMASh	Reversible.	Only for newly synthesized protein.
HaloTag-HyT; HaloPROTACs	Tagged ORFs and PROTAC commercially available; *in vivo* applications.	Covalent 33 kDa target modification; non-catalytic.
Nanobodies: deGradFP and AID nanobody	Suitable for GFP-tagged ORFs available commercially.	Target modification; substrate receptor engineering.

The broad applicability of emerging modalities such as AUTACs, ENDTACs/LYTACs, and RIBOTACs briefly mentioned in this Review remains to be determined and hence is not summarized here. SAR, structure-activity relationship; SMs, small molecules; BBB, blood-brain barrier; CNS, central nervous system; ORFs, open reading frames.

soon as the chimeric protein is produced, releasing the POI in an unfused, stable state. A potent and specific clinical-grade inhibitor (asunaprevir) exists for NS3 and the addition of asunaprevir blocks processing, resulting in rapid degradation of the intact fusion protein (Figure 7). An advantage of this method is that NS3 has no known endogenous substrates and the *in vivo* pharmacology and pharmacokinetics of asunaprevir are well characterized. A disadvantage is that the degradation pathway for the unprocessed fusion protein is not known.

Creating Hydrophobic Degrons with Adamantyl-Based Probes: HaloTag-HyT and HaloPROTACs

A systematic genetic screen in yeast for endogenous degrons yielded a set of hydrophobic peptides that normally reside in the core of globular proteins or are inserted into membranes (Gilon et al., 1998). The realization that exposed hydrophobic sequences lead to degradation led to the repurposing of the HaloTag as an inducible degron. HaloTag is a modified haloalkane dehalogenase that covalently binds chloroalkane ligands. Attaching the highly hydrophobic adamantyl moiety to a chloroalkane ligand and adding it to cells expressing HaloTagged fusion proteins evoked the unfolded protein response (UPR) and degradation of the tagged proteins in cell lines, zebra fish, and mice (Neklesa et al., 2011). Disadvantages specific to this technique include limited bioavailability of ligand due to binding to membranes and plasma proteins as well as induction of the UPR, which could complicate phenotypic analyses. The responsible Ub ligases and chaperones remain unknown.

HaloTag fusion proteins can additionally be degraded using HaloPROTACs that recruit VHL (Buckley et al., 2015) or cIAP (Tomoshige et al., 2015) ligases. They are available commercially (e.g., HaloPROTAC-3 from Promega) and have undergone further optimization allowing researchers to assess target depletion phenotypes *in vitro* (Tovell et al., 2019a) and *in vivo* (BasuRay et al., 2019).

TPD of Tagged Proteins with Biologics

Exploiting Intrabodies and Nanobodies for Proteolysis

Intracellular antibodies or intrabodies are single chain fragment antibodies expressed in the cytoplasm. In the earliest application of this technology to deplete proteins, an ScFv selective for the cytoskeletal protein τ was fused to IkBα (Melchionna and Cattaneo, 2007). Following administration of TNFα, the entire complex of τ-ScFv-IkBα was degraded.

A similar but more broadly applicable approach makes use of genetically encoded nanobodies that recognize GFP-tagged proteins. Nanobodies are single domain antibodies derived from camels or sharks and characterized by high stability, solubility, and low molecular weight. GFP-tagged ORFs (open reading frames) are widely available for model systems including yeast, flies, and human cells. In a technique called deGradFP (degrade Green Fluorescent Protein), the authors created a novel substrate receptor for an SCF ubiquitin ligase by fusing an F-box domain to a nanobody that binds GFP (Caussinus et al., 2011). This chimeric receptor targets both cytosolic and nuclear GFP-tagged proteins for degradation in *Drosophila* cells. Subsequent reports found deGradFP to be inefficient in degrading nuclear proteins in mammalian cells but proficiency was increased by switching the F-box for the CRL3 substrate receptor SPOP (Shin et al., 2015) or recruiting the bacterial *Shigella flexneri* E3 ligase IpaH9.8 (Ludwicki et al., 2019). In zebrafish, on the other hand, the most consistent results were obtained with the F-box domain from Fbxw11b (Yamaguchi et al., 2019). Yet

another permutation on the deGradFP technology was reported by Daniel et al. (2018), in which they fused an AID to GFP nanobody sequences to generate an AID-nanobody chimera. Ectopic expression of TIR1 is required for the AID-nanobody to work, but now all GFP-tagged ORFs can potentially be degraded in an auxin-dependent manner. An advantage of these methods is their broad utility for inducing degradation of any GFP- or YFP-tagged protein. A disadvantage of all the variations described above is the requirement to express rather large engineered fusion proteins.

Conclusion

TPD is rapidly coming of age both as a therapeutic approach to eliminate specific proteins in disease to achieve a salubrious effect and as a research approach to eliminate specific proteins in a temporally controlled manner to evaluate the consequences of acute loss-of-function (see Table 2 for a summary). Although the power of TPD has been demonstrated in numerous studies, the approach is still in its infancy and the next few years should bear witness to tremendous strides in the development of new tools and methods for TPD and its implementation in both therapeutic and research contexts.

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DECLARATION OF INTERESTS

R.V., D.M., and R.J.D. are employees and stock holders of AMGEN. R.J.D. is additionally SVP of AMGEN Global Research.

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