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Protein degradation for drug discovery

Identification and characterization of cancer vulnerabilities via targeted protein degradation

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Target(ed) protein degradation (TPD) is a novel paradigm in drug discovery and a promising therapeutic strategy. TPD is based on small-molecules that catalyze the degradation of proteins by re-directing the ubiquitination activity of ubiquitin E3 ligases. Its unique molecular pharmacology enables robust, selective and fast elimination of proteins in cellular assays and *in vivo*. In addition to possible clinical applications, TPD is also emerging as an attractive alternative to traditional pharmacologic or genetic perturbation strategies. Directly acting degraders, as well as chemical-genetics derivatives offer unique opportunities in the pre-clinical identification, characterization and mechanistic validation of therapeutic targets.

Introduction

Most drug development efforts rely on the *a priori* identification of robust and mechanistically understood targets for therapeutic intervention. Identification and characterization of cancer vulnerabilities is facilitated by a plethora of perturbation-technologies and downstream experimental readouts. Nevertheless, the field of cancer research faces a crisis where a high percentage of published findings are either not sufficiently robust to warrant ensuing drug-development efforts, or simply fail to be reproduced [1–3]. Different sources and pitfalls, including experimental design and lack of suitable controls can contribute to this problem, and are covered in an excellent recent perspective [4]. Of particular relevance to the validation of dependencies in cancer is the choice of perturbation strategies, as well as an awareness of the associated strengths and shortcomings. Here, we will specifically focus on targeted protein degradation (TPD) as a novel validation strategy that combines advantages of both traditional pharmacologic and genetic perturbations. We will discuss how the unique characteristics inherent to TPD can support pre-clinical target validation, and differentiate TPD from commonly used validation strategies. Moreover, we will showcase recent examples where TPD was applied to inform on the mechanism of action of known therapeutic targets, or to identify and validate novel cancer vulnerabilities.

Targeted protein degradation

Target(ed) protein degradation is based on small-molecules, generally called "degraders", that induce the degradation of

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proteins by modulating the ubiquitination activity of E3 ligases. Overall, there are two types of small-molecule degraders: (i) non-chimeric compounds that act as molecular glues, or (ii) hetero-bifunctional molecules, often referred to as ''proteolysis-targeting chimeras'' (PROTACs) [5]. Both function by inducing associations of a target protein of interest (POI) and an E3 ubiquitin ligase. Induced molecular proximity consequently prompts ubiquitin transfer to the POI and its ensuing proteasomal degradation (Fig. 1).

(i) Examples of molecular glues include thalidomide, lenalidomide and the related family of ''immunomodulatory drugs'' (IMiDs), certain aryl sulfonamides, as well as the plant hormone auxin. All of these small-molecules bind substrate receptors (SR) of the cullin-RING family of E3 ligases (CRL). Binding induces or complements a neomorphic interaction surface that leads to the cooperative binding of a given neosubstrate, ubiquitin transfer, and its subsequent degradation by the proteasome. In brief, IMiDs induce tripartite binding of the SR CRBN to a spectrum of C2H2 zinc fingers proteins, most notably the transcription factors IKZF1 and IKZF3 [6–11]. Certain anti-cancer sulfonamides such as indisulam or tasisulam were similarly shown to degrade the splicing factor RBM39 by modulating the SR DCAF15 [12,13]. Finally, the phytohormone auxin employs a highly similar mechanism by inducing the degradation of the AUX/IAA transcriptional regulators via re-directing the E3 ligase activity of the E3 ligase TIR1 [14,15]. Notably, lenalidomide is a widely used anti-myeloma drug, thus providing evidence that induction of TPD via a glue-like mechanism

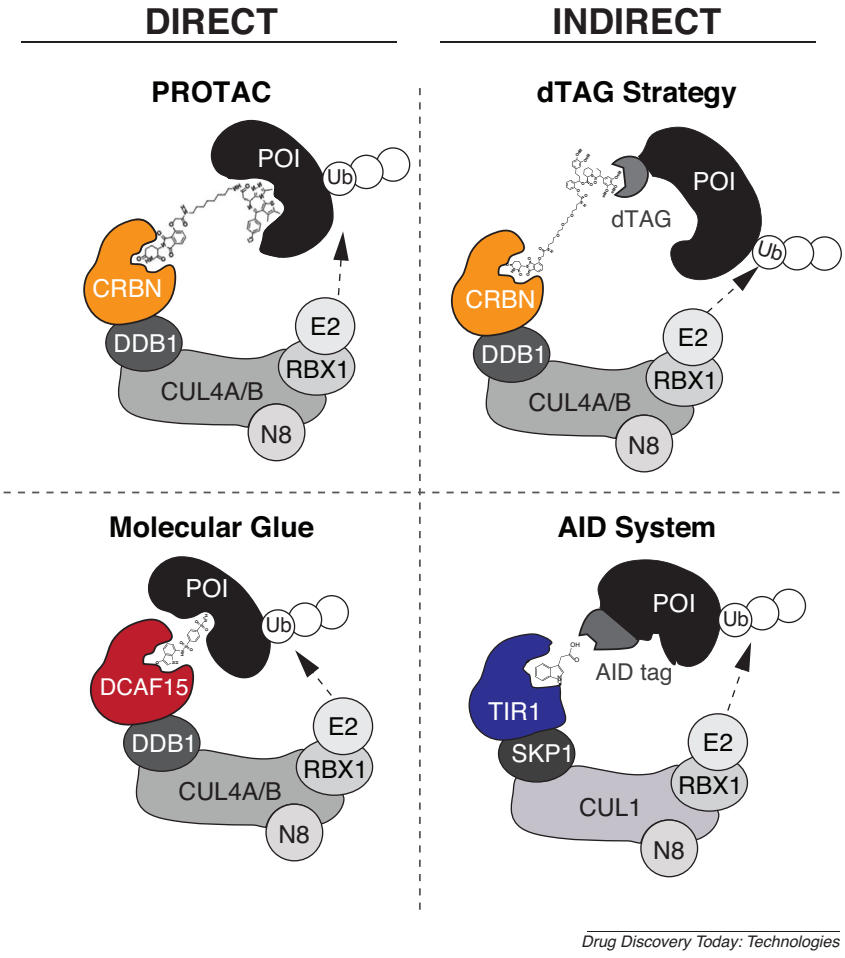

Fig. 1. Target(ed) protein degradation: direct and indirect strategies.
Schematics of approaches based on small-molecules (``degraders'') to induce degradation of target proteins, by re-directing the activity of ubiquitin E3 cullin RING ligases (CRLs). Direct degradation strategies are depicted on the left: hetero-bifunctional compounds (**PROTACs**/``proteolysis-targeting chimeras'') are exemplified by the BET protein degrader dBET6 (upper left), and non-chimeric chemicals (**molecular glues**) are illustrated by the RBM39 degrader indisulam (bottom left). Two examples of indirect, chemical-genetics strategies are represented on the right: the **dTAG-strategy** builds on a $FKBP12^{F36V}$-based tag (dTAG) which can be fused to a POI, allowing ligand-induced degradation by endogenous $CRL4^{CRBN}$ (upper right). Standardized degrader dTAG-7 is shown as an example. The auxin inducible degron **(AID) system** is based on AID tagging of the POI, enabling its inducible degradation upon auxin addition via re-directing $CRL1^{TIR1}$, where TIR1 needs to be ectopically expressed (bottom right). CRL complexes recruited by the exemplified degraders are composed of a cullin scaffold (CUL4A/B or CUL1), an adaptor protein (DDB1 or SKP1), a substrate receptor (CRBN, DCAF15 or TIR1) and the RING protein RBX1 recruiting an E2 ligase. POI: protein of interest. Ub: ubiquitin. N8: NEDD8 (active CRLs).

can be an efficacious therapeutic strategy [16]. Unfortunately, a lack of general design principles has thus far limited the scalable discovery of molecular glues to degrade additional disease-relevant targets in a rational manner.

(ii) PROTACs are based on a modular design where a targeting warhead and an E3-ligase binder are connected via a flexible linker [5]. PROTACs can thus simultaneously bind to an E3 ligase and a POI, enforce their molecular proximity, and induce POI ubiquitination and degradation. Due to this modular architecture, PROTACs can in principle be adapted to different POIs simply by exchanging the targeting warhead. This potential versatility rendered PROTACs particularly interesting for the pharmaceutical development, despite potential challenges related to their relatively high molecular weight that often ranges from 700 to 1200 Da.

The attention given to PROTACs led to a recent increase in the number of probes available for preclinical target exploration. Over the last three years, (a) CRBN and (b) VHL emerged as versatile E3 ligases that are ideally suited for PROTAC design:

(a) Inspired by the identification of CRBN as the cellular target of thalidomide [17], we have recently reported the first *in vivo* compatible heterobifunctional degrader [18]. Conjugation of a competitive BET-bromodomain antagonist to an IMiD-like phthalimide moiety afforded the heterobifunctional compound dBET1. dBET1 induced fast, potent and selective degradation of BET proteins BRD2, BRD3 and BRD4 in cell lines and in mouse xenograft experiments, and continuous degrader treatment outperformed competitive BET inhibition in disseminated acute myeloid leukemia (AML) xenografts [18]. Phthalimide conjugation was shown to be a generalizable approach by additionally reporting potent heterobifunctional degraders of the prolyl isomerase FKBP12 [18]. Moreover, BET PROTACs with a different warhead- and linker design showed potent and selective degradation in Burkitt's lymphoma cell lines [19].

(b) In contrast, VHL-based PROTACs did not evolve from know-how on E3 binding molecular glues, but initially relied on peptidic VHL binding ligands [20]. Proof of concept with peptidic ligands subsequently motivated the rational design of small-molecule binders of VHL [21], and enabled the design of heterobifunctional small-molecule degraders of Halo-tagged proteins, BRD4, ERRα or the kinase RIPK2 [22–24]. Similar to CRBN-based degraders, VHL-PROTACs were found to be highly selective, and compatible with *in vivo* applications [24,25].

While PROTAC design has a strong emphasis on $CRL4^{CRBN}$ and $CRL2^{VHL}$, it is not limited to these two ligases. Functional degraders were also developed based on ligands that engage non-CRL ligases such as MDM2 [26,27], or cIAP1 [28–30]. Additionally, recent studies based on chemoproteomics and affinity-based protein profiling have unlocked RNF4, RNF114 and DCAF16 as additional E3 ligases actionable for PROTAC development [31–33]. Moreover, work with artificial recruitment systems suggested that a wide variety of E3s is in principle amenable for chemical hijacking, and that future extension to additional ligases will only be limited by the identification of suitable ligands [34]. Due to space considerations, we will here mostly focus on CRBN and VHL-based degraders, molecular glues and chemical-genetic strategies derived thereof.

In many aspects, PROTACs and molecular glues behave similar to each other, but very different from traditional competitive inhibitors. Most notably, they follow a mechanism of catalytic turnover where a single small-molecule equivalent can induce the degradation of multiple target protein equivalents [24]. Thus, degraders can operate at sub-stoichiometric levels and induce near-complete proteolysis at concentrations below equilibrium target occupancy. Here, we will attempt to discuss how this unique molecular pharmacology can be applied to validate and characterize cancer targets and vulnerabilities in a pre-clinical setting.

Conventional target validation strategies

Currently, target validation ideally relies on integrative use of genetic or pharmacologic strategies. Genetic approaches are typically either based on RNA-interference (RNAi) or CRISPR/Cas9-mediated genetic disruption. Conceptually, genetic perturbations interfere with all functions associated with a particular protein as they effectively reduce overall cellular protein abundance. Conversely, target validation by small-molecule antagonists is limited to individual protein functions encoded by the chemically addressable domain. However, pharmacologic target validation offers some intriguing advantages. This includes dose-dependent control of effect size, reversibility, as well as a higher kinetic resolution as the actionable activity of most targets can typically be blocked within minutes to a few hours after ligand exposure. Comparatively, genetic perturbations are typically less dynamically controlled, sometimes entirely irreversible, and most often do not allow titration of effect size.

One of the biggest challenges of both strategies is the often uncertain issue around off-target activities, where the effect of an shRNA/sgRNA or a small-molecule extends to unknown target proteins. Confounding factors can particularly occur if small-molecules are (i) poorly characterized, and/or (ii) used at concentrations that are agnostic to target engagement and thus at levels that exceed the concentrations needed to block the target activity [4,35]. Off-target action of functional genomics-based approaches is well-documented, extensively

reviewed elsewhere, and addressed via continuous optimizations (Fig. 2) [36–39].

TPD as target-validation approach: opportunities and challenges

How does target protein degradation compare to these traditional target validation approaches? In principle, it combines key advantages of conventional chemical and genetic perturbation strategies (see also Fig. 2). Degradation typically occurs within a few hours after cellular treatment, thus conserving the high kinetic resolution of traditional antagonists. Simultaneously, TPD affects overall target protein abundance in a comparable manner to genetic perturbations. Similar to chemical inhibition, degradation enables dose-dependent control, albeit following a parabolic concentration-dependence where elevated degrader concentrations preferentially trigger the formation of binary complexes over a fully functional tripartite assembly. This effect is commonly referred to as the ''hook effect''. Upon washout of the degrader, the effect on protein levels is reversible. As reversal depends on active protein re-synthesis, the associated kinetics are however target-specific and correlate with the rate of protein production.

The question of specificity of small-molecule degraders comes in different layers. For PROTACs, the general notion is that converting a small-molecule binder into a heterobifunctional degrader provides means to increase selectivity. Among others, this has been shown by converting multi-targeted kinase inhibitor warheads into CRBN- and VHL-based degraders [40–43]. Resulting PROTACs degrade only a subset of the bound target proteins. Notably, the ability to induce degradation is not correlated with binding affinity to the respective target protein. Future work will be required to predict the specificity of a given PROTAC, or to rationally design selective degraders. Towards that goal, first important insights were gained by structural and biophysical studies that attribute degrader selectivity to differential cooperativity in ternary complex formation, or plastic interprotein contacts [44–46].

One notable caveat of the PROTAC approach are off-targets that are independent of the targeting warhead, but emerge from inadvertent ligase modulation through the E3-binding moiety. For instance, we have recently observed that CRBN-based degraders with a multi-targeted kinase inhibitor warhead can induce degradation of the translation factor GSPT1 [47]. Of note, GSPT1 is not bound by the promiscuous kinase inhibitor scaffold. It is degraded via the phthalimide-linker conjugate that closely matches the mechanism of the known CRBN modulator CC-885 functioning as molecular glue [47,48]. Notably, recent comprehensive profiling of the full ''degrome'' of CRBN modulation via molecular glues revealed a spectrum of possibly degradable targets [11]. This target-spectrum will inform the selectivity profiling of novel CRBN-based PROTACs to restrict warhead-independent E3 modulation. Generally, the identification of off-targets for PROTACs is relatively straightforward. Given their sub-stoichiometric

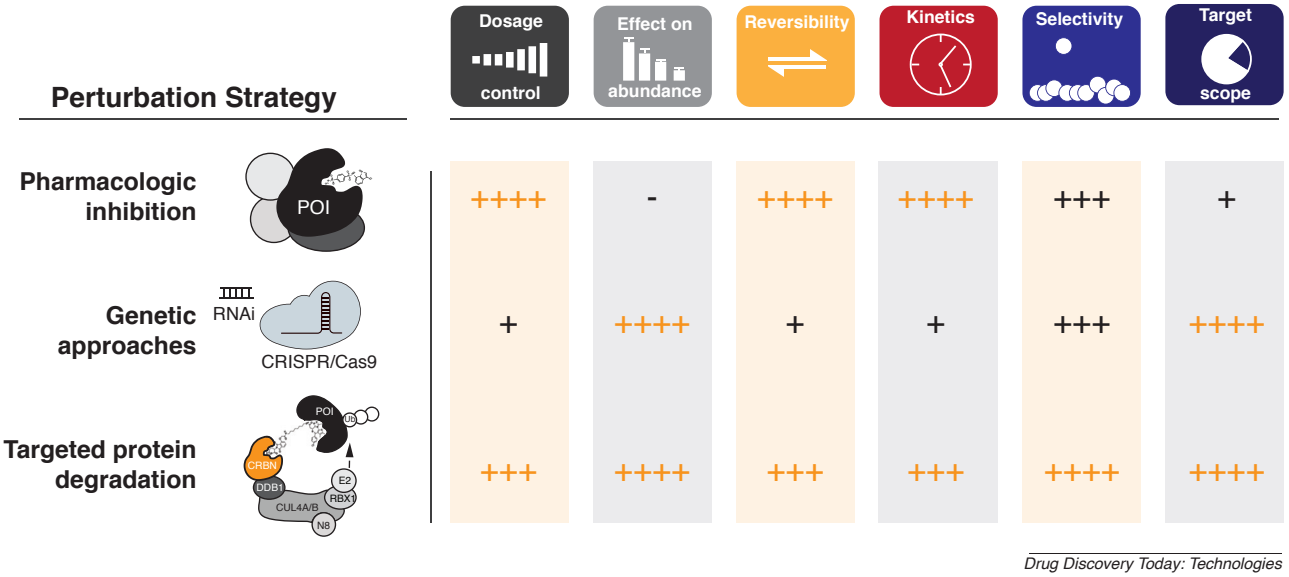

Fig. 2. Comparison of target validation strategies.
Targeted protein degradation (TPD) is compared to genetic and traditional pharmacologic approaches with regard to key features discussed in this review. The presented scoring is a semi-quantitative attempt aimed to highlight the particular strengths and weaknesses of each perturbation strategy, and should be considered as a rough approximation. This is particularly true for the evaluation of selectivity, as both genetic perturbants (RNAi and CRISPR/Cas9) as well as small-molecule probes show a wide inherent variability. Comparative studies of small-molecule inhibitors and their degrader derivatives have however indicated that the degraders are generally of higher specificity. Among others, this is explained by the fact that induction of degradation depends on productive ternary complex assembly and can thus be dictated by structural constraints outside of the particular ligand binding pocket. Noteworthy, all features assigned to pharmacologic inhibitors assume non-covalent inhibitors.

activity, off-target inhibitory or agonistic events are often negligible at cellular concentrations required to induce target proteolysis. Hence, coupling PROTAC treatment with downstream quantitative expression proteomics typically gives a concise picture of the overall target space. This makes the characterization of degraders more manageable compared to traditional small-molecule inhibitors where target-identification requires integration of multiple experimental strategies. Often, this also involves unbiased drug-affinity chromatography that requires compound immobilization and thus relies on medicinal chemistry support.

Interpreting and controlling cellular target degradation

Key to the proper interpretation of any target validation strategy is the implementation of experiments powered to rationally ablate the effect of a perturbation. Of particular importance in cancer target discovery, the prevailing use of ''down assays'' puts a high burden on ensuing rescue experiments [4]. To validate on-target effect of PROTACs, a set of rescue strategies is frequently used in the literature. This includes proving reversal of the degrader effect by pharmacologic inhibition of the proteasome, or by blocking cullin neddylation (and thus CRL activity) via pharmacologic NAE1 inhibition. Degrader-specific control experiments also extend to comparative studies in isogenic cell lines that are either pro- or deficient in expression of the respective E3. Further controls relate to chemical competition experiments with E3 ligase binders as well as experiments with chemically matched PROTAC-derivatives that are incapable of E3 binding.

Importantly, while all of these strategies are essential to validate the mechanism of targeted proteolysis, they do not necessarily prove causality of degradation of the known target and the observed cellular phenotype (e.g. cell death) as they would also prevent the destabilization of (possibly unknown) off-targets. To unequivocally link cellular PROTAC effects to the degradation of a specific target, experimental strategies need to affect target engagement. For instance, degrader efficacy can be mitigated upon ectopic expression of a target protein mutated in key-residues required for degrader binding, or via chemical competition with a (benign) binding ligand. Alternatively, it is conceivable to attempt a functional rescue by introducing mutations of amino acid residues at the dimerization interface with the E3 ligase to prevent ternary complex formation, or by mutating key lysine residues known to be ubiquitinated. However, the identification of critical residues is nontrivial in the absence of structural information.

TPD-based target validation *in vivo*

Small-molecule degraders have successfully been applied for *in vivo* target validation. In standard xenograft- or PDX models, it is however important to consider the obtained effects and the measured therapeutic window also in light of species differences. It is for instance conceivable that a degrader can very effectively induce target proteolysis in a human cellular background, but that potency and specificity do not translate when degraders are tested in mouse cells where subtle differences in the amino-acid sequence of binding sites or protein surface interfaces can significantly abrogate degrader efficacy [49]. If indeed degradation of the mouse protein requires higher drug concentrations compared to its human counterpart, it is important to investigate the extent to which this falsifies the interpretation of a determined therapeutic window. Currently, such species differences are best documented for IMiDs. It is however reasonable to assume that PROTACs can similarly be affected. While the availability of mouse models with humanized E3 ligases will be helpful in certain instances [50], recent data focusing on the thalidomide target SALL4 underscores that in some cases, efficient degradation only occurs when both involved proteins (target and E3) are from the same species [10]. However, the issue of species selectivity is not unique to PROTACs or molecular glues, but represents a prevalent challenge in drug discovery which is for instance well-documented for antibody therapies [51]. Collectively, while chemical degraders are well suited for *in vivo* target validation studies, the unique pharmacology of TPD requires careful consideration and interpretation of obtained results.

Chemical-genetic derivatives of TPD

As outlined in specific examples below, target validation by TPD can either occur via directly acting small-molecule degraders, or via chemical-genetic derivatives thereof (Fig. 1). Chemical-genetics approaches have the advantage that a standardized compound can be used to prompt the degradation of a wide spectrum of target proteins. A key limitation is however that targets need to be amenable to the introduction of low molecular weight tags that are required for degrader binding. Here, we will focus on two chemical-genetics approaches, (i) the degradation tag (dTAG) strategy, as well as (ii) auxin-inducible degron (AID) tagging.

(i) The dTAG approach (Fig. 1) is based on a previously described, mutant-selective, heterobifunctional degrader of an engineered variant of FKBP12 [52]. A defined amino-acid substitution in FKBP12 (F36V) extends the ligand binding pocket so that it can selectively be exploited by AP1867, a 'bumped' synthetic ligand [52]. Consequently, expression of a protein of interest as a fusion to $FKBP12^{F36V}$ (from here on referred to as dTAG) renders this fusion 'degradable' via a standardized ligand. Mechanistically, the dTAG approach works in close analogy to aforementioned degraders. The heterobifunctional dTAG-ligand recruits dTAG fusion proteins to the endogenous $CRL4^{CRBN}$ ligase complex, and induces fast and complete proteasomal degradation of the fusion protein

[53]. The dTAG approach was first employed to identify the mechanistic underpinnings of a novel vulnerability of acute leukemia to the transcriptional regulator ENL (described in detail below) [53]. Fusion proteins can either be ectopically expressed, or endogenously engineered via CRISPR/Cas9 mediated knock-in [54]. Notably, recent studies showcased that dTAG-enabled target degradation can also be achieved in vivo [54].

(ii) The AID-tag represents complementary alternative to the dTAG approach (Fig. 1D). Mechanistically, it is inspired by the mode of action of the aforementioned phytohormone auxin [14,55,56]. The use in mammalian cells thus requires ectopic expression of the Tir1 E3 ligase along with ectopic expression or endogenous tagging of the POI via the AID-tag. AID-tagged proteins can subsequently be recruited to a functional $CRL1^{TIR1}$ ligase complex in an auxin-dependent manner, again leading to their ubiquitination and proteasomal degradation [55,56]. Given the high concentrations of auxin that are required to induce degradation, it has thus far not been possible to perform AID experiments *in vivo*.

Based on reported data, both dTAG and AID-tagging enable exquisitely selective perturbations, and off-targets based on inadvertent CRBN modulation have not been reported for the standardized dTAG ligands [53,54,57]. In addition, based on the reported cases, simultaneous degradation of protein interactors of the POI has not been reported even though it is conceivable that interacting proteins might face destabilization as a downstream consequence of initial POI degradation. Therefore, both approaches allow for a fast, near-complete, and highly selective degradation of their respective target proteins. In addition, both approaches permit a straightforward, phenotypic rescue via ectopic expression of untagged cDNAs that are resistant to drug-induced degradation, and are thus expected to revert phenotypes induced by degrader treatment.

Notable other chemical-genetics approaches well-suited for target identification and validation include the Shield system [58], HaloPROTACs [22], induced displacement of cryptic degrons [59], and small-molecule-assisted shutoff (SMASh) [60]. Due to space limitations, these approaches will not be discussed in detail. As all these approaches require genetic manipulation, their direct applicability in cancer therapy is limited. A notable exception might be the use of degradation approaches as off-switches for synthetic- or gene-therapy based approaches. In the following, we want to discuss recent examples where TPD was applied to validate and characterize known targets, to identify novel cancer vulnerabilities, or to de-validate target hypotheses. Examples will relate to directly acting degraders, chemical-genetics strategies, as well as their integrated use.

Characterization and validation of cancer targets

BRD4 and BET proteins have received a lot of attention as therapeutic targets for different cancers [61]. Initial drug development efforts were motivated by recurrent translocations of BRD4 observed in NUT midline carcinoma [62]. Subsequently, BRD4 was identified as a non-oncogene addiction in AML via *in vivo* RNAi screens [63]. Mechanistically, BRD4 emerged as a transcriptional co-activator that binds to acetylated histones and transcription factors at promoters and enhancers via two N-terminal bromodomains [64]. BRD4 binding to chromatin was found to be highly asymmetrical, where clusters of regulatory regions (referred to as super-enhancers) are bound by a disproportional amount of BRD4 [65]. Bromodomain inhibition by competitive small-molecules such as JQ1 was shown to displace BET proteins from chromatin, outlining a strategy for pharmacologic inhibition of BET-dependent gene control [62,66]. Given that super-enhancers were found to be hyper-sensitive to BET inhibitor treatment, BET proteins were mechanistically linked to transcriptional control of super-enhancer associated genes such as c-MYC, extending their therapeutic potential also to other malignancies [67,68].

In an effort to dissect the increased cellular efficacy of BET protein degraders compared to BET inhibitors, we set out to compare the transcriptional consequences of the BET inhibitor JQ1 with the second-generation BET degrader dBET6 [69]. We identified that BET degradation led to a global displacement of BRD4 from all regulatory regions including transcriptional start sites. This led to a widespread ablation of mRNA transcription, which we could link to a global disruption of transcriptional pause release. Collectively, this established BET proteins as master regulators of transcription elongation. Notably, acute BET degradation was inconsequential for P-TEFb recruitment to transcriptional start sites and enhancers, thus challenging the paradigmatic role of BRD4 as a P-TEFb recruitment factor [69,70]. Notably, given that employed degraders lacked selectivity for BRD4 over other BET proteins, our experimental setup did not allow assigning these transcriptional effects exclusively to BRD4. This was addressed by ensuing work that applied a BRD4-AID knock-in strategy compatible with selective BRD4 degradation [57]. BRD4 degradation in K562 and HCT116 cells induced a very comparable, widespread inhibition of mRNA synthesis that is consistent with a general role of BRD4 as a RNA Pol II co-activator. Notably, also in this model, BRD4 degradation appeared to be inconsequential for chromatin-bound P-TEFb levels [57]. Further research will be required to delineate the mechanism of action of BRD4 in Pol II transcription, including proposed roles as an atypical kinase that phosphorylates the carboxyterminal domain of Pol II [71].

Beyond BET proteins, targeted protein degradation was furthermore successfully applied to validate the bromodomain containing protein BRD9 as a promising, context-spe-

cific cancer target. BRD9 is a component of the recently identified non-canonical BAF (ncBAF) complex, a variant of the canonical BAF chromatin remodeler complex [72]. BRD9 was initially shown to be required for the proliferation of AML cell lines via shRNA experiments [73]. This spurred the development of competitive BRD9 bromodomain antagonists [73,74]. BRD9 inhibitor treatment caused transcriptional consequences reminiscent of BRD9 knockdown, but cellular efficacy of BRD9 bromodomain inhibition was relatively subtle [73]. Notably, phthalimide-based conversion of the same BRD9 bromodomain ligand into a BRD9 degrader yielded a potent and highly selective PROTAC that exerted a potent anti-proliferative effect in AML cell lines. Cellular potency of BRD9 degradation was increased up to 100-fold compared to parental BRD9 bromodomain inhibitors [75]. Hence, ligand-induced degradation provided definitive pharmacologic evidence of a dependency of AML cells on BRD9 [75]. More recently, pharmacologic degradation was also employed to identify and validate BRD9 as a synthetic-lethal target in cancers harboring canonical BAF complex mutations [76,77].

TPD-enabled identification and validation of novel cancer targets

The YEATS domain containing protein ENL is part of a multiprotein assembly called the super elongation complex (SEC) that orchestrates gene activity at the level of transcriptional elongation [78]. ENL was identified as a dependency in acute leukemia via an unbiased genome-scale CRISPR/Cas9 dropout screen [53], or via hypothesis-driven, focused investigation [79]. To explain the acute mechanistic role for ENL in leukemia pathogenesis and transcription regulation, Erb et al. implemented the aforementioned dTAG approach [53]. Ectopic expression of dTAG-ENL combined with genetic depletion of endogenous ENL allowed for rapid ENL ablation in sensitive AML cell lines, and thus for a mechanistic understanding of ENL-dependent, essential transcriptional circuits. The authors identified a core set of genes with disproportionally high levels of promoter-bound ENL, including well-known leukemic driver genes such as MEIS1, MYB, and RUNX1. ENL degradation suppressed transcription initiation and elongation genome-wide, but led to pronounced transcriptional defects at ENL^{high} genes at early time points. Mechanistically, transcriptional impairment of ENL^{high} genes was caused by attenuated SEC recruitment and activity. Rescue experiments finally validated that the YEATS reader domain is essential for ENL-dependent leukemic growth. Therefore, this study also provided mechanistic support for inhibiting the ENL YEATS domain as a promising strategy in leukemia [53].

An additional leukemic dependency was recently identified via a VHL-based heterobifunctional degrader of TRIM24 [80]. TRIM24 is a bromodomain-containing transcriptional regulator that has been implicated as specific vulnerability in breast and prostate cancers [81,82]. However, potent and selective ligands for the TRIM24 bromodomain were mostly found inconsequential for cancer cell proliferation suggesting that bromodomain inhibition may not be sufficient as an anti-cancer strategy. This found further support in domain-scanning CRISPR/Cas9 experiments, which highlighted the RING domain of TRIM24 as the functional domain linked to the essentiality of TRIM24 in AML [80]. In line with this, pharmacologic TRIM24 degradation exerted an anti-proliferative response that outperformed competitive bromodomain inhibitors, thus confirming that the sensitivity of AML cell lines to loss of TRIM24 is likely independent of the TRIM24 bromodomain.

Applications of TPD for target de-validation

In addition to identifying and characterizing novel targets, TPD was also applied to invalidate previously postulated cancer vulnerabilities.

MELK is a serine/threonine kinase that has been reported to be overexpressed in various cancers, most notably breast cancers [83]. MELK expressing cells were shown to have higher tumor-initiating potential, which was reported to be MELK dependent [84,85]. RNAi-based studies have established a role for MELK in the proliferation of breast-cancer cells [86]. This was further supported via a competitive MELK kinase inhibitor that showed pronounced activity in cancer models of different origins, but lacked sufficient specificity for MELK over other kinases [87]. Employing the dTAG system, a recent study could however show that chemical degradation of MELK was inconsequential in breast cancer cells [88]. This was supported by additional, selective MELK kinase inhibitors, CRISPR/Cas9 mediated MELK knockout and shRNA knockdown of MELK. An independent study that mostly relied on CRISRP/Cas9 mediated mutagenesis came to a similar conclusion [89].

An additional kinase that was a long-sought after target is TANK-binding kinase 1 (TBK1). It was an attractive target due to its reported role in tumorigenesis, especially in KRAS-mutant ($KRAS^{mut}$) tumors where RNAi based genetic screens suggested a synthetic-lethal relationship between $KRAS^{mut}$ and TBK1. However, the notion that $KRAS^{mut}$ cancer cells selectively depend on TKB1 activity was challenged by subsequent reports, and pharmacologic TBK1 kinase inhibition failed to recapitulate the synthetic lethal interaction with $KRAS^{mut}$ [90]. Arguably, this could still be explained by an involvement of molecular functions that are independent of the kinase activity of TBK1, such as molecular scaffolding functions. However, a recently published, VHL-based TBK1 degrader was similarly incapable of selectively exerting anti-proliferative effects in $KRAS^{mut}$ cancer cell lines [91]. Given that potent and selective TBK1 degradation was achieved over a spectrum of cell lines,

these findings corroborate that TBK1 does not constitute a selective dependency in $KRAS^{mut}$ cancers.

Conclusions

Target(ed) protein degradation (TPD) is a promising novel paradigm in chemical probe- and drug development. Small-molecule degraders follow a unique mode of action based on catalytic target turnover that enables the degradation of super-stoichiometric amounts of target protein. Ablation of proteins via small-molecule degraders is potent, selective, and typically occurs within the first hours after treatment. Therefore, TPD is particularly informative when coupled with unbiased technologies that measure acute phenotypes and thus capitalize on the high kinetic resolution. To study causal mechanisms of targets involved in gene-control, it can for instance be highly informative to couple degradation with nascent RNA-sequencing approaches such as NET-seq or SLAM-seq [57,69]. This high kinetic resolution, along with a suite of well-defined control and rescue experiments positions TPD as a technology well-suited for the identification of cancer vulnerabilities and associated mechanistic studies.

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