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Opportunities and challenges of protein-based targeted protein degradation

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In the 20 years since the first report of a proteolysis targeting chimeric (PROTAC) molecule, targeted protein degradation (TPD) technologies have attempted to revolutionize the fields of chemical biology and biomedicine by providing exciting research opportunities and potential therapeutics. However, they primarily focus on the use of small molecules to recruit the ubiquitin proteasome system to mediate target protein degradation. This then limits protein targets to cytosolic domains with accessible and suitable small molecule binding pockets. In recent years, biologics such as proteins and nucleic acids have instead been used as binders for targeting proteins, thereby expanding the scope of TPD platforms to include secreted proteins, transmembrane proteins, and soluble but highly disordered intracellular proteins. This perspective summarizes the recent TPD platforms that utilize nanobodies, antibodies, and other proteins as binding moieties to deplete challenging targets, either through the ubiquitin proteasome system or the lysosomal degradation pathway. Importantly, the perspective also highlights opportunities and remaining challenges of current protein-based TPD technologies.

Introduction

Recent technological advancements have greatly expanded molecular level understanding of disease pathologies, revealing a multitude of potential protein targets for therapeutic

intervention.^{1–5} While traditional therapeutics such as small molecules and monoclonal antibodies are effective at blocking protein function through occupancy-driven strategies, they are often limited in targeting proteins such as transcription factors, non-enzymatic proteins, and scaffolding proteins that lack suitable binding pockets. Moreover, achieving complete protein inhibition often requires high drug dosages and continuous exposure, which can lead to increased off-target effects and resistance.⁶ Traditional targeted protein degradation (TPD)

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discover the drivers of biomolecular interactions and (2) leverage that information to modulate disease relevant proteins. She holds appointments in the Departments of Chemistry and of Microbiology & Immunology at Stanford University and is an Institute Scholar at Sarafan ChEM-H. (Photo credit: Do Pham/Stanford University).

and facilitating access to the cellular degradation machinery; and (2) a summary of intracellular protein degraders comprising nanobodies, antibodies, or other proteins as POI and degradation machinery binders. For this aspect, we cover the identification and engineering of antibody/nanobody binders for various POIs, the degrader design strategy and protein delivery methods. We also discuss remaining challenges of protein-based degraders, such as unknown pharmacokinetics and the absence of informed design. Throughout this Perspective, we offer an extensive examination of the latest advancements in protein-based degraders, while further enhancing our understanding of essential biological factors that govern degradation processes. Moreover, we also provide insights into the potential for strategically designing these types of degraders for use in therapeutic applications and in exploring biological pathways.

Fundamentals of TPD

There are two major types of degradation systems in cells: the lysosomal pathway and the proteasomal pathway.^{41,42} In the lysosomal pathway, lysosomes degrade both extracellular and intracellular substances (proteins, nucleic acids, polysaccharides, and others).⁴³ The extracellular substances and plasma membrane components (such as receptors or channels) enter the cell through the endocytic pathway⁴⁴ and become degraded in the highly acidic endosome/lysosome.⁴⁵ This contributes to plasma membrane repair, immune response, bone resorption, and pathogen elimination.⁴⁶ Some of the internalization process is highly specific, which allows cells to efficiently internalize specific substances while ignoring others. For instance, in receptor-mediated endocytosis, cells selectively take up substances (*e.g.*, extracellular proteins) that bind to specific receptors on the cell membrane.⁴⁷ This binding causes a conformational change in the receptor, which in turn triggers the formation of a vesicle containing the ligand–receptor complex.⁴⁸ The vesicle then travels to the early endosome, where many endocytosed substances dissociate from their receptors in the mildly acidic compartment and are selectively sorted *via* transvesicular compartments (like multivesicular bodies, MVB, or endosomal carrier vesicles, ECVs)⁴⁹ to fuse with the lysosome for subsequent degradation by acidic hydrolases in that compartment. Meanwhile, upon the release of substances, the receptors are recycled back to the plasma membrane through recycling endosomes (Fig. 1a). By harnessing the receptor-mediated endocytic pathway for lysosomal degradation, LYTACs have been used to degrade extracellular or transmembrane proteins.³⁵ Lysosomes are also responsible for degrading intracellular damaged proteins or organelles through autophagy; autophagy allows the wrapping of damaged proteins by autophagosomes.⁵⁰ Upon fusion of autophagosomes and lysosomes, autolysosomes are formed to degrade the cargo and release the breakdown products (amino acids) into the cytosol for reuse (Fig. 1b). The regulation of intracellular substance levels through lysosomes is of great significance for maintaining the normal metabolic activities of cells and for defending against microbial infections.⁵¹

In this Perspective, we review the progress made in protein-based degrader systems, focusing on two key aspects: (1) an overview of recent developments to deplete extracellular or transmembrane proteins using LYTACs, GluTACs, AbTACs, PROTABs, and KineTACs, in order to highlight the unique design enabling the uptake of extracellular or transmembrane proteins

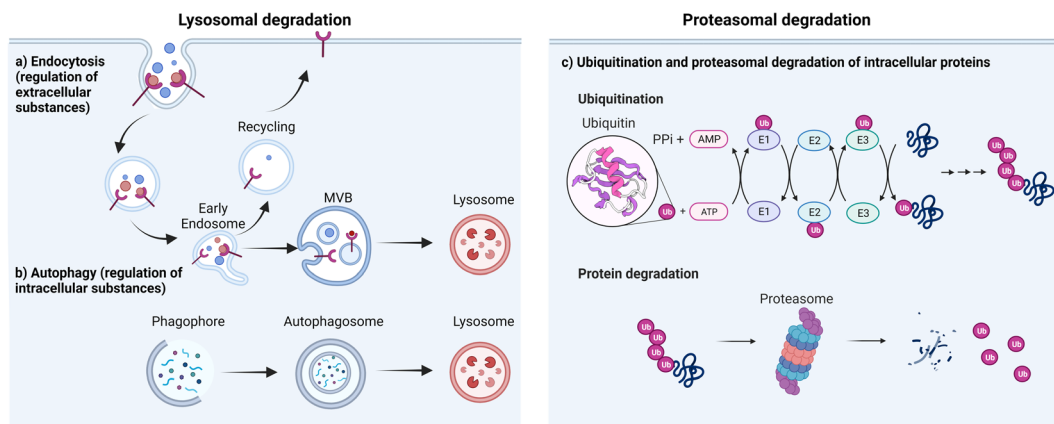


Fig. 1 Major cellular platforms for protein degradation. Degradation of (a) extracellular substances through endocytosis, (b) intracellular substances through autophagosome/lysosome fusion and (c) intracellular substances through proteasomal pathway.

The second major degradation system is the proteasomal system. In this system, proteins are degraded by the 26S proteasome, a complex of proteolytic enzymes that break down proteins into small peptides.⁵² This system has three main components: the proteasome, various ubiquitin ligases, and multiple ubiquitinating or de-ubiquitinating enzymes. Ubiquitin is a regulatory protein that is highly conserved across all forms of life. It consists of 76 amino acids with a molecular weight of 8.5 kDa. It is linked to proteins that are targeted for degradation through a multistep process called ubiquitination. Ubiquitination involves the action of three enzymes to mediate the covalent modification of proteins. In the first step, an E1 (ubiquitin activating enzyme) binds to the ubiquitin molecule through its active site. In the next step, ubiquitin is transferred to the active site of E2 (ubiquitin conjugating enzyme). In the third step, E3 (ubiquitin ligase enzyme) recognizes the substrate protein to be degraded and catalyzes the transfer of ubiquitin from the E2 protein to the substrate (Fig. 1c).

There are different types of E3 ligases, including the really interesting new gene (RING) E3s, homologous to E6-AP C terminus (HECT) E3s, and RING-between-RING (RBR) E3s.⁵³ The mechanism of substrate ubiquitination differs among each type.⁵⁴ The RING E3 ligases, most notably the Cullin–RING ligase (CRL) multi-subunit superfamily, comprise the largest known class of ubiquitin ligases.^{55,56} Human cells express seven different cullins (CUL1, 2, 3, 4A, 4B, 5 and 7) that each nucleate a multisubunit ubiquitin ligase.⁵⁷ CUL1 CRLs, which are known commonly as SKP1, CUL1, and F-box (SCF) proteins, recruit substrates through the adaptor protein SKP1 and an F-box-protein substrate receptor;⁵⁸ CUL2 CRLs such as VHL, and CUL5 CRLs recruit substrates through an Elongin-BC adaptor and a suppressor of cytokine signaling/Elongin-BC (SOCS/BC)-box-protein substrate receptor;⁵⁹ CUL3 CRLs, such as speckle type BTB/POZ protein (SPOP), recruit substrates through BTB-domain-containing substrate-receptor proteins;⁶⁰ CUL4A CRLs, which include DCAF, recruit substrates through the adaptor protein DNA-damage-binding protein-1 (DDB1).⁶¹ HECT E3 ligases first form a thioester intermediate with ubiquitin, then subsequently transfer it to the substrate.⁶² RBR E3 ligases use one RING domain to bind the

ubiquitin loaded E2, then transfer ubiquitin to the second RING domain, which facilitates ubiquitin ligation to the substrate.⁵⁷ In all cases, the substrate specificity of the system is determined by the E3 ligase, which binds directly to the target substrate. Following the initial ubiquitination event, the substrate-linked ubiquitin can be ubiquitinated on one of several lysine residues, leading to the formation of polyubiquitin chains.⁶³

Ubiquitin has several lysine residues (K6, K11, K27, K29, K33, K48 and K63) and the free amino group located at its N-terminus that are involved in the formation of polyubiquitin linkages. Different homo- and heterotypic linkages are employed during the modification of target proteins to subsequently induce different cellular functions. For example, K63-linked ubiquitination serves as a docking site to mediate protein–protein interactions or conformational changes while the K48-linked polyubiquitin chains are involved in proteasomal degradation.^{64,65} The proteasomal degradation pathway plays a central role in removing abnormal or misfolded proteins and in regulating a variety of protein functions that allow control of the physiological behavior of the cell. Small molecule PROTACs, molecular glue degraders, and protein-based degraders for intracellular POIs all primarily utilize this pathway to induce protein degradation.

As the two most significant degradation pathways in cells, the proteasomal pathway and the lysosomal system independently regulate the degradation of substances in cells and interact with each other to achieve compensatory or synergistic outcomes. By harnessing the power of these two degradation systems, TPD technologies mediate the depletion of both intracellular, transmembrane, and extracellular proteins with high specificity and efficiency.

Protein-based degraders that target extracellular or transmembrane proteins

Extracellular and transmembrane proteins constitute 40% of all protein-encoding genes and comprise those that are critical in



cancer, ageing-related diseases, and autoimmune disorders.²⁰ However, small molecule PROTACs and molecular glue degraders rely on the intracellular protein degradation machinery, which limits their use to proteins with intracellular domains. To deplete extracellular or transmembrane proteins, new strategies by employing engineered antibodies or nanobodies that traffic extracellular or transmembrane proteins to intracellular degradation machineries have been developed.

Lysosome targeting receptor mediated extracellular or transmembrane protein degradation

Receptor mediated endocytosis provides a means to achieve specific protein degradation *via* the lysosomal pathway. LYTACs hijack the interaction of glycosylated proteins with lysosome-targeting receptors (LTRs) at the plasma membrane to shuttle transmembrane glycoproteins to the lysosome for degradation. LYTACs are constructed with two components: a POI binder and a LTR binder (Fig. 2a). The first generation of LYTACs harnessed the cation-independent mannose-6-phosphate receptor (CI-M6PR) as the LTR.³⁵ By conjugating synthetic, nonhydrolyzable mannose-6-phosphonate (M6Pn) glycopeptides to antibodies, Ab-M6Pn conjugates were used to direct antibody targets to the lysosome *via* endocytosis. In the late endosome, the lower pH enabled dissociation of the targets and their procession to the lysosome for degradation, while the Ab-M6Pn was recycled back to the plasma membrane or Golgi. It was first demonstrated that conjugation of M6Pn ligand to biotin increased the internalization of the biotin binding protein NeutrAvidin in several cell lines. The strategy was then expanded to antibody binders, beginning with a polyclonal anti-mouse IgG non-specifically

labeled with M6Pn glycopolypeptides on lysine residues to generate the IgG-M6Pn. Coincubation of mouse primary antibodies and IgG-M6Pn accelerated the degradation of a neurodegenerative disease target, ApoE4 and a cancer therapeutic target, CD71 (transferrin receptor-1). To further demonstrate the scope of LYTACs, direct M6Pn modification of the clinically approved antibodies cetuximab (Ctx) and Atezolizumab was performed. These two antibodies are known binders for cancer therapeutic targets epidermal growth factor receptor (EGFR) and programmed death ligand 1 (PD-L1), respectively. EGFR is a driver of cancer proliferation that harbors intrinsic tyrosine kinase function, with high abundance of EGFR uniquely found in some brain, lung, liver and other cancers.⁶⁶ It performs multiple scaffolding functions regardless of inhibition of its receptor tyrosine kinase activity. PD-L1 is a transmembrane protein that is over-expressed on tumor cells; it binds to PD-1 receptors on activated T cells and mediates the inhibition of cytotoxic T cells; these deactivated T cells remain inhibited in the tumor microenvironment. The PD-1/PD-L1 pathway represents an adaptive immune resistance mechanism exerted by tumor cells for immune escape and has a high impact on the efficacy of cancer therapy.⁶⁷ LYTACs created with Ctx and atezolizumab mediated selective loss of EGFR and PD-L1 by more than 70% within 48 h of treatment in several cell lines. The degradation was dependent on M6P binding and on lysosomal acidification. Additionally, a CRISPR interference screen uncovered an exocyst complex as a previously unidentified but essential component of biochemical pathway for CI-M6PR-mediated cargo internalization in cell lines. CI-M6PR is broadly expressed in most tissues, ensuring that the approach works in many settings.³⁵

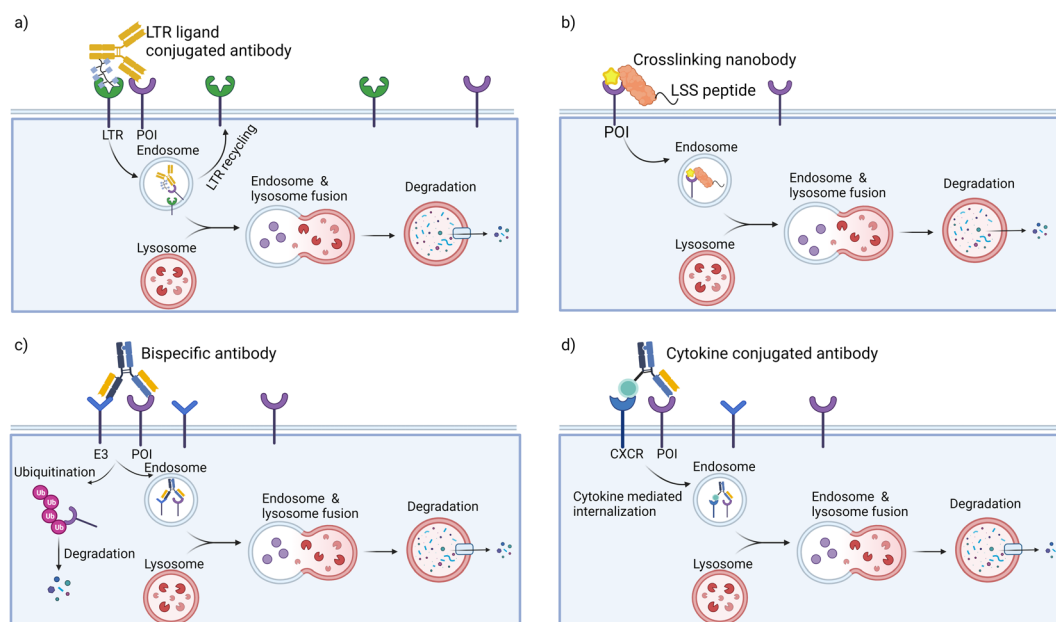
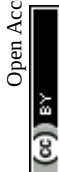


Fig. 2 Targeted protein degradation platforms for transmembrane proteins. (a) LYTACs use antibodies modified with LTR binders to direct the transmembrane POI to the lysosome for degradation; LTR is recycled to the cell membrane. (b) Nanobodies functionalized with crosslinking moieties and lysosomal sequence to induce the lysosomal degradation of transmembrane proteins. (c) AbTACs use recombinant bispecific antibodies that engage transmembrane E3 ligases and proteins for lysosomal degradation. (d) KineTACs also employ recombinant bispecific antibodies that consist of one cytokine arm and one POI binding arm to induce internalization and degradation of transmembrane proteins.



To achieve tissue specificity, second generation LYTACs were independently developed by the Bertozzi³⁶ and Tang groups;⁶⁸ these molecules engage the liver-specific ASGPR as the LTR (the Spiegel group has also targeted ASGPR with small molecule binders in a platform termed molecular degraders of extracellular proteins).⁶⁹ ASGPR has exclusive expression in hepatocytes and functions to traffic glycoproteins bearing *N*-acetylgalactosamine (GalNAc) or galactose to the lysosome *via* clathrin-mediated endocytosis. Following internalization and endosomal acidification, ASGPR releases GalNAc and recycles back to the plasma membrane, while glycoproteins proceed to the lysosome for degradation. GalNAc-LYTACs efficiently ablated EGFR in HCC cells but not in cells lacking ASGPR. Whereas Ctx-M6Pn did not have cell-type selectivity, GalNAc-LYTACs are capable of cell-specific POI degradation. This could have implications for their development into therapeutics, as the ability to restrict POI loss to particular tissues could improve the efficiency of degraders.

LYTACs are capable of degrading both secreted and transmembrane proteins in several cell types. The flexible construct and modular design enable adaptation to binders that include antibodies, peptides, aptamers, and small molecules. The liver-specific GalNAc-LYTAC has presented the possibility of cell type-specific LYTACs. In depth studies to elucidate the spatial orientation required for LTR recognition, kinetics of LTR turnover, the rates of target trafficking, and sorting through the endocytic pathway will be critical for translational applications.

Inspired by LYTACs, GlueTAC⁷⁰ was developed by fusing a nanobody with a lysosomal-sorting sequence so as to target transmembrane proteins (Fig. 2b). In this approach, the PD-L1 nanobody (Nb-PD-L1) was engineered to incorporate a fluorosulfate-*L*-tyrosine (FSY) for covalently labeling of POIs. By conjugating the covalent nanobody to a cell penetrating peptide consisting of a nona-*D*-arginine peptide and a lysosomal-sorting sequence (NPGY), the resultant GlueTAC led to the internalization and lysosomal degradation of more than 89% of PD-L1 and showed sustained T-cell activation and tumor growth inhibition. Without the FSY modification, only 32% of PD-L1 was degraded. The irreversible covalent binders have the potential to improve the degradation efficiency of POIs and to avoid off-target effects during endocytosis. However, specific screening to identify unnatural amino acid incorporation and modification sites is required for each target, which may hamper the broad application of GlueTACs.

Bi-specific antibodies mediating transmembrane and extracellular protein degradation

To target transmembrane proteins, the Wells group developed fully recombinant bi-specific antibodies known as AbTACs, which bring the transmembrane E3 ligase RNF43 into close proximity with transmembrane POIs to mediate their degradation (Fig. 2c).³⁷ Their work utilized phage display to identify a binder for the ectodomain of RNF43. To target PD-L1 specifically, AbTACs were constructed using a “knobs-into-holes” technique with two half-IgGs aimed at RNF43 and PD-L1, leading to a 63% degradation of PD-L1. In contrast to small

molecules and LYTACs, the fully recombinant bi-specific IgG scaffold has favorable pharmacokinetic properties.

In normal cells, the expression level of RNF43 is highly constrained. However, hyperactivation of Wnt signaling in colorectal cancer results in an elevated expression of RNF43 and ZNRF3. This abnormally high expression level enabled scientists at Genentech to develop the colorectal cancer (CRC)-specific degraders by adopting bi-specific antibodies.³⁸ A series of antibodies with picomolar to nanomolar affinities against the ectodomains of RNF43 and ZNRF3 were obtained. Focusing on the insulin growth factor 1 receptor (IGF1R), a receptor tyrosine kinase that mediates growth factor signaling in various tissues and cancers, ZNRF3*IGF1R bispecific antibodies showed clearance up to 80% with specificity for CRC both *in vitro* and *in vivo*. Mechanistic studies revealed that ZNRF3*IGF1R antibodies can engage both proteasomal and lysosomal degradative pathways in a catalytic manner. The CRC-specific degradation seen with ZNRF3*IGF1R was demonstrated to be applicable to other cell membrane targets (such as HER2 and PD-L1) and transmembrane E3 ubiquitin ligases.

Genetic optimization was used to investigate the structure-activity relationship (SAR) between different bi-specific antibody scaffolds and their degradation efficiencies, with the aim of understanding the properties necessary for efficient degradation.^{38,71} The study found that degradation efficiency is highly dependent on the specific epitopes of the E3 and the POI rather than binding affinity. However, the optimal epitope for an E3 ligase is not universal to all targets, as it varies due to the different complexes formed between POIs and E3 ligases. The flexibility, valency, and orientation of the binding arms in IgG scaffolds also affect degradation efficiency. Therefore, careful optimization of the epitope, affinity, orientation, format, and combination of antibodies is necessary to achieve efficient degradation.

The above-mentioned bi-specific antibodies rely on transmembrane E3 ligases and intracellular ubiquitin transfer, limiting their scope to transmembrane proteins and leaving the secreted proteome out of reach. To address this limitation, the Wells group developed a new technology called KineTACs, constructed with genetically encoded human bi-specific antibodies that contain a cytokine arm to target a cytokine receptor, and a target-binding arm for POIs (Fig. 2d).³⁹ By focusing on CXCL12, they demonstrated that KineTACs can efficiently utilize CXCR7 internalization for lysosomal degradation. KineTACs are generalizable against other cytokines (*e.g.*, CXCL11, vMIP2, and IL-2) and have been used to degrade eight therapeutically relevant transmembrane and secreted proteins (PD-L1, HER-2, EGFR, and CDCP1, VEGF, TNF- α , CXCL11, and vMIP2). Compared to LYTACs and AbTACs, CXCL12-based KineTACs are exquisitely selective in degrading target proteins with minimal off-target effects, as revealed by proteomic analyses. However, KineTACs require the target protein to be internalized by a cytokine receptor, which may not be available or functional in all cell types or under all conditions. Additionally, the cytokine arm of KineTACs may activate signaling

pathways and induce cytokine-related toxicities *in vivo*, which needs to be carefully evaluated during preclinical development.

Protein-based PROTACs for targeting intracellular proteins

Ubiquitin-dependent proteolysis is a crucial pathway for regulating the levels of intracellular proteins as part of normal cellular maintenance processes. There are more than 600 E3 ligases encoded by the human genome,⁷² with each E3 ligase having a dedicated type of substrate.⁷³ The substrate specificity of the UPS is determined by the substrate-recognition domains within the E3 ubiquitin ligase complexes. To manipulate intracellular E3 ligases, protein-based PROTAC strategies primarily focus on engineering substrate-specific nanobodies or other binding moieties within E3 ligases to redirect the UPS system towards non-substrate protein targets. In contrast to small-molecule PROTACs, which frequently use small molecule binders for cereblon (CRBN), VHL, MDM2, and cIAP in their designs, protein-based PROTAC platforms use a much broader range of E3 ligases. These platforms include, but are not limited to, ubiquibodies,⁷⁴ TRIM21-nanobody fusion proteins,⁷⁵ chimeric F-box fusion proteins,⁷⁶ the affinity-directed protein missile system (AdPROM),⁷⁷ and the antibody RING-mediated destruction (ARMeD)⁷⁸ system. In this section, we provide a brief introduction to these technologies and focus on several examples that demonstrate their specificity in degrading protein isoforms (Fig. 3). Additionally, we highlight protein delivery methods, particularly the recent development of cell-permeant protein-based PROTACs for intracellular targets.

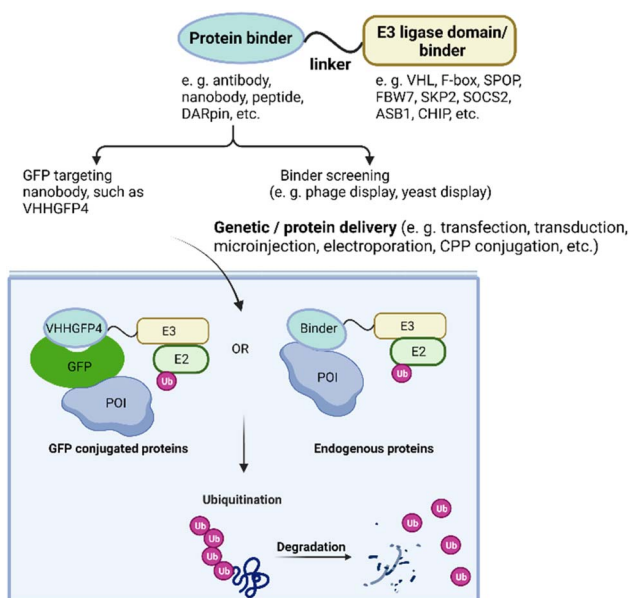


Fig. 3 Overview of protein-based PROTAC design. With protein or gene delivery methods, protein-based PROTACs enter cells and engage with both POI and E3 ligase, leading to polyubiquitination subsequent degradation of the POI.

Protein-based PROTACs for targeting green fluorescent protein (GFP)-tagged proteins

A series of pioneer protein-based PROTACs demonstrated their degradation abilities using intracellular POIs fused with GFP. In these designs, the substrate recognition module of the E3 ligases was replaced with a GFP binder such as the anti-GFP nanobody vhhGFP4. For instance, Affolter and colleagues developed the deGradeFP technique in 2012, which used the N-terminal domain of Slmb, a F-box protein identified in *Drosophila melanogaster*, fused to vhhGFP4 to induce the degradation of proteins with GFP fusion in both mammalian cells and *Drosophila*.⁷⁹ In 2016, the Sapkota group engineered the AdPROM system by modifying VHL to target GFP-tagged proteins for proteolysis in mammalian cells.⁸⁰

To assess the impact of different binders and E3 ligases on target degradation, the Partridge group characterized various E3 ligases and GFP binding scaffolds on GFP-tagged histone 2B.⁸¹ The tested E3 ligases included CUL1 ligases beta-transducin repeat containing protein (β -TRCP), F-box, and WD repeat domain-containing 7 (FBW7), and S-phase Kinase-associated Protein 2 (SKP2); CUL2 ligase VHL; CUL3 ligase speckle type BTB/POZ protein (SPOP); CUL5 ligases suppressor of cytokine signaling 2 (SOCS2), ankyrin repeat and SOCS box-containing 1 (ASB1); and U-box E3 ligase carboxy-terminus of Hsc70-interacting protein (CHIP). The results revealed that at least half of the E3 ligases tested led to more than 70% depletion of the POI. Additionally, a range of binding scaffolds such as peptides, nanobodies, designed ankyrin repeat proteins (DARPin), alpha repeat proteins, and monobodies, can successfully target GFP-tagged proteins. By replacing the GFP-binding domain with the 16-amino-acid Con1 peptide, which contains the conserved PIP sequence common to proliferating cell nuclear antigen (PCNA) binding partners, the protein-based PROTAC Con1-SPOP was developed and rapidly induced the degradation of PCNA upon doxycycline-induced expression. These discoveries indicate that protein-based PROTACs offer significant advantages in terms of linker and E3 ligase flexibility.

Protein-based PROTACs incorporating specific binders to target intracellular protein isoforms

Protein-based PROTACs provide researchers with a high level of flexibility when selecting binders for degradation targets and E3 ligases, thereby enabling the degradation of “intractable” unmodified therapeutic targets (*i.e.*, proteins for which small molecule binders have been difficult to obtain). However, extending the protein-based PROTAC platform to endogenous proteins requires the identification of highly specific binders, which can be challenging due to high sequence similarity between homologs, variants, and isoforms of proteins. Here, we highlight successful examples of protein-based PROTACs that distinguish the target protein from closely related homologs.

The downstream signaling of RHO small GTPases is mediated by its ability to switch between guanosine diphosphate (GDP)-bound and guanosine triphosphate (GTP)-bound states. A phage display selection protocol was used to identify an



Another example of a protein-based PROTAC targeting KRAS mutations is the monobody 12VC1 reported by the Koide

One strategy to deliver protein-based PROTACs into cells uses genetic approaches. The delivery methods are typically

(within hours).⁷⁸ The accelerated rate of degradation is attributed to the timing of protein production, with transfection of mRNA or DNA requiring a longer time to produce sufficient amounts of degrader proteins. Zeng *et al.* also used electroporation to deliver antibodies for a Trim-Away application.¹⁰² An advantage of electroporation is that it avoids accumulation in endosomes or lysosomes, and maintains cell viability for further studies, making this method particularly useful for delivering protein-based degraders. However, it is largely relegated to *in vitro* applications.

An alternative approach to overcome the translational limitations of the above-mentioned delivery methods is the development of cell-permeant protein-based PROTACs. This is done by conjugating or appending cell penetrating peptides (CPPs) to the protein-based degrader.¹⁰³ CPPs are short amino acids that can translocate cargo across the plasma membrane and facilitate their delivery to the cytoplasm. Since the mid-1990s, thousands of putative CPPs such as supercharged proteins, cyclic peptides, hydrocarbon stapled peptides, and miniature proteins, have been identified.^{103,104} Although their mechanisms of delivery are not all fully understood, it is believed that the positively charged residues (either Lys or Arg) in most CPPs form electrostatic interactions with sulfated proteoglycans on the cell membrane and induce endocytosis. Developing a universal CPP that can deliver diverse protein cargos without disrupting cell function would offer a more efficient platform for further translational studies.

Very recently, our group created a protein-based PROTAC to deplete endogenous BCL11A, a transcription factor validated as a therapeutic target for the hemoglobin disorders sickle cell disease and β -thalassemia.⁴⁰ Our design fused a nanobody binder specific for BCL11A to the cell-permeant miniature protein ZF5.3 (ref. 105 and 106) and to tSPOP, the catalytic domain of the E3 adapter protein SPOP. This protein-based PROTAC depleted cellular BCL11A in differentiated primary erythroid precursor cells and induced the expression of fetal hemoglobin (HbF).

As with other transcription factors, developing PROTACs for BCL11A is difficult because of BCL11A's intrinsic structural disorder and its close similarity to a paralog, BCL11B.¹⁰⁷ Despite being largely unstructured, BCL11A contains several well-ordered regions, including a CCHC zinc finger (ZnF0) and six C2H2 zinc fingers (ZnF1–6). The ZnF0 domain is involved in self-association of BCL11A,¹⁰⁸ while ZnF2–6 are critical for its function in suppressing HbF expression.¹⁰⁹ Targeting these domains with specific binders holds great potential for modulating the activity of BCL11A and its role in regulating HbF expression. However, the zinc finger regions of BCL11A and its paralog BCL11B share high sequence similarity, which may lead to binders recognizing both proteins. To address this, we created fragments of the well-ordered ZnF domains but included “extended”, presumptive disordered regions where the sequence differs between paralogs. For example, in targeting the extended ZnF23 (exZnF23) domain,

An alternative method to deliver protein-based PROTACs is by introducing nucleic acids or pure proteins into cells through microinjection and electroporation. For example, with microinjection, antibodies and Trim-21 proteins were directly introduced into individual cells to enable Trim-Away mediated degradation.⁷⁵ Hwang and colleagues injected degrader mRNA into the embryo zebrafish to deplete Hmga2-Citrine.¹⁰⁰ However, microinjection is a labor-intensive and skilled approach that can only be used on a small scale, and its potential for clinical application is limited. On the other hand, electroporation creates pores in the cell membrane using a short electrical pulse, allowing proteins to pass into the cell. This method is easy, fast, and enables a large number of cells to be transfected in a short time. For example, Clift *et al.* used electroporation to confirm the efficacy of Trim-Away in bulk populations of cells and to adapt the method for larger-scale assays.^{75,101} Similarly, Ibrahim *et al.* electroporated degrader proteins into cells to test the ARMeD system and found that electroporation of pure proteins resulted in a much higher degradation rate (within minutes) than that of expression

Reducing BCL11A expression has been shown to alleviate symptoms in individuals with hemoglobin disorders *via* reactivation of HbF expression.^{110,111} However, only the genetic approaches clustered regularly interspaced short palindromic repeats (CRISPR) – Cas9 and RNA interference are used in patients.¹¹² The sustained BCL11A degradation observed upon protein-based PROTAC treatment in HUDEP-2 cells prompted an investigation into the effect of ZF5.3-2D9-tSPOP on HbF induction in differentiated HUDEP-2 and primary human hematopoietic stem cells (CD34⁺ cells). During erythropoiesis, hemoglobin (Hb) synthesis increases from low levels in early progenitors to significantly higher amounts in mature enucleated erythrocytes. Consistent with its functional role as the Hb regulator, BCL11A levels also change during differentiation. As such, the timing of degrader delivery is critical. In both HUDEP-2 and CD34⁺ cells, significant HbF induction was observed during differentiation after treatment with the protein-based PROTAC. The treatment with ZF5.3-2D9-tSPOP had no impact on erythropoiesis of CD34⁺ cells, as demonstrated by comparable expression levels of differentiation surface markers CD235a and CD36 in treated and control cells. However, treated CD34⁺ cells exhibited slower proliferation compared to control, possibly due to BCL11A loss – this observation is consistent with studies using CRISPR-Cas9 deletion and shRNA silencing of BCL11A.¹¹³ The observed loss of BCL11A and induction of HbF

In parallel with this study, nanobodies targeting another fragment of BCL11A were also obtained.¹¹⁴ In contrast to exZnF23, the ZnF6 fragment is well-ordered but also distinct from the paralog BCL11B. Using the same strategy for ligand discovery, an initial nanobody binder for ZnF6 was evolved from a ligand with K_d of 2.75 μ M to one with a K_d < 700 nM *via* error prone mutagenesis. A crystallographic structure of the nanobody-BCL11A ZnF6 complex (Nb_6101-ZnF6) revealed that ZnF6 interacts with complementarity determining regions 2 and 3 of the nanobody. Site No. 45 on the nanobody Nb_6101 was shown to be critical because of its interaction with Lys806 on ZnF6; mutagenesis to convert Met45 of Nb_6101 (Fig. 4) to either an aspartic acid, a glutamic acid, a serine, or a threonine allowed for the formation of a hydrogen bond with Lys806, which improved binding affinity by approximately 6-fold. Computational modeling provided additional insights to enable the optimization of regions near site No. 45, and subsequent mutagenesis produced nanobody binders with significantly improved affinities ($K_d \sim 20$ nM). The approaches used here to guide the evolution of nanobodies led to ~ 100 -fold enhancement of their affinities for the target. With these evolved high-affinity binders, conjugation to Trim21 also led to the Trim-Away mediated degradation of BCL11A but not BCL11B. These two studies highlight the differences of targeting a well-ordered protein (ZnF6) *versus* one with structural disorder (exZnF23) but show that the latter is feasible.

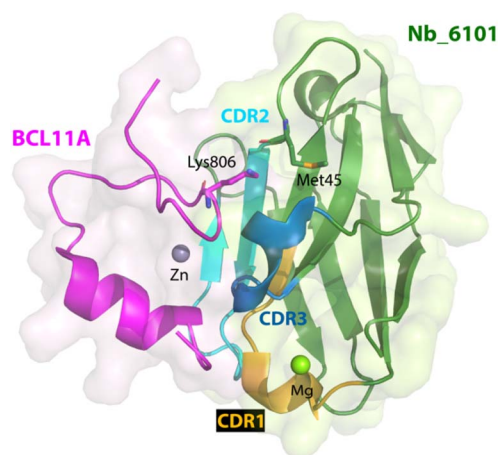


Fig. 4 X-ray crystallographic structure of nanobody Nb_6101 in complex with ZnF6 of BCL11A (PDB 8DTN). Nb_6101 is shown in green and ZnF6 in magenta. Methionine 45 in Nb_6101 close to Lysine 806 in ZnF6 could be replaced to increase binding affinity.

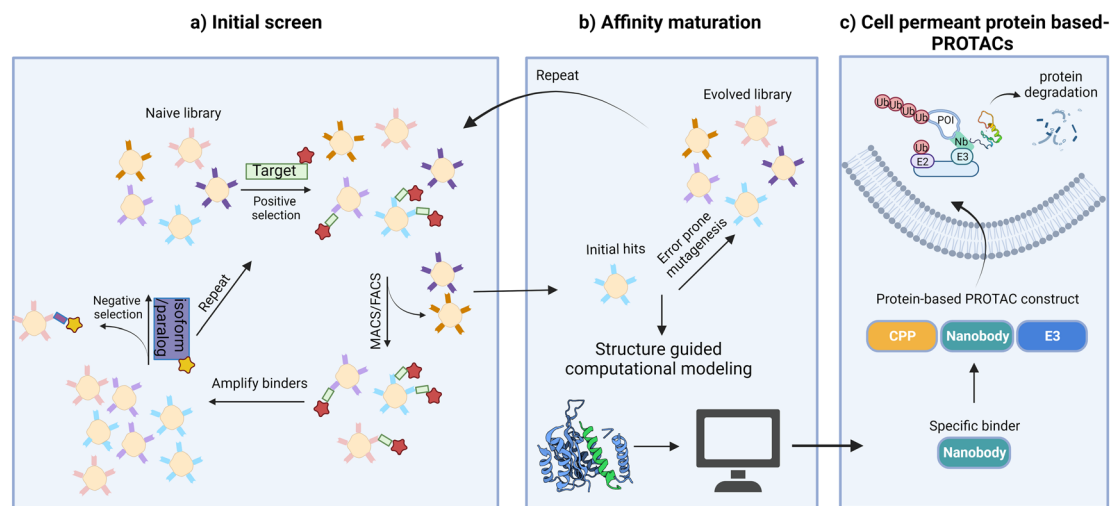


Fig. 5 Strategies to target “undruggable” soluble proteins with cell-permeant protein-based PROTACs. (a) Screen to identify initial nanobody binders; (b) mutagenesis or structure guided computational modeling to improve binding affinities; (c) functionalization of nanobody binders for cell penetration and E3 ligase recruitment leads to POI degradation.

Our strategy of systemic ligand identification, cell penetration functionalization, and protein-based PROTAC construction provides a new possibility for the targeted degradation of other difficult-to-drug proteins (Fig. 5). Additional studies to understand the details of protein translocation, ligand orientation, and structure–activity relations of binders and E3 ligases are critical for improving the degradation efficiencies and broadening the applications of protein-based PROTACs. Finally, strategies to achieve cell specificity *in vivo* will be crucial for future clinical applications of protein-based degraders.

Conclusions and outlook

TPD has had a tremendously positive impact on the fields of chemical biology, cancer biology, immunobiology, and drug discovery.¹¹⁵ Studies on protein degradation pathways have yielded valuable insights into the development of diverse TPD technologies, which all function by exploiting endogenous systems. The development of protein-based PROTACs has revealed exciting possibilities for targeting a wide range of proteins, including membrane proteins, secreted proteins, and highly disordered proteins. Additionally, a wider array of E3 ligases has been employed, providing higher flexibility compared to small molecule degraders. The success in the past two decades and recent developments in both academia and industry suggest that TPD could become a key modality for developing therapeutics.

Despite this, the widespread clinical applications of protein-based PROTACs face significant challenges. Although protein-based PROTACs offer greater flexibility in terms of binders and E3 ligases selection, it is important to note that some examples have failed for unknown reasons,^{71,81} and that the rules for achieving efficient degradation of a particular POI are still not clear. The degradation appears to be influenced more by the epitope of the POI/paratope of the binder than by affinity. Additionally, synergy of ligand, target POI, and E3 ligase is critical for

efficient degradation, but molecular control of that synergy is yet to be understood. As such, achieving successful and efficient POI degradation requires careful optimization of ligand affinity, specificity, and orientation, and may require the screening of multiple E3 ligases. The direct screening of protein-based degraders using combinatorial domain libraries can also be considered.

Finally, the application of protein-based PROTACs *in vivo* is challenging due to limitations in achieving selective cell type delivery. However, the recent development of protein delivery methods such as the bacterial contractile injection system,¹¹⁶ the botulinum neurotoxin platform,¹¹⁷ and functionalized nanoparticles¹¹⁸ provide exciting possibilities that might accelerate the clinical applications of protein-based PROTACs. It is our hope that efforts from our group directed at expanding target scope to intrinsically disordered protein regions, performing in-depth studies of structure activity relationships, achieving tissue- and organ-specific delivery, coupled with investigations on pharmacokinetics, pharmacodynamics, off-target side effects and toxicities, will enable the translational applications of protein-based PROTACs.

Author contributions

F. S. and L. M. K. D. conceptualized, wrote, and edited the Perspective.

Conflicts of interest

There are no conflicts to declare.

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References

- 1 A. Buzdin, M. Sorokin, A. Garazha, A. Glusker, A. Aleshin, E. Poddubskaya, M. Sekacheva, E. Kim, N. Gaifullin, A. Giese, A. Seryakov, P. Rumiantsev, S. Moshkovskii and A. Moiseev, RNA sequencing for research and diagnostics in clinical oncology, *Semin. Cancer Biol.*, 2020, **60**, 311–323.
- 2 A. Henry, M. Gordillo-Marañón, C. Finan, A. F. Schmidt, J. P. Ferreira, R. Karra, J. Sundström, L. Lind, J. Ärnlov, F. Zannad, A. Mälarstig, A. D. Hingorani and R. T. Lumbers, Therapeutic Targets for Heart Failure Identified Using Proteomics and Mendelian Randomization, *Circulation*, 2022, **145**, 1205–1217.
- 3 M. Schenone, V. Dančák, B. K. Wagner and P. A. Clemons, Target identification and mechanism of action in chemical biology and drug discovery, *Nat. Chem. Biol.*, 2013, **9**, 232–240.
- 4 T. Wang, K. Birsoy, N. W. Hughes, K. M. Krupczak, Y. Post, J. J. Wei, E. S. Lander and D. M. Sabatini, Identification and characterization of essential genes in the human genome, *Science*, 2015, **350**, 1096–1101.
- 5 A. L. Hopkins and C. R. Groom, The druggable genome, *Nat. Rev. Drug Discovery*, 2002, **1**, 727–730.
- 6 P. Cohen, D. Cross and P. A. Jänne, Kinase drug discovery 20 years after imatinib: progress and future directions, *Nat. Rev. Drug Discovery*, 2021, **20**, 551–569.
- 7 D. P. Bondeson, A. Mares, I. E. D. Smith, E. Ko, S. Campos, A. H. Miah, K. E. Mulholland, N. Routly, D. L. Buckley, J. L. Gustafson, N. Zinn, P. Grandi, S. Shimamura, G. Bergamini, M. Faelth-Savitski, M. Bantscheff, C. Cox, D. A. Gordon, R. R. Willard, J. J. Flanagan, L. N. Casillas, B. J. Votta, W. den Besten, K. Famm, L. Kruidenier, P. S. Carter, J. D. Harling, I. Churcher and C. M. Crews, Catalytic *in vivo* protein knockdown by small-molecule PROTACs, *Nat. Chem. Biol.*, 2015, **11**, 611–617.
- 8 J. Krönke, N. D. Udeshi, A. Narla, P. Grauman, S. N. Hurst, M. McConkey, T. Svinkina, D. Heckl, E. Comer, X. Li, C. Ciarlo, E. Hartman, N. Munshi, M. Schenone, S. L. Schreiber, S. A. Carr and B. L. Ebert, Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells, *Science*, 2014, **343**, 301–305.
- 9 G. Lu, R. E. Middleton, H. Sun, M. Naniong, C. J. Ott, C. S. Mitsiades, K. K. Wong, J. E. Bradner and W. G. Kaelin Jr, The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins, *Science*, 2014, **343**, 305–309.
- 10 G. E. Winter, D. L. Buckley, J. Paulk, J. M. Roberts, A. Souza, S. Dhe-Paganon and J. E. Bradner, Phthalimide conjugation as a strategy for *in vivo* target protein degradation, *Science*, 2015, **348**, 1376–1381.
- 11 M. Békés, D. R. Langley and C. M. Crews, PROTAC targeted protein degraders: the past is prologue, *Nat. Rev. Drug Discovery*, 2022, **21**, 181–200.
- 12 M. Pettersson and C. M. Crews, PROteolysis TARgeting Chimeras (PROTACs) — past, present and future, *Drug Discovery Today: Technol.*, 2019, **31**, 15–27.
- 13 M. Naito, N. Ohoka and N. Shibata, SNIPERs—Hijacking IAP activity to induce protein degradation, *Drug Discovery Today: Technol.*, 2019, **31**, 35–42.
- 14 H. Wang, R. Wang, K. Cai, H. He, Y. Liu, J. Yen, Z. Wang, M. Xu, Y. Sun, X. Zhou, Q. Yin, L. Tang, I. T. Dobrucki, L. W. Dobrucki, E. J. Chaney, S. A. Boppart, T. M. Fan, S. Lezmi, X. Chen, L. Yin and J. Cheng, Selective *in vivo* metabolic cell-labeling-mediated cancer targeting, *Nat. Chem. Biol.*, 2017, **13**, 415–424.
- 15 B. Nabets, F. M. Ferguson, B. K. A. Seong, M. Kuljanin, A. L. Leggett, M. L. Mohardt, A. Robichaud, A. S. Conway, D. L. Buckley, J. D. Mancias, J. E. Bradner, K. Stegmaier and N. S. Gray, Rapid and direct control of target protein levels with VHL-recruiting dTAG molecules, *Nat. Commun.*, 2020, **11**, 4687.
- 16 D. Takahashi, J. Moriyama, T. Nakamura, E. Miki, E. Takahashi, A. Sato, T. Akaike, K. Itto-Nakama and H. Arimoto, AUTACs: Cargo-Specific Degraders Using Selective Autophagy, *Mol. Cell*, 2019, **76**, 797–810.
- 17 E. De Vita, D. Lucy and E. W. Tate, Beyond targeted protein degradation: LD ATTECs clear cellular lipid droplets, *Cell Res.*, 2021, **31**, 945–946.
- 18 T. Ito, H. Ando, T. Suzuki, T. Ogura, K. Hotta, Y. Imamura, Y. Yamaguchi and H. Handa, Identification of a Primary Target of Thalidomide Teratogenicity, *Science*, 2010, **327**, 1345–1350.
- 19 A. Mullard, Targeted protein degraders crowd into the clinic, *Nat. Rev. Drug Discovery*, 2021, **20**, 247–250.
- 20 M. Uhlén, L. Fagerberg, B. M. Hallström, C. Lindskog, P. Oksvold, A. Mardinoglu, Å. Sivertsson, C. Kampf, E. Sjöstedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani, C. A. Szizyarto, J. Odeberg, D. Djureinovic, J. O. Takanen, S. Hober, T. Alm, P. H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J. M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen and F. Pontén, Proteomics. Tissue-based map of the human proteome, *Science*, 2015, **347**, 1260419.
- 21 K. M. Sakamoto, K. B. Kim, A. Kumagai, F. Mercurio, C. M. Crews and R. J. Deshaies, Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 8554–8559.
- 22 W.-C. Hon, M. I. Wilson, K. Harlos, T. D. W. Claridge, C. J. Schofield, C. W. Pugh, P. H. Maxwell, P. J. Ratcliffe, D. I. Stuart and E. Y. Jones, Structural basis for the recognition of hydroxyproline in HIF-1 α by pVHL, *Nature*, 2002, **417**, 975–978.
- 23 J.-H. Min, H. Yang, M. Ivan, F. Gertler, W. G. Kaelin and N. P. Pavletich, Structure of an HIF-1 α -pVHL Complex: Hydroxyproline Recognition in Signaling, *Science*, 2002, **296**, 1886–1889.
- 24 R. K. Henning, J. O. Varghese, S. Das, A. Nag, G. Tang, K. Tang, A. M. Sutherland and J. R. Heath, Degradation of Akt using protein-catalyzed capture agents, *J. Pept. Sci.*, 2016, **22**, 196–200.



- 25 T.-T. Chu, N. Gao, Q.-Q. Li, P.-G. Chen, X.-F. Yang, Y.-X. Chen, Y.-F. Zhao and Y.-M. Li, Specific Knockdown of Endogenous Tau Protein by Peptide-Directed Ubiquitin-Proteasome Degradation, *Cell Chem. Biol.*, 2016, **23**, 453–461.
- 26 J. Qu, X. Ren, F. Xue, Y. He, R. Zhang, Y. Zheng, H. Huang, W. Wang and J. Zhang, Specific Knockdown of α -Synuclein by Peptide-Directed Proteasome Degradation Rescued Its Associated Neurotoxicity, *Cell Chem. Biol.*, 2020, **27**, 751–762.
- 27 J. Hines, J. D. Gough, T. W. Corson and C. M. Crews, Posttranslational protein knockdown coupled to receptor tyrosine kinase activation with phosphoPROTACs, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 8942–8947.
- 28 K. Montrose and G. W. Krissansen, Design of a PROTAC that antagonizes and destroys the cancer-forming X-protein of the hepatitis B virus, *Biochem. Biophys. Res. Commun.*, 2014, **453**, 735–740.
- 29 K. T. G. Samarasinghe, S. Jaime-Figueroa, M. Burgess, D. A. Nalawansa, K. Dai, Z. Hu, A. Bebenek, S. A. Holley and C. M. Crews, Targeted degradation of transcription factors by TRAFACs: TRANscription Factor TARgeting Chimeras, *Cell Chem. Biol.*, 2021, **28**, 648–661.
- 30 J. Shao, Y. Yan, D. Ding, D. Wang, Y. He, Y. Pan, W. Yan, A. Kharbanda, H.-y. Li and H. Huang, Destruction of DNA-Binding Proteins by Programmable Oligonucleotide PROTAC (O'PROTAC): Effective Targeting of LEF1 and ERG, *Adv. Sci.*, 2021, **8**, 2102555.
- 31 J. Liu, H. Chen, H. Kaniskan, L. Xie, X. Chen, J. Jin and W. Wei, TF-PROTACs Enable Targeted Degradation of Transcription Factors, *J. Am. Chem. Soc.*, 2021, **143**, 8902–8910.
- 32 A. Ghidini, A. Cléry, F. Halloy, F. H. T. Allain and J. Hall, RNA-PROTACs: Degradation of RNA-Binding Proteins, *Angew. Chem., Int. Ed.*, 2021, **60**, 3163–3169.
- 33 K. M. Patil, D. Chin, H. L. Seah, Q. Shi, K. W. Lim and A. T. Phan, G4-PROTAC: targeted degradation of a G-quadruplex binding protein, *Chem. Commun.*, 2021, **57**, 12816–12819.
- 34 S. He, F. Gao, J. Ma, H. Ma, G. Dong and C. Sheng, Aptamer-PROTAC Conjugates (APCs) for Tumor-Specific Targeting in Breast Cancer, *Angew. Chem., Int. Ed.*, 2021, **60**, 23299–23305.
- 35 S. M. Banik, K. Pedram, S. Wisnovsky, G. Ahn, N. M. Riley and C. R. Bertozzi, Lysosome-targeting chimeras for degradation of extracellular proteins, *Nature*, 2020, **584**, 291–297.
- 36 G. Ahn, S. M. Banik, C. L. Miller, N. M. Riley, J. R. Cochran and C. R. Bertozzi, LYTACs that engage the asialoglycoprotein receptor for targeted protein degradation, *Nat. Chem. Biol.*, 2021, **17**, 937–946.
- 37 A. D. Cotton, D. P. Nguyen, J. A. Gramespacher, I. B. Seiple and J. A. Wells, Development of Antibody-Based PROTACs for the Degradation of the Cell-Surface Immune Checkpoint Protein PD-L1, *J. Am. Chem. Soc.*, 2021, **143**, 593–598.
- 38 H. Marei, W. K. Tsai, Y. S. Kee, K. Ruiz, J. He, C. Cox, T. Sun, S. Penikalapati, P. Dwivedi, M. Choi, D. Kan, P. Saenz-Lopez, K. Dorigi, P. Zhang, Y. T. Kschonsak, N. Kljavin, D. Amin, I. Kim, A. G. Mancini, T. Nguyen, C. Wang, E. Janezic, A. Doan, E. Mai, H. Xi, C. Gu, M. Heinlein, B. Biehs, J. Wu, I. Lehoux, S. Harris, L. Comps-Agrar, D. Seshasayee, F. J. de Sauvage, M. Grimmer, J. Li, N. J. Agard and E. M. F. de Sousa, Antibody targeting of E3 ubiquitin ligases for receptor degradation, *Nature*, 2022, **610**, 182–189.
- 39 K. Pance, J. A. Gramespacher, J. R. Byrnes, F. Salangsang, J.-A. C. Serrano, A. D. Cotton, V. Steri and J. A. Wells, Modular cytokine receptor-targeting chimeras for targeted degradation of cell surface and extracellular proteins, *Nat. Biotechnol.*, 2023, **41**, 273–281.
- 40 F. Shen, G. Zheng, M. Setegne, K. Tenglin, M. Izadi, H. Xie, L. Zhai, S. H. Orkin and L. M. K. Dassama, A Cell-Permeant Nanobody-Based Degradation That Induces Fetal Hemoglobin, *ACS Cent. Sci.*, 2022, **8**, 1695–1703.
- 41 I. Dikic, Proteasomal and Autophagic Degradation Systems, *Annu. Rev. Biochem.*, 2017, **86**, 193–224.
- 42 C. de Duve, The lysosome turns fifty, *Nat. Cell Biol.*, 2005, **7**, 847–849.
- 43 J. P. Luzio, P. R. Pryor and N. A. Bright, Lysosomes: fusion and function, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 622–632.
- 44 J. Gruenberg, The endocytic pathway: a mosaic of domains, *Nat. Rev. Mol. Cell Biol.*, 2001, **2**, 721–730.
- 45 G. J. Doherty and H. T. McMahon, Mechanisms of endocytosis, *Annu. Rev. Biochem.*, 2009, **78**, 857–902.
- 46 R. M. Perera and R. Zoncu, The lysosome as a regulatory hub, *Annu. Rev. Cell Dev. Biol.*, 2016, **32**, 223–253.
- 47 M. Kaksonen and A. Roux, Mechanisms of clathrin-mediated endocytosis, *Nat. Rev. Mol. Cell Biol.*, 2018, **19**, 313–326.
- 48 B. T. Kelly, S. C. Graham, N. Liska, P. N. Dannhauser, S. Höning, E. J. Ungewickell and D. J. Owen, AP2 controls clathrin polymerization with a membrane-activated switch, *Science*, 2014, **345**, 459–463.
- 49 R. C. Piper and D. J. Katzmman, Biogenesis and function of multivesicular bodies, *Annu. Rev. Cell Dev. Biol.*, 2007, **23**, 519–547.
- 50 C. He and D. J. Klionsky, Regulation Mechanisms and Signaling Pathways of Autophagy, *Annu. Rev. Genet.*, 2009, **43**, 67–93.
- 51 J. Gruenberg and F. G. van der Goot, Mechanisms of pathogen entry through the endosomal compartments, *Nat. Rev. Mol. Cell Biol.*, 2006, **7**, 495–504.
- 52 A. Ciechanover, A. Orian and A. L. Schwartz, Ubiquitin-mediated proteolysis: biological regulation via destruction, *Bioessays*, 2000, **22**, 442–451.
- 53 F. E. Morreale and H. Walden, Types of Ubiquitin Ligases, *Cell*, 2016, **165**, 248.
- 54 C. E. Berndsen and C. Wolberger, New insights into ubiquitin E3 ligase mechanism, *Nat. Struct. Mol. Biol.*, 2014, **21**, 301–307.



- 55 R. Budhaidarmo, Y. Nakatani and C. L. Day, RINGs hold the key to ubiquitin transfer, *Trends Biochem. Sci.*, 2012, **37**, 58–65.
- 56 R. J. Deshaies and C. A. Joazeiro, RING domain E3 ubiquitin ligases, *Annu. Rev. Biochem.*, 2009, **78**, 399–434.
- 57 D. M. Wenzel and R. E. Klevit, Following Ariadne's thread: a new perspective on RBR ubiquitin ligases, *BMC Biol.*, 2012, **10**, 1–8.
- 58 C. Cenciarelli, D. S. Chiaur, D. Guardavaccaro, W. Parks, M. Vidal and M. Pagano, Identification of a family of human F-box proteins, *Curr. Biol.*, 1999, **9**, S1–S3.
- 59 W. Cai and H. Yang, The structure and regulation of Cullin 2 based E3 ubiquitin ligases and their biological functions, *Cell Div.*, 2016, **11**, 7.
- 60 P. Genschik, I. Sumara and E. Lechner, The emerging family of CULLIN3-RING ubiquitin ligases (CRL3s): cellular functions and disease implications, *EMBO J.*, 2013, **32**, 2307–2320.
- 61 M. D. Petroski and R. J. Deshaies, Function and regulation of cullin–RING ubiquitin ligases, *Nat. Rev. Mol. Cell Biol.*, 2005, **6**, 9–20.
- 62 M. B. Metzger, V. A. Hristova and A. M. Weissman, HECT and RING finger families of E3 ubiquitin ligases at a glance, *J. Cell Sci.*, 2012, **125**, 531–537.
- 63 N. Zheng and N. Shabek, Ubiquitin Ligases: Structure, Function, and Regulation, *Annu. Rev. Biochem.*, 2017, **86**, 129–157.
- 64 R. B. Damgaard, The ubiquitin system: from cell signalling to disease biology and new therapeutic opportunities, *Cell Death Differ.*, 2021, **28**, 423–426.
- 65 M. Tracz and W. Bialek, Beyond K48 and K63: non-canonical protein ubiquitination, *Cell. Mol. Biol. Lett.*, 2021, **26**, 1.
- 66 M. L. Uribe, I. Marrocco and Y. Yarden, EGFR in Cancer: Signaling Mechanisms, Drugs, and Acquired Resistance, *Cancers*, 2021, **13**, 2748.
- 67 A. H. Sharpe and K. E. Pauken, The diverse functions of the PD1 inhibitory pathway, *Nat. Rev. Immunol.*, 2018, **18**, 153–167.
- 68 Y. Zhou, P. Teng, N. T. Montgomery, X. Li and W. Tang, Development of Triantennary N-Acetylgalactosamine Conjugates as Degradors for Extracellular Proteins, *ACS Cent. Sci.*, 2021, **7**, 499–506.
- 69 D. F. Caianiello, M. Zhang, J. D. Ray, R. A. Howell, J. C. Swartzel, E. M. J. Branham, E. Chirkin, V. R. Sabbasani, A. Z. Gong, D. M. McDonald, V. Muthusamy and D. A. Spiegel, Bifunctional small molecules that mediate the degradation of extracellular proteins, *Nat. Chem. Biol.*, 2021, **17**, 947–953.
- 70 H. Zhang, Y. Han, Y. Yang, F. Lin, K. Li, L. Kong, H. Liu, Y. Dang, J. Lin and P. R. Chen, Covalently Engineered Nanobody Chimeras for Targeted Membrane Protein Degradation, *J. Am. Chem. Soc.*, 2021, **143**, 16377–16382.
- 71 J. A. Gramespacher, A. D. Cotton, P. W. W. Burroughs, I. B. Seiple and J. A. Wells, Roadmap for Optimizing and Broadening Antibody-Based PROTACs for Degradation of Cell Surface Proteins, *ACS Chem. Biol.*, 2022, **17**, 1259–1268.
- 72 W. Li, M. H. Bengtsson, A. Ulbrich, A. Matsuda, V. A. Reddy, A. Orth, S. K. Chanda, S. Batalov and C. A. Joazeiro, Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling, *PLoS One*, 2008, **3**, e1487.
- 73 G. M. Burslem and C. M. Crews, Proteolysis-Targeting Chimeras as Therapeutics and Tools for Biological Discovery, *Cell*, 2020, **181**, 102–114.
- 74 A. D. Portnoff, E. A. Stephens, J. D. Varner and M. P. DeLisa, Ubiquibodies, synthetic E3 ubiquitin ligases endowed with unnatural substrate specificity for targeted protein silencing, *J. Biol. Chem.*, 2014, **289**, 7844–7855.
- 75 D. Clift, W. A. McEwan, L. I. Labzin, V. Konieczny, B. Mogessie, L. C. James and M. Schuh, A Method for the Acute and Rapid Degradation of Endogenous Proteins, *Cell*, 2017, **171**, 1692–1706.
- 76 K. M. Sakamoto, K. B. Kim, A. Kumagai, F. Mercurio, C. M. Crews and R. J. Deshaies, Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 8554–8559.
- 77 L. M. Simpson, T. J. Macartney, A. Nardin, L. J. Fulcher, S. Röth, A. Testa, C. Maniaci, A. Ciulli, I. G. Ganley and G. P. Sapkota, Inducible Degradation of Target Proteins through a Tractable Affinity-Directed Protein Missile System, *Cell Chem. Biol.*, 2020, **27**, 1164–1180.
- 78 A. F. M. Ibrahim, L. Shen, M. H. Tatham, D. Dickerson, A. R. Prescott, N. Abidi, D. P. Xirodimas and R. T. Hay, Antibody RING-Mediated Destruction of Endogenous Proteins, *Mol. Cell*, 2020, **79**, 155–166.
- 79 E. Caussinus, O. Kanca and M. Affolter, Fluorescent fusion protein knockout mediated by anti-GFP nanobody, *Nat. Struct. Mol. Biol.*, 2012, **19**, 117–121.
- 80 L. J. Fulcher, T. Macartney, P. Bozatzi, A. Hornberger, A. Rojas-Fernandez and G. P. Sapkota, An affinity-directed protein missile system for targeted proteolysis, *Open Biol.*, 2016, **6**, 160255.
- 81 S. Lim, R. Khoo, K. M. Peh, J. Teo, S. C. Chang, S. Ng, G. L. Beilhartz, R. A. Melnyk, C. W. Johannes, C. J. Brown, D. P. Lane, B. Henry and A. W. Partridge, bioPROTACs as versatile modulators of intracellular therapeutic targets including proliferating cell nuclear antigen (PCNA), *Proc. Natl. Acad. Sci. U. S. A.*, 2020, **117**, 5791–5800.
- 82 N. Bery, L. Keller, M. Soulié, R. Gence, A. L. Iscache, J. Cherier, S. Cabantous, O. Sordet, I. Lajoie-Mazenc, J. D. Pedelacq, G. Favre and A. Olichon, A Targeted Protein Degradation Cell-Based Screening for Nanobodies Selective toward the Cellular RHOB GTP-Bound Conformation, *Cell Chem. Biol.*, 2019, **26**, 1544–1558.
- 83 Y. Jia, T. Jiang, X. Li, C. Zhao, L. Zhang, S. Zhao, X. Liu, M. Qiao, J. Luo, J. Shi, H. Yang, Y. Wang, L. Xi, S. Zhang, G. Gao, C. Su, S. Ren and C. Zhou, Characterization of distinct types of KRAS mutation and its impact on first-line platinum-based chemotherapy in Chinese patients with advanced non-small cell lung cancer, *Oncol. Lett.*, 2017, **14**, 6525–6532.

- 84 M. Malumbres and M. Barbacid, RAS oncogenes: the first 30 years, *Nat. Rev. Cancer*, 2003, **3**, 459–465.
- 85 A. R. Moore, S. C. Rosenberg, F. McCormick and S. Malek, RAS-targeted therapies: is the undruggable drugged?, *Nat. Rev. Drug Discovery*, 2020, **19**, 533–552.
- 86 J. M. L. Ostrem and K. M. Shokat, Direct small-molecule inhibitors of KRAS: from structural insights to mechanism-based design, *Nat. Rev. Drug Discovery*, 2016, **15**, 771–785.
- 87 N. Bery, S. Legg, J. Debreczeni, J. Breed, K. Embrey, C. Stubbs, P. Kolasinska-Zwiercz, N. Barrett, R. Marwood, J. Watson, J. Tart, R. Overman, A. Miller, C. Phillips, R. Minter and T. H. Rabbitts, KRAS-specific inhibition using a DARPIn binding to a site in the allosteric lobe, *Nat. Commun.*, 2019, **10**, 2607.
- 88 N. Bery, A. Miller and T. Rabbitts, A potent KRAS macromolecule degrader specifically targeting tumours with mutant KRAS, *Nat. Commun.*, 2020, **11**, 3233.
- 89 K. W. Teng, S. T. Tsai, T. Hattori, C. Fedele, A. Koide, C. Yang, X. Hou, Y. Zhang, B. G. Neel, J. P. O'Bryan and S. Koide, Selective and noncovalent targeting of RAS mutants for inhibition and degradation, *Nat. Commun.*, 2021, **12**, 2656.
- 90 S. Lim, R. Khoo, Y.-C. Juang, P. Gopal, H. Zhang, C. Ye, K. M. Peh, J. Teo, S. Ng, B. Henry and A. W. Partridge, Exquisitely Specific anti-KRAS Biodegraders Inform on the Cellular Prevalence of Nucleotide-Loaded States, *ACS Cent. Sci.*, 2021, **7**, 274–291.
- 91 N. Bery, L. Keller, M. Soulie, R. Gence, A. L. Iscache, J. Cherier, S. Cabantous, O. Sordet, I. Lajoie-Mazenc, J. D. Pedelacq, G. Favre and A. Olichon, A Targeted Protein Degradation Cell-Based Screening for Nanobodies Selective toward the Cellular RHOB GTP-Bound Conformation, *Cell Chem. Biol.*, 2019, **26**, 1544–1558.
- 92 A. F. M. Ibrahim, L. Shen, M. H. Tatham, D. Dickerson, A. R. Prescott, N. Abidi, D. P. Xirodimas and R. T. Hay, Antibody RING-Mediated Destruction of Endogenous Proteins, *Mol. Cell*, 2020, **79**, 155–166.
- 93 Y. Ju Shin, S. Kyun Park, Y. Jung Jung, Y. Na Kim, K. Sung Kim, O. Kyu Park, S.-H. Kwon, S. Ho Jeon, L. A. Trinh, S. E. Fraser, Y. Kee and B. Joon Hwang, Nanobody-targeted E3-ubiquitin ligase complex degrades nuclear proteins, *Sci. Rep.*, 2015, **5**, 14269.
- 94 M. B. Ludwicki, J. Li, E. A. Stephens, R. W. Roberts, S. Koide, P. T. Hammond and M. P. DeLisa, Broad-Spectrum Proteome Editing with an Engineered Bacterial Ubiquitin Ligase Mimic, *ACS Cent. Sci.*, 2019, **5**, 852–866.
- 95 Z. X. Chong, S. K. Yeap and W. Y. Ho, Transfection types, methods and strategies: a technical review, *PeerJ*, 2021, **9**, e11165.
- 96 T. K. Kim and J. H. Eberwine, Mammalian cell transfection: the present and the future, *Anal. Bioanal. Chem.*, 2010, **397**, 3173–3178.
- 97 J. T. Bulcha, Y. Wang, H. Ma, P. W. L. Tai and G. Gao, Viral vector platforms within the gene therapy landscape, *Signal Transduction Targeted Ther.*, 2021, **6**, 53.
- 98 D. VanDyke, J. D. Taylor, K. J. Kao, J. Hunt and J. B. Spangler, Biologics-based degraders—an expanding toolkit for targeted-protein degradation, *Curr. Opin. Biotechnol.*, 2022, **78**, 102807.
- 99 G. Gallardo, C. H. Wong, S. M. Ricardez, C. N. Mann, K. H. Lin, C. E. G. Leyns, H. Jiang and D. M. Holtzman, Targeting tauopathy with engineered tau-degrading intrabodies, *Mol. Neurodegener.*, 2019, **14**, 38.
- 100 W. Y. Hwang, Y. Fu, D. Reyon, M. L. Maeder, S. Q. Tsai, J. D. Sander, R. T. Peterson, J. R. J. Yeh and J. K. Joung, Efficient genome editing in zebrafish using a CRISPR-Cas system, *Nat. Biotechnol.*, 2013, **31**, 227–229.
- 101 D. Clift, C. So, W. A. McEwan, L. C. James and M. Schuh, Acute and rapid degradation of endogenous proteins by Trim-Away, *Nat. Protoc.*, 2018, **13**, 2149–2175.
- 102 J. Zeng, A. F. Santos, A. S. Mukadam, M. Osswald, D. A. Jacques, C. F. Dickson, S. H. McLaughlin, C. M. Johnson, L. Kiss, J. Luptak, N. Renner, M. Vaysburd, W. A. McEwan, E. Morais-de-Sá, D. Clift and L. C. James, Target-induced clustering activates Trim-Away of pathogens and proteins, *Nat. Struct. Mol. Biol.*, 2021, **28**, 278–289.
- 103 E. Böhmová, D. Machová, M. Pechar, R. Pola, K. Venclíková, O. Janoušková and T. Etrych, Cell-penetrating peptides: a useful tool for the delivery of various cargoes into cells, *Physiol. Res.*, 2018, **67**, S267–S279.
- 104 F. Heitz, M. C. Morris and G. Divita, Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics, *Br. J. Pharmacol.*, 2009, **157**, 195–206.
- 105 R. F. Wissner, A. Steinauer, S. L. Knox, A. D. Thompson and A. Schepartz, Fluorescence Correlation Spectroscopy Reveals Efficient Cytosolic Delivery of Protein Cargo by Cell-Permeant Miniature Proteins, *ACS Cent. Sci.*, 2018, **4**, 1379–1393.
- 106 A. Steinauer, J. R. LaRochelle, S. L. Knox, R. F. Wissner, S. Berry and A. Schepartz, HOPS-dependent endosomal fusion required for efficient cytosolic delivery of therapeutic peptides and small proteins, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 512–521.
- 107 E. Satterwhite, T. Sonoki, T. G. Willis, L. Harder, R. Nowak, E. L. Arriola, H. Liu, H. P. Price, S. Gesk, D. Steinemann, B. Schlegelberger, D. G. Oscier, R. Siebert, P. W. Tucker and M. J. S. Dyer, The BCL11 gene family: involvement of BCL11A in lymphoid malignancies, *Blood*, 2001, **98**, 3413–3420.
- 108 P. Grabarczyk, P. Winkler, M. Delin, P. K. Sappa, S. Bekeschus, P. Hildebrandt, G. K. Przybylski, U. Völker, E. Hammer and C. A. Schmidt, The N-Terminal CCHC Zinc Finger Motif Mediates Homodimerization of Transcription Factor BCL11B, *Mol. Cell Biol.*, 2018, **38**, e00317–e00368.
- 109 N. Liu, V. V. Hargreaves, Q. Zhu, J. V. Kurland, J. Hong, W. Kim, F. Sher, C. Macias-Trevino, J. M. Rogers, R. Kurita, Y. Nakamura, G. C. Yuan, D. E. Bauer, J. Xu, M. L. Bulyk and S. H. Orkin, Direct Promoter Repression by BCL11A Controls the Fetal to Adult Hemoglobin

- 110 J. Yin, X. Xie, Y. Ye, L. Wang and F. Che, BCL11A: a potential diagnostic biomarker and therapeutic target in human diseases, *Biosci. Rep.*, 2019, **39**, BSR20190604.
- 111 S. H. Orkin, Molecular Medicine: Found in Translation, *Med*, 2021, **2**, 122–136.
- 112 H. Frangoul, D. Altshuler, M. D. Cappellini, Y.-S. Chen, J. Domm, B. K. Eustace, J. Foell, J. de la Fuente, S. Grupp, R. Handgretinger, T. W. Ho, A. Kattamis, A. Kernytsky, J. Lekstrom-Himes, A. M. Li, F. Locatelli, M. Y. Mapara, M. de Montalembert, D. Rondelli, A. Sharma, S. Sheth, S. Soni, M. H. Steinberg, D. Wall, A. Yen and S. Corbacioglu, CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β -Thalassemia, *N. Engl. J. Med.*, 2020, **384**, 252–260.
- 113 S. Luc, J. Huang, J. L. McEldoon, E. Somuncular, D. Li, C. Rhodes, S. Mamoor, S. Hou, J. Xu and S. H. Orkin, Bcl11a Deficiency Leads to Hematopoietic Stem Cell Defects with an Aging-like Phenotype, *Cell Rep.*, 2016, **16**, 3181–3194.
- 114 M. Yin, M. Izadi, K. Tenglin, T. Viennet, L. Zhai, G. Zheng, H. Arthanari, L. M. K. Dassama and S. H. Orkin, Evolution of nanobodies specific for BCL11A, *Proc. Natl. Acad. Sci. U. S. A.*, 2023, **120**, e2218959120.
- 115 L. Zhao, J. Zhao, K. Zhong, A. Tong and D. Jia, Targeted protein degradation: mechanisms, strategies and application, *Signal Transduction Targeted Ther.*, 2022, **7**, 113.
- 116 J. Kreitz, M. J. Friedrich, A. Guru, B. Lash, M. Saito, R. K. Macrae and F. Zhang, Programmable protein delivery with a bacterial contractile injection system, *Nature*, 2023, **616**, 357–364.
- 117 H. Roh, B. G. Dorner and A. Y. Ting, Cell-Type-Specific Intracellular Protein Delivery with Inactivated Botulinum Neurotoxin, *J. Am. Chem. Soc.*, 2023, **145**, 10220–10226.
- 118 M. J. Mitchell, M. M. Billingsley, R. M. Haley, M. E. Wechsler, N. A. Peppas and R. Langer, Engineering precision nanoparticles for drug delivery, *Nat. Rev. Drug Discovery*, 2021, **20**, 101–124.

