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Review

Bifunctional modalities for repurposing protein function

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SUMMARY

Nature takes advantage of induced proximity to perform various functions. Taking inspiration from nature, we can also trigger desired biological processes using bifunctional small molecules that artificially induce proximity. For example, bifunctional small molecules have been designed to trigger the ubiquitin-dependent proteasomal degradation of intracellular proteins. Now, recent classes of bifunctional compounds have been developed to degrade extracellular targets, membrane proteins, damaged organelles, and RNA by recruiting alternative degradation pathways. In addition to inducing degradation, bifunctional modalities can change phosphorylation and glycosylation states to evoke a biological response. In this review, we highlight recent advances in these innovative classes of compounds that build on a rich history of chemical inducers of dimerization. We anticipate that more bifunctional molecules, which induce or remove posttranslational modifications, to endow neo-functionalities will emerge.

INTRODUCTION

Cellular homeostasis, a key hallmark of living organisms, arises from interactions between biomolecules—especially enzyme-substrate interactions—within and outside the cell. Conventionally, the function of a particular enzyme has been investigated and controlled through the use of an inhibitor, and such molecules (e.g., ATP-competitive kinase inhibitors) have provided a conceptual pathway toward many FDA-approved drugs (Figure 1A). However, enzyme inhibition is only one pathway for altering biological function, and many desired cellular changes cannot be accomplished through inhibition alone. Recently, there has been a resurgence of another class of molecules that operate by inducing proximity between the target proteins, evoking a number of functions beyond inhibition (Stanton et al., 2018; Gerry and Schreiber 2020; Schreiber 2021). Like conventional inhibitors, these bifunctional proximity inducers also allow for the investigation of biological functions and are propelling the development of cutting-edge therapeutic modalities. These bifunctional molecules can be classified as "dumbbells" or "molecular glues" (Figure 1B), where dumbbells link two molecules that individually bind to their target proteins, while the molecular glues induce similar ternary complexes without an obvious linker.

Conventional inhibitors form binary complexes with the target protein and exhibit different features compared with bifunctional molecules that form ternary complexes (Hughes and Ciulli 2017; Lai and Crews 2017). In binary complexes, the activators or inhibitors target a functional site, either orthosterically or allosterically, to modulate the target (Figure 1A). In ternary complexes, the bifunctional molecules can bind to various sites, including active or allosteric sites (Figure 1B). While conventional inhibitors are occupancy driven, bifunctional molecules are often event driven. As a result, conventional inhibitors are stoichiometric, while bifunctional molecules can be sub-stoichiometric and catalytic. Furthermore, conventional inhibitors require strong binding affinities, whereas bifunctional molecules may exhibit low-to-moderate binding affinities to targeted proteins, as some ternary complexes rely on cooperativity. Another noteworthy comparison is that, compared with enzyme inhibitors, which can globally affect enzyme targets, these bifunctional molecules can be used to localize enzymatic activity to a given target. In addition, binary complexes have a saturation binding effect, where at high concentrations, the binding site is occupied. In contrast, ternary complexes can exhibit a hook effect, where high concentrations of the small molecule can saturate the two binding partners into individual binary complexes, resulting in loss of efficacy at a higher dose (Rodbard et al., 1978). Mathematical frameworks to describe the three-body equilibria have been developed to support experimental and theoretical findings of these ternary complexes (Douglass et al., 2013).

Bifunctional molecules can increase the effective molarity of two components (e.g., enzyme and target protein) and arrange them into meaningful orientations to evoke the desired biological response. This underlying principle is employed by cells to produce a biological output in many scenarios and is the *modus operandi* of many natural products (e.g., cyclosporin A) that involve proximity induction of two proteins (Schreiber 2021). Leveraging this principle, Spencer et al. "synthesized a signaling pathway" by rationally developing a synthetic bifunctional molecule that induced proximity between signal transducers (Spencer et al., 1993). This work laid the foundation for the development of

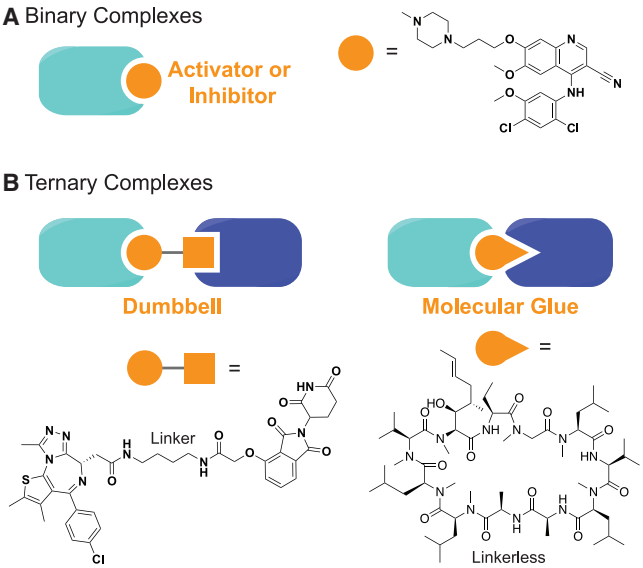

Figure 1. A comparison of binary and ternary complexes
(A) Traditional small-molecule inhibition or activation strategies target one protein or enzyme and form binary complexes.
(B) Ternary complexes are formed with small-molecule "dumbbell" compounds (left) or "molecular-glue" compounds (right), which recruit enzymes or proteins to native or non-native targets.

synthetic bifunctional molecules with a wide array of activities ranging from the degradation of proteins and RNA to the induction of specific posttranslational modifications (PTMs). Furthermore, large high-throughput genomic sequencing (e.g., TCGA and ICGC) (ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium 2020) and RNAi and CRISPR screening (DepMap) (Tsherniak et al., 2017) efforts suggest that many genetic variants drive human diseases via PTMs (Iqbal et al., 2020), and in some instances, haploinsufficiency and gene dosage of enzymes could be the drivers. Such genetic alterations can cause changes in biochemical activities of proteins, inducing hyperactivation of kinase activities in receptor tyrosine kinases (RTKs; e.g., PI3KCA, MET, and EGFR), which are frequently mutated in cancer. Bifunctional molecules with the ability to modulate the biochemical changes that are caused by genetic mutation drivers will provide more therapeutic options for treating serious diseases.

Here, we review recent advances in the development of bifunctional molecules that induce native and neo-functions, beginning with those that target degradation and moving to those that add or remove phosphoryl and glycosyl groups on proteins. Finally, we describe potential biological applications of this emerging class of molecules to complement and expand current therapeutic strategies.

DEGRADATION-INDUCING BIFUNCTIONAL MOLECULES

Traditional genetic methods for protein removal utilize knockdown or knockout strategies (e.g., small interfering RNA, antisense oligonucleotides, CRISPR-associated nucleases), although delivery and efficacy remain a challenge. More recently, small molecules have been developed to degrade proteins or RNA by recruiting endogenous cellular machinery to the desired targets. For additional reviews on triggered degradation and how these modes of action differ from those of their predecessors, please see Valeur et al. (2019); Ding et al. (2020); and Luh et al. (2020).

Degradation via the ubiquitin-proteasomal system

One way to degrade a target protein is by recruiting ubiquitin E3 ligases, which can be done using heterobifunctional small molecules termed proteolysis-targeting chimeras (PROTACs) (Sakamoto et al., 2001; Lai and Crews 2017; Pettersson and Crews 2019; Sun et al., 2019). PROTACs are formed by linking two compounds: one that binds to the target protein and one that binds to an E3 ligase, such as the Von Hippel-Lindau disease tumor suppressor protein (VHL), cereblon (CRBN), or mouse double minute 2 (MDM2). With PROTACs, the target protein is tagged with ubiquitin and shuttled to the proteasome for degradation (Figure 2A). These bifunctional degraders have long-lasting effects since the target protein must be resynthesized after degradation, as opposed to classic occupancy-driven inhibitors, whereby the target protein is reactivated upon inhibitor dissociation. Importantly, the PROTAC is often recycled and, therefore, can be catalytic. In 2001, Crews, Deshaies, and colleagues reported an example of a PROTAC that induced the degradation of methionine aminopeptidase-2 (MetAP2) (Sakamoto et al., 2001). Since then, PROTACs have been used to degrade a variety of protein targets, including those involved in cancer and diseases, and have been reviewed extensively (Lai and Crews 2017; Pettersson and Crews 2019; Sun et al., 2019).

Several challenges still exist in the design and efficacy testing of PROTACs. To improve the efficacy of PROTACs, work is also being done to increase the number of targetable E3 ligases, considering that only a handful of E3 ligases are commonly used, while there are over 600 known E3 ligases (Clague et al., 2015), which could add selectivity, as some ligases may be enriched in specific disease states (Wang et al., 2017; Paiva and Crews 2019). Zhang et al. used cysteine-reactive electrophiles to identify the once poorly characterized DCAF16 as a component of an E3 ubiquitin ligase complex (Zhang et al., 2019). In a similar study, Spradlin et al. used activity-based protein profiling to identify that the natural product nimbolide reacts with a cysteine on the E3 ligase RNF114 (Spradlin et al., 2019; Ward et al., 2019). Both efforts highlight the potential of proteomic platforms to uncover new enzymes involved in targeted protein degradation. In another example of expanding the E3 ligase space, Cotton et al. developed an extracellular antibody-based PROTAC (AbTAC) that recognizes both the cell-surface E3 ligase RNF43 and the membrane-bound programmed death ligand 1 (PD-L1) by binding to their extracellular domains to induce degradation (Cotton et al., 2021). A necessary avenue for further studies involves delineation of general principles for linker design and attachment site (exit vector). While online databases and computational tools such as PROTAC-DB (Weng et al., 2020) and PRosetTAC (Zaidman et al., 2020) are emerging, modeling the three-component systems can be difficult. An additional challenge involves developing cell-based assays for detecting ternary complexes (e.g., HiBiT/LgBiT assays; Riching et al., 2018), cooperativity, and turnover in cells. Furthermore, most off-target studies of PROTACs have focused on quantifying the alteration in off-target protein levels, when the readout should

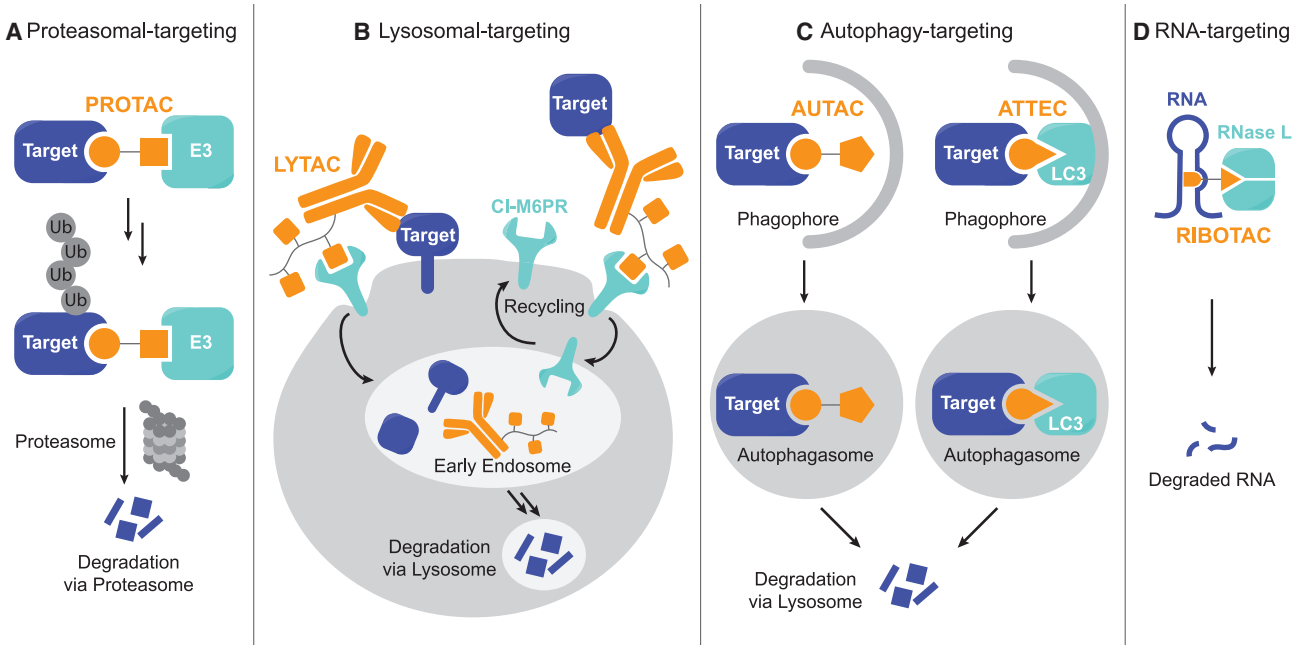

Figure 2. Methods for targeted degradation
(A) Proteasomal-targeting methods with proteolysis-targeting chimeras (PROTACs).
(B) Lysosomal-targeting methods with lysosomal-targeting chimeras (LYTACs).
(C) Autophagy-targeting methods with autophagy-targeting chimeras (AUTACs) and autophagosome-tethering compounds (ATTECs).
(D) RNA-targeting methods with ribonuclease-targeting chimeras (RIBOTACs).

be off-target protein ubiquitination using mass spectrometry methods (Xu et al., 2010; Udeshi et al., 2013).

The applications of PROTACs are far reaching, and several PROTACs are in preclinical and clinical trial studies that are underway (e.g., the orally bioavailable ARV-110 compound that targets the androgen receptor in prostate cancer; Petrylak et al., 2020). Further studies evaluating off-target effects and the pharmacokinetic and pharmacodynamic properties of PROTACs, which may be different from traditional therapeutics, are needed for future clinical applications.

Degradation via the lysosomal pathway

While bifunctional molecules that recruit the proteasomal pathway have had substantial success and have pioneered the field of small-molecule-targeted degradation, their mechanism of action is mostly limited to intracellular targets, with the exception of the aforementioned AbTACs. Motivated by this limitation, lysosomal-targeting chimeras (LYTACs) were developed for membrane-bound or extracellular targets to broaden the scope of protein degradation (Figure 2B) (Banik et al., 2020). The Bertozzi group hypothesized that creating a molecule that binds both a lysosomal-targeting receptor, such as cation-independent mannose-6-phosphate receptor, CI-M6PR, and a membrane-bound or extracellular target could trigger the target to enter the lysosomal degradation pathway. Here, they identified a first-generation stable derivative of mannose-6-phosphate, which is recognized by the receptor CI-M6PR. They demonstrated that their glycopeptide polymer linked to antibodies could successfully degrade exogenously supplied proteins (e.g., mCherry) and membrane-bound targets (e.g., EGFR). The key to their findings was the multivalency of their glycopeptide and the shuttling and recycling of CI-M6PR back to the membrane after it delivered the tagged protein cargo to the lysosomal compartments. Like PROTACs, there is much room to expand this technology, including the exploration of other lysosomal targeting receptors or small molecules that target these receptors instead of a large polymer. To this effort, Bertozzi and co-workers developed a second-generation LYTAC tag, bearing a smaller glycosyl motif, that targets the liver-specific asialoglycoprotein receptor (Ahn et al., 2021).

Degradation via the autophagy pathway

While PROTACs can target folded proteins, the autophagy pathway can degrade a greater range of intracellular substrates, including damaged organelles, aggregated proteins, intracellular debris, and non-proteinaceous biomolecules. Thus, autophagy-targeting chimeras (AUTACs) and autophagosome-tethering compounds (ATTECs) have been developed to recruit this potentially more robust degradation pathway (Figure 2C). However, a major challenge here has been creating bifunctional molecules to target autophagy, as autophagy-targeting small molecules are limited and the autophagy pathway is not as well understood as the ubiquitin-proteasomal system. Previous papers demonstrated that in antibacterial autophagy (xenophagy), cGMP (*S*-guanylation) can trigger ubiquitination and autophagosomal clearance. The Arimoto group hypothesized that a tag that resembles this *S*-guanylation could serve as a stand-alone tag to trigger autophagy (Takahashi et al., 2019). In doing so, they successfully degraded MetAP2, one of the earliest PROTAC targets (Sakamoto et al., 2001), and FKBP12 by linking their tag to known binders of those proteins. They also applied this

technology to target a mitochondrial-fused EGFP-HaloTag protein. They destabilized the mitochondria, and upon treatment with their compound linked to a chloroalkane, the mitochondrial potential was restored by clearing out the damaged organelle. Finally, their AUTAC technology was utilized to target dysfunctional mitochondria in Detroit532 cells to show mitochondrial restoration. While this autophagy-triggered degradation increases the scope of these technologies beyond proteins to organelles and other macromolecules, further work can be done to identify and optimize ligands that bind to key proteins involved in autophagy. In addition, since the autophagy pathway is in the cytosol, nuclear protein degradation can be a challenge. For instance, the authors attempted to degrade nucleus-residing BRD4, but were less successful than with their cytosolic targets.

While AUTACs rely on ubiquitination for the target to be recognized by the autophagy pathway, ATTECs interact directly with LC3, an autophagosome precursor involved in phagophore formation. This side-steps the requirement for ubiquitin, thus expanding its range of targetable proteins (Li et al., 2019; Li et al., 2020). Moreover, ATTEC compounds are smaller in comparison to LYTACs and AUTACs. As an example of an ATTEC, Li et al. used a mutant protein in Huntington's disease (HD) to target a stretch of glutamines to the phagophore. Small-molecule screening found molecular-glue-like compounds that interacted with both the mutant HD protein and LC3, but not the wild-type HD protein. *In vivo* models were able to reverse the phenotype in mutant HD without affecting the wild-type protein. In addition, the authors were able to target disease-relevant glutamine repeats found in other mutant proteins, like those that cause spinocerebellar ataxia type 3, demonstrating its generalizability.

RNA degradation

Inspired by induced protein degradation, ribonuclease-targeting chimeras (RIBOTACs) combine an RNA binder with a ribonuclease binder to recruit nucleases to degrade RNA (Figure 2D). Alternative RNA degradation methods, such as antisense oligonucleotides and small interfering RNAs, have extremely poor uptake properties due to their large size and charge (Meyer et al., 2020). RIBOTACs are medium-sized molecules that overcome some uptake and permeability limitations, serving as an alternative method for RNA degradation. First reported in 2018 by Costales et al., a RIBOTAC was developed to recruit ribonuclease L (RNase L) to dimerize and degrade the precursor of pri-miR-96, an oncogenic RNA (Costales et al., 2018). Now, there are several other examples using RIBOTACs to target other disease-relevant RNAs via recruitment of RNase L, including pre-miR-210 (Costales et al., 2019), pre-miR-21 (Costales et al., 2020), and, more recently, SARS-CoV-2 RNA (Haniff et al., 2020). As a small drawback, appending the RNase L binding molecule to RNA-targeting molecules typically weakens the affinity to the target RNA. However, this loss in binding affinity is compensated for in activity, as RNA target degradation is more effective than simply binding the RNA with a small molecule (Meyer et al., 2020). One of the major limitations here is a lack of specific, high-affinity, small-molecule binders to RNA. For more information on RIBOTACs and their predecessors, please see Meyer et al. (2020). In addition, three-body equilibria for "RNA-small molecule-enzyme" interactions may be different than for "protein-small molecule-enzyme" interactions and have not been substantially developed. RNA stability, compared with protein stability, can also affect the ternary complexes and effectiveness of the small molecules.

NON-DEGRADATION-INDUCING BIFUNCTIONAL MOLECULES

Complementary to degradation-inducing small molecules, non-degradation-inducing bifunctional molecules can induce induce or remove PTMs, and have important applications in chemical biology and medicine.

Bifunctional molecules to induce or remove phosphorylation

Numerous cellular processes require phosphorylation (the addition of a phosphoryl group, PO_3^{2-}) by a kinase to its substrate protein, typically on serine (Ser), threonine (Thr), and tyrosine (Tyr) residues. The majority of cellular phosphorylation is due to Ser/Thr-specific protein kinases, although there are also Tyr-specific kinases classified as RTKs or non-receptor tyrosine kinases. The converse—protein dephosphorylation via the removal of a phosphoryl group as facilitated by protein phosphatases—is also an area of interest in the regulation of cellular processes and functions. Phosphatases, although classically known to be highly promiscuous and less well studied than kinases, can enforce a surprisingly high degree of specificity through docking interactions, like hydrophobic grooves, with certain protein substrates (Roy and Cyert 2009; Chen et al., 2017). Taken together, protein kinases and phosphatases complement one another and regulate a multitude of cellular processes in eukaryotes.

Protein phosphorylation is a common cellular PTM, and abnormal phosphorylation activity can trigger leukemias, gastrointestinal stromal tumors, hematologic malignancies, prostate cancer, and pancreatic cancer. Therefore, there is great interest in both inducing and removing phosphorylation through small-molecule technology. Over a thousand kinase expression alterations have been observed in human tumors, designating them as cancer biomarkers (Ardito et al., 2017). Genetic drivers and passenger mutations are often connected to abnormal PTM states. Since many cancers arise from aberrant kinase and/or phosphatase activity and phospho-site mutations, designing bifunctional molecules that could modulate the specific phosphorylation events has become an increasingly attractive approach to target proteins as therapeutic tools or to study biological pathways (Deshaies 2020; Gerry and Schreiber 2020). Thus, using bifunctional molecules to induce targeted, non-native PTMs (termed neo-PTMs) can have a number of therapeutic benefits that may complement PROTAC-based protein degradation. First, appending phosphoryl or different glycosylation groups to specific signaling proteins of interest with dose and temporal control can rewire signaling pathways. For instance, serine phosphorylation of oncogenic RTKs, key components of major growth factor signaling pathways such as EGFR and FGFR1, may result in RTK endocytosis and subsequent lysosomal degradation (Nadratowska-Wesolowska et al., 2014). Likewise, the fucosylation of cell-surface receptors, such as LRP6, an oncogene and component of WNT signaling, may lead to receptor endocytosis (Hong et al., 2020). Immune signaling pathways may also be induced by neo-PTMs for cancer immunotherapy. Second, the addition of negatively charged

phosphoryl groups or several N- or O-glycans capped with negatively charged sialic acid to aggregation-prone proteins, which are insoluble or hydrophobic, may increase solubility and reduce protein self-aggregation. This is particularly important for proteins involved in neurodegenerative diseases such as amyloid-β, α-synuclein, and TDP-43 (Peng et al., 2020). Third, depositing multiple negatively charged phosphoryl groups or bulky glycosylation groups to undruggable targets, such as transcription factors, can hinder the ability of target proteins to interact with DNA, RNA, and other proteins. Fourth, neo-phosphorylation or glycosylation on oncogenic targets may be presented by major histocompatibility complexes and activate T cells for an enhanced immune response against cancer cells. In fact, both phosphorylated and glycosylated peptides, such as from LSP1 and MUC1, respectively, have been shown to elicit immune responses (Apostolopoulos et al., 2003; Cobbold et al., 2013). Fifth, phosphorylation or glycosylation can modulate the stability and longevity of target proteins. For instance, several phosphorylation sites may be able to recruit ubiquitin ligases to trigger oncogene degradation. In contrast, certain types of glycosylation can shield proteins, such as tumor suppressors, from proteolytic cleavage to enhance their stability. In addition, while PROTACs target Lys, kinases can target Ser, Thr, Tyr, and His. Taken together, the induction of neo-PTMs by bifunctional molecules has potential to accomplish a variety of biological applications and, if successful, will help accelerate the development of needed therapies for unmet medical conditions.

Similar to degradation-inducing small molecules, kinases phosphorylate specific substrates based on proximity-mediated reactivity (e.g., local interactions). Therefore, in order for a kinase to phosphorylate a non-substrate protein, it must be rewired to alter its interactome. Hobert and Schepartz demonstrated this concept by creating a synthetic adaptor protein to bring together the Src family kinase Hck and the otherwise poor substrate hDM2. Their synthetic adaptor fuses the miniature protein YY2, which activates Hck via interaction with its SH3 domains, and the miniature protein 3.3, which binds to hDM2, via a $(GGST)_n$ linker. The application of this adaptor led to the successful phosphorylation of hDM2 (Hobert and Schepartz 2012).

Small molecules have also been employed to phosphorylate proteins. The best described class of such small molecules is chemical inducers of dimerization (CIDs) for RTKs engineered to contain fusion proteins. For example, the protein FKBP12 fused to the cytoplasmic domains of an RTK (e.g., FGFR2) can be homodimerized via a dimer of its small-molecule binding partner, FK506, to induce subsequent phosphorylation and activation of the fused RTK and its corresponding signaling cascade (Jin et al., 1998; Yang et al., 1998; Muthuswamy et al., 1999; Li et al., 2002; Freeman et al., 2003; Nguyen et al., 2018). These CID-induced phosphorylation events profoundly influence many fundamental cellular processes, ranging from embryonic development to the progression of diseases such as cancer. This demonstrates that it is feasible to induce heterodimerizations between two different non-receptor species, such as an enzyme and a non-substrate target protein. The underlying principle of proximity-mediated reactivity remains the same as for homodimers and heterodimers, through native or non-native mechanisms.

Phosphorylation-inducing small molecules (PHICS) are bifunctional molecules that can recruit a kinase to phosphorylate a target protein through the induction of proximity (Figure 3A) (Siriwardena et al., 2020). They usually contain an AMPK or PKC kinase binder conjugated to a target protein binder and, as such, can redirect kinases to phosphorylate non-substrate proteins. PHICS can also be used to induce signaling-relevant phosphorylation in cells, as evidenced by PHICS generated for BTK that demonstrate phosphorylation at S180. Phosphorylation at S180 is normally performed by PKC to inhibit the membrane translocation of BTK. PHICS can also induce neo-phosphorylation events, including neo-phosphorylations of BRD4 *in vitro*. Importantly, these bifunctional small molecules are generally event driven rather than occupancy driven, and this catalytic mechanism ensures turnover. However, the required formation of a ternary complex rather than a binary complex implies that these compounds suffer from the hook effect, wherein high concentrations of the bifunctional molecule form binary complexes that autoinhibit the intended function. Currently, there are PHICS to phosphorylate Ser/Thr residues, and PHICS that phosphorylate Tyr residues are now being developed.

Furthermore, the development of molecules that are capable of removing PTMs, in addition to those that can induce PTMs, was inspired by the fact that cellular processes are regulated by both PTM inducers (e.g., kinases) and PTM removers (e.g., phosphatases). Therefore, just as heterobifunctional PHICS exist to induce phosphorylation, their direct opposite, phosphatase-recruiting chimeras (PhoRCs), have also been developed to control dephosphorylation (Figure 3B). These PhoRC bifunctional molecules consist of a protein phosphatase binding motif (e.g., PP1) linked to a target protein binding motif (e.g., AKT and EGFR). While classical kinase inhibitors prevent a kinase from phosphorylating all of its substrate targets, which can be highly disruptive to a cell, PhoRCs remove phosphoryl groups only from specific target substrates.

As a proof of concept, HaloTag constructs of PP1 were transfected into human prostate cancer LNCaP cells and treated with HaloTag-reactive chloroalkyl-containing molecules conjugated with an AKT or EGFR binder. A decrease in pAKT or pEGFR was observed in cells expressing HaloTag-PP1 only. Next, a short PP1-activating tetrapeptide (RVSF, which belongs to the PP1 motif RVXF) was used to build a bifunctional molecule that can recruit PP1 to AKT, decreasing $pAKT^{308}$ and $pAKT^{S473}$ levels more than with an AKT inhibitor alone (Yamazoe et al., 2020). The use of an RVXF motif leads to dephosphorylation only at high concentrations, due to the peptidic nature of the binder (poor drug-like qualities); nonetheless, this work represents the first example of using synthetic molecules to induce the removal of a PTM through the recruitment of non-native enzyme-substrate pairs. With more small-molecule discovery efforts put into kinase and phosphatase binders, phosphorylation-modulating molecules can be developed that use many types of kinase/phosphatase binders, paralleling the number of E3 ligase recruiters now available for PROTAC design. In addition, it would expand the current space of PTM-inducing molecules to include PTM removal and addition/subtraction of PTMs beyond ubiquitin.

Bifunctional molecules to induce or remove glycosylation

Glycosylation is another common PTM essential for cell-cell interactions on the surfaces of cells, and it is fundamental for

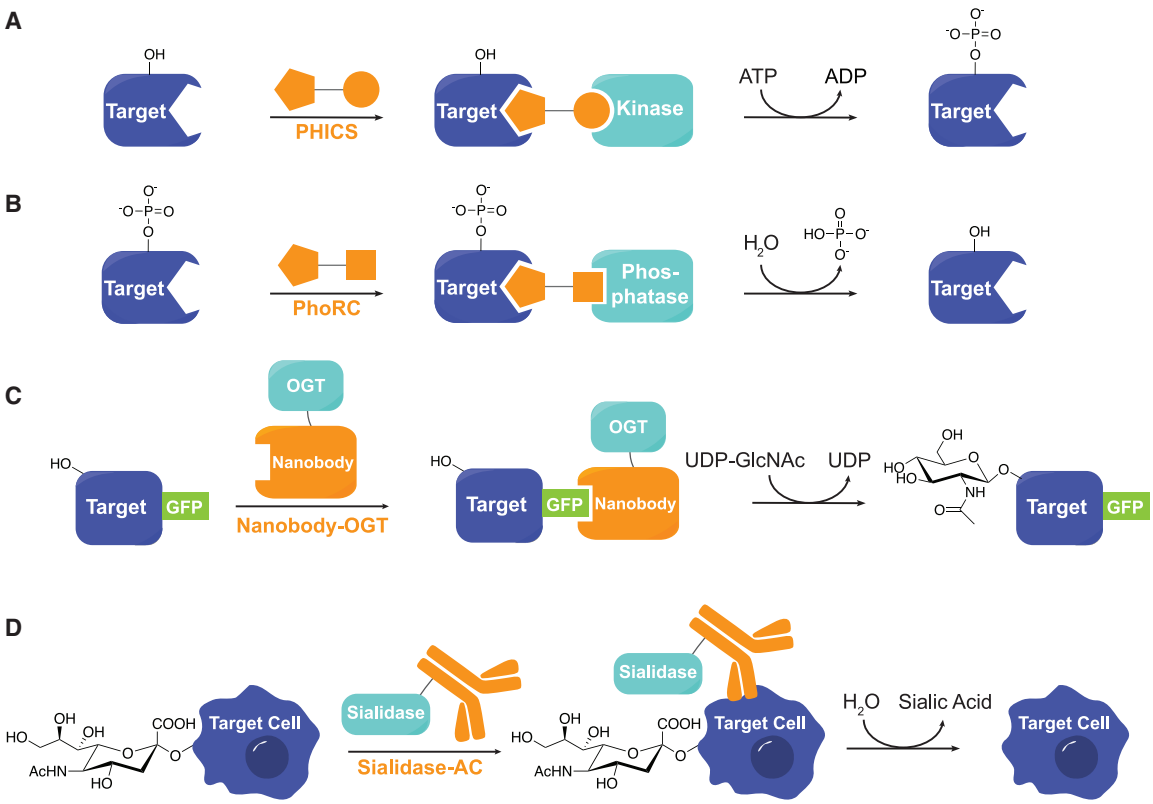

Figure 3. Methods for inducing or removing post-translational modifications
(A) Bifunctional molecules to add phosphoryl groups (PHICS), (B) bifunctional molecules to remove phosphoryl groups (PhoRCs), (C) nanobodies that recognize GFP fused to OGT to glycosylate GFP-tagged fusion proteins, and (D) sialidase antibody conjugates to cleave sialic acid on cell surfaces.

protein-protein interactions within the cell, including mitochondrial and cytoskeletal functions, signaling cascades, and transcription regulation. Glycosylation is frequently deregulated in diseases due to its high sensitivity to changes in physiological states. For example, the DepMap genetic screening effort across a panel of hundreds of cancer cell lines identified cancer cells that depend on both PTK2 tyrosine kinase and the glycosyltransferase RPN2 for survival (Tsherniak et al., 2017), suggesting that glycosyltransferases may play roles in driving cancer development in addition to tyrosine kinases. In cancer, N-glycosylation of PD-L1 has been shown to suppress T cell activity by reducing PD-L1 proteasomal degradation (Li et al., 2016). The fucosyltransferase FUT8 facilitates tumor invasion and metastasis partly by fucosylating L1CAM, a regulator of cell invasion and migration, and reducing its proteolytic cleavage (Agrawal et al., 2017). Furthermore, glycosylated MUC1, a glycoprotein frequently expressed in metastatic cancers, has been demonstrated to yield glycosylated peptides that are recognized by glycoform-specific T cell receptors (Apostolopoulos et al., 2003), with potential implications in cancer immunotherapy development. In infectious diseases, bacteria can inject their glycosyltransferases or glycoside hydrolases to rewire host proteins' functions. For instance, an enteropathogenic *Escherichia coli* strain evades host defenses using a protein with *N*-acetylglucosamine transferase activity. This introduced glycosylation on the death domain of TRADD protein disrupts TRADD oligomerization, NF-κB signaling, apoptosis, and necroptosis in infected cells (Li et al., 2013), such that deglycosylating bifunctional molecules may have antibacterial benefits.

Compared with phosphorylation, glycosylation can be more diverse, in that a variety of sugars are added to asparagine, hydroxylysine, and tryptophan in addition to serine, threonine, and tyrosine (Schjoldager et al., 2020). Most secretory proteins and the majority of nuclear and cytoplasmic proteins are glycosylated in the endoplasmic reticulum (ER) and the Golgi apparatus (Steentoft et al., 2013). For the reverse process, the removal of a glycosyl group is carried out by glycoside hydrolases, more commonly known as glycosidases, and each glycosidase has an affinity for breaking down certain sugar types. These enzymes typically process glycoproteins in the ER and Golgi apparatus and are also present in the lysosome for further sugar degradation.

Not surprisingly, glycosylation is frequently deregulated in disease due to its high sensitivity to changes in physiological states. Thus, methods to chemically induce or remove glycosyl groups on select targets would have a major impact on the understanding of the involvement of these PTMs in disease. As a first example of the artificial induction of these PTMs, the *O*-linked β-*N*-acetylglucosamine (O-GlcNAc) glycosylation of specific proteins in cells has recently been demonstrated using bifunctional molecules through proximity-directed O-GlcNAc transferase (OGT), a protein that directs the initiation of O-GlcNAcylation (Figure 3C). Here, constructs of OGT fused with a nanobody recognizing GFP or the four amino acid sequence

EPEA as a proximity-directing agent could selectively glycosylate GFP/EPEA-fused target proteins, including the transcription factors JunB and cJun, as well as Nup62 and α-synuclein (Ramirez et al., 2020). Although OGT engineering was required, the study suggests the feasibility of using bifunctional molecules to induce glycosylation on specific proteins for therapeutic purposes. The active development of selective binders for OGT and glycosyltransferase, in general, will advance glycosylation-modifying bifunctional small molecules in the future, and this is currently being actively explored due to the role of glycosylation in diseases (Ortiz-Meoz et al., 2015; Zhang et al., 2020).

Another recent innovation demonstrated that a bifunctional molecule using an enzyme involved in the capping process of glycosylation, *Helicobacter pylori*-derived fucosyltransferase, could enrich for tumor antigen-reactive T cells for cancer immunotherapy (Liu et al., 2020). The fucosyltransferase-fucose-GDP conjugate can self-catalyze to install a fucosyltransferase on the *N*-acetyllactosamine of bait cells, which function as tumor-antigen-presenting cells to capture reactive T cells. The installed fucosyltransferase on bait cells can then use proximity-directed fucosylation to label T cells with biotin-fucose-GFP, which can then be pulled down with streptavidin beads for expansion and cancer treatment. Collectively, this study suggests that bifunctional molecules can induce glycosylation extracellularly for capturing specific populations of immune cells for cell therapy. Advances in glycosylation profiling to understand unique sugar modification patterns on specific cell types will expedite the development of similar strategies for cellular enrichment with potential applications in cell therapy.

Similar to glycosylation, deglycosylation also has potential therapeutic benefits. Ge et al. created a generalizable nanobody-fused split *O*-GlcNAcase, which removes O-GlcNAc from a target protein in cells. Importantly, their system allows for investigating site-specific O-GlcNAc at the protein level (Ge et al., 2021). In addition, a cancer immunotherapy study developed antibody-enzyme conjugates by fusing trastuzumab, a HER2-targeting antibody, with sialidase from *Vibrio cholerae* (Xiao et al., 2016). The sialidase removes sialic acids from cell-surface glycans on natural killer (NK) cells, which normally suppress NK cell activation. Therefore, this antibody-enzyme conjugate enhances the ability of NK cells to kill HER2-overexpressing breast cancer cells. The study suggests the potential for bifunctional molecules to alter tumor immunity by removing sugar(s) from the cell surface. A second generation of the anti-HER2-sialidase conjugate with reduced off-target sialidase activity was recently developed using the *Salmonella typhimurium* sialidase, which lacks domains that enable binding independent of anti-HER2-targeting activity (Figure 3D) (Gray et al., 2020). Although examples of bifunctional modalities that can deglycosylate targets are limited, a better understanding of deglycosylation mechanisms and the development of specific binders for glycoside hydrolase will accelerate the development of specific classes of small molecules that can deglycosylate proteins.

CONCLUSIONS AND FUTURE DIRECTIONS

Here, we reviewed applications of synthetic bifunctional modalities that induce native and neo-modifications on biomolecules. Compared with traditional inhibitors, which inhibit enzyme function, the bifunctional molecules reviewed here can induce a function on a particular target. Used initially to tag a protein with ubiquitin for degradation, there are now bifunctional molecules that recruit the lysosomal degradation pathway, the autophagy pathway, ribonucleases, kinases, phosphatases, glycosyltransferases, and glycosylases to desired targets. Beyond PTMs, Shokat and colleagues created bifunctional compounds that recruit a non-native protein binder (FKBP12 or cyclophilin A) to Ras, which ultimately inhibits Ras's interactions with its native substrates (Zhang and Shokat, 2019). These examples demonstrate that the possibility of redirecting enzymes or proteins to perform non-native actions on substrates can reveal other biological applications.

Before these synthetic bifunctional molecules can be consistently used in real-world applications, the field must address several key considerations. Traditional compounds that target only one protein or enzyme need to evaluate the kinetics and thermodynamic interactions for only that single target. For bifunctional molecules, multiple interactions must be deeply explored, and three-body equilibrium models can be challenging. The differential binding affinities of both sides of the bifunctional molecule can negatively affect the efficacy of the molecule as a whole to induce an effective ternary complex due to the hook effect. For instance, if there is a significant difference in binding affinity, where one side is much stronger at binding (e.g., nanomolar) to its target than the other (e.g., micromolar), then the half-maximal concentration of ternary inhibition may be unfavorably low (Douglass et al., 2013). Furthermore, the inherent stability and lifetime of the targets can affect the complex formation (e.g., RNA targets versus protein targets differ in stability and the time required for resynthesis). In addition, there are two sides of the compound that can exhibit off-target effects, which can lead to unfavorable interactions or unpredictable ternary complexes. Moreover, these "small" molecules often defy Lipinski's rule of five for druglikeness; they are generally larger in molecular weight and contain several additional hydrogen bond donors and acceptors, which traditionally indicates they might not perform well *in vivo*. Thus, there is a significant interest in identifying molecular glues, which are smaller and more druglike than bifunctional molecules with linkers (Schreiber 2021). These compounds are typically harder to rationally design because a portion of the two linked protein interfaces also interacts, instead of only two moieties separately binding to their respective targets.

Despite these challenging limitations, we are starting to see some successes with these bifunctional molecules, which is beginning to reverse the notion that they will not be useful *in vivo*. Initial studies from the Yang lab showed that event-driven PROTACs can have superior drug-resistant properties compared with occupancy-driven inhibitors, often performing well even in the presence of acquired mutations (de Wispelaere et al., 2019). In addition, while we have described bifunctional molecules and proteins that degrade, phosphorylate, and glycosylate targets, there are untapped PTMs. For example, acetylation and methylation are important PTMs, and tools to better study these pathways will be valuable for further understanding biological systems. Furthermore, as with RIBOTACs that target RNA, we hope to see additional classes of biomolecular targets in the future, since the field is largely focused on proteins. In addition

to degrading RNA, we anticipate that we will soon be able to use bifunctional molecules to add PTMs to RNA, specific loci on DNA, or histones. This should provide further insight into site-specific modifications by allowing the precise addition or removal of specific PTMs. Overall, synthetic bifunctional small molecules are starting to prove their worth in the field, and the development of these molecules can help us better elucidate the biological functions of numerous pathways and inspire the generation of therapeutic tools.

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DECLARATION OF INTERESTS

Broad Institute has filed patent applications for some of the work described in this article.

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