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Review

Screening for molecular glues – Challenges and opportunities

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ABSTRACT

Molecular glues are small molecules, typically smaller than PROTACs, and usually with improved physicochemical properties that aim to stabilise the interaction between two proteins. Most often this approach is used to improve or induce an interaction between the target and an E3 ligase, but other interactions which stabilise interactions to increase activity or to inhibit binding to a natural effector have also been demonstrated. This review will describe the effects of induced proximity, discuss current methods used to identify molecular glues and introduce approaches that could be adapted for molecular glue screening.

1. Introduction

Interaction between molecules is an essential part of the processes controlling the reactions of life, and being able to regulate interactions between target proteins and other macromolecules represents an attractive therapeutic opportunity. Molecular glues are small molecules that induce proximity between a target protein and an effector macromolecule, resulting in a new interaction, or which stabilise an existing interaction, to bring about a change in the resulting function of the target protein. Molecular glues may therefore potentially alter a range of biological processes, including transcription, translation, protein folding and degradation [1]. The concept of molecular glues is not novel, with the term being used in the late 1980′s for small molecules or proteins that induced protoplast fusion or allowed platelet plug formation [2,3]. The term was also used for the immunosuppressants cyclosporin A and FK506, describing their ability to induce proximity between the

Abbreviations: Alpha, amplified luminescent proximity homogeneous assay; AML, acute myeloid leukemia; BioID, proximity-dependent biotin identification; BRD4, bromodomain-containing protein 4; BRD9, bromodomain-containing protein 9; BVdU, bromovinyldeoxyuridine; Cas9, CRISPR associated protein 9; CCNK, CDK12-interacting protein cyclin K; CDK12, cyclin-dependent kinase 12; CDO1, cysteine dioxygenase type1; CELMoDs, cereblon modulators; CK1α, casein kinase 1 alpha; CN, calcineurin; CRBN, cereblon; CRISPR, clustered regularly interspaced short palindromic repeats CRL, cullin-RING ligases; CsA, cyclosporin A; Cub, C-terminal half of ubiquitin CYPA, cyclophilin A; dCK, deoxycytidine kinase; DDB1, DNA damage-binding protein 1; DEL, DNA-encoded library; Derlin-1, degradation in endoplasmic reticulum protein 1; DMSO, dimethyl sulfoxide; EFC, enzyme fragment complementation; ER, estrogen receptor; ERRγ DBD, estrogen related receptor gamma's DNA binding domain; FA, fluorescence anisotropy; FKBP, FK506 binding protein; GFP, green fluorescent protein; GSPT1, G1 to S phase transition 1; HA, hemagglutinin IKZF1, IKAROS Family Zinc Finger protein 1; IMiDs, immunomodulatory drugs; IP, immunoprecipitation; KSR, kinase suppressor of Ras; MAPPIT, mammalian protein-protein interaction trap; MASPIT, mammalian small molecule-protein interaction trap) MDMX, murine double minute X; MEK1, mitogen-activated protein kinase kinase; MS, mass-spectrometry; mTOR, the mammalian target of rapamycin also referred to as the mechanistic target of rapamycin; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NanoBRET, nanoluciferase bioluminescence resonance energy transfer; nESI, nano electrospray ionisation NF-AT, nuclear factor of activated T cells; NFKB1, Nuclear factor NF-kappa-B p105 subunit; NRF2, Nuclear factor erythroid 2-related factor 2; Nub, N-terminal half of ubiquitin; PBMC, peripheral blood mononuclear cell; POI, protein of interest; PPI, protein-protein interaction; PROTAC, proteolysis targeting chimeras; RBM39, RNA-binding protein 39; RBX1, RING-box protein 1; rePPI-G, rapid evolution of protein–protein interaction glues RTKs, receptor tyrosine kinases SALL4, sal-like protein 4; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sgRNA, single guide RNA; SH2, Src homology 2; SHP2, Src homology-2 domain containing protein tyrosine phosphatase-2; SMAD4, mothers against decapentaplegic homolog 4; SOD1, superoxide dismutase 1; SPR, surface plasmon resonance; TF, transcription factor; TP53, tumor protein P53 or cellular tumor antigen p53; TR-FET, time resolved Förster resonance energy transfer; UBR7, ubiquitin protein ligase E3 component N-recognin 7; VHL, von hippel–lindau; WT, wild type; β-TrCP, β-transducin repeat-containing protein.

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common target, calcineurin and cyclophilin and FKBP respectively [4]. Following the success of PROTACs in inducing protein proximity between target proteins and E3 ligases to bring about target protein degradation [5], recently, efforts have been made to extend this approach to identify molecular glues that are able to induce protein proximity, but without the requirement for individual warheads and linkers. Thus far, most of the identified glues stabilise an interaction between the target protein and an E3 ligase, leading to degradation. It is believed that the mechanism is primarily by enhancing pre-existing weak interactions, although binding through a binary complex with one protein partner and inducing the formation of a ternary complex is possible. Where molecular glues enhance existing, weak interactions [6], or induce new interactions between molecules that do not usually interact [7], they do not necessarily require a binding pocket on the individual target protein but operate to provide improved complementarity between the interacting protein surfaces [8]. Thus, molecular glues have the exciting prospect for improving the druggability of previously intractable targets. The challenge is in identifying molecular glues, as many of the known compounds have not been identified via rational screening, but rather from serendipitous findings after traditional phenotypic screening approaches, Table 1 [9]. However, focus is shifting towards methods that do allow rational approaches including both diversity screening and rational design, exploiting a range of binding partners that will enhance our ability to exploit this novel mechanism. Here, we will highlight the opportunities for induced protein proximity and discuss the current and future methods for cell-based and cell free screening that illustrate the scope for exploiting molecular glues in drug discovery. For a review of molecular glues targeting protein degradation in the clinic see Sasso *et al.* [10].

2. Induced protein proximity

Biochemical processes occurring between molecules in the cell are controlled by the degree of physical separation between the two interacting species. Only when in a proximal location can two molecules interact to produce the desired biological effect. For many years, drug discovery focussed on interrupting the interactions between proteins by searching for molecules that could disrupt protein-protein interactions (PPIs) or inhibit / antagonise protein-ligand interactions [11,12]. Perhaps the recognition that artificial small molecules (molecular glues) could be used to induce protein proximity was established in the early 1990s, with recognition of the action of the immunophilins [13], the developing understanding of the function of FK506 in forming the FKBP12-FK506-calcineurin complex [14], and the realisation that natural glue processes could also be targeted, for example the Src homology 2 (SH2) domain of tyrosine kinases facilitates signal transduction by binding phosphotyrosine without the requirement for catalysis [15].

Molecular glues function by inducing or enhancing interactions between two proteins, bridging the surfaces to allow for complementarity enabling the formation of a ternary complex. It has been suggested that molecular glues may predominantly function by binding only to one binding partner, or only to the binary complex formed between the interacting proteins [16] (Fig. 1). Where the two binding partners have some level of intrinsic affinity, the action of the molecular glue to stabilise this interaction further has led to the description as a chemical stabiliser [17,18]. This hypothesis distinguishes molecular glues from PROTACs, which are bifunctional molecules having affinity for both protein partners. Molecular glues thus, lack the issue often experienced by PROTACs, whereby at higher concentrations the formation of the ternary complex is prevented due to both proteins being complexed to the PROTAC – the so-called hook effect. This effect is not experienced by molecular glues, and they do not demonstrate saturable binding behaviour. Rational design of PROTACs has focussed on firstly identifying ligands for the target and effector proteins, which typically uses traditional hit finding methods and then on optimisation of the linker between these two warheads to improve affinity and functionality. In contrast, molecular glue discovery and design requires new screening strategies that induce proximity. Most molecular glues are expected to form interactions between protein molecules (intermolecular), but interactions within a single protein (intramolecular) have also been described, which show glue-like behaviour. In this case, different domains of the protein are brought into close proximity, altering the function. An example is the allosteric SHP2 inhibitor, SHP099 [19]. The functional outcome of induced protein proximity may be varied, leading to stabilisation, inhibition, activation or even degradation (Fig. 2). In the subsections below we describe the role of induced proximity in several settings.

Table 1
Molecular glues discovered by serendipity and rational design. Target proteins and binding partners (where known) are shown.

Name	Target Protein	Binding partner	Approach	Reference
Thalidomide	IKZF1, IKZF3	CRBN	Serendipity	[39]
Indisulam	RBM39	DCAF15	Serendipity	[118]
HQ461	CDK12	Cyclin K	Serendipity	[119]
Rapamycin	FKBP12	FRB (mTOR)	Serendipity	[120]
Cyclosporine A	Calcineurin	Cyclophilin	Serendipity	[4]
Asukamycin	TP53	UBR7	Serendipity	[65]
Lenalidomide	IKZF1, IKZF3	CRBN	Serendipity	[121]
FK506	FKPB12	Calcineurin	Serendipity	[4]
Paclitaxel	β -tubulin	β-tubulin	Serendipity	[122]
Pomalidomide	IKZF1, IKZF3	CRBN	Serendipity	[123]
Sanglifehrin	IMPDH2	Cyclophilin A	Serendipity	[124]
BI-3802	BCL6	BCL6	Serendipity	[125]
CCT369260	BCL6	BCL6	Serendipity	[126]
NRX-252,114	β -catenin	β -TRCP	Rational design	[41]
NRX-252,262	β -catenin	β -TRCP	Rational design	[41]
BTX-1188	GSPT1, IKZF1/3	CRBN	Rational design	[127]
CCT369260	BCL6	BCL6	Rational design	[126]
dCeMM1	RBM39	DCAF15	Rational design	[40]
dCeMM2/3/4	Cyclin K	CDK12	Rational design	[40]
CR8	CDK12	Cyclin K	Rational design	[128]

2.1. Protein stabilisation

Molecular glues may bind to an existing protein-protein complex and enhance the affinity of the interaction. This may give the molecular glue a distinct advantage, as it may not be required to have as tight an affinity compared to inhibitory compounds that may have to compete with a native binder partner to achieve the desired biological modulation. Enhancing a previously occurring interaction is thus an attractive strategy. Additionally, molecular glues may bind to a transient and specific intermolecular surface or pocket, formed between interacting proteins. Only when the relevant binding partners are present would the ternary complex be formed. This potentially provides a greater degree of selectivity that could assist in reducing off-target effects [20,21]. Indeed, studies of potential pockets at protein-protein interfaces have been undertaken, suggesting mechanisms for pocket formation [22,23], and some have attempted to describe what chemical requisites make a good stabiliser [24,25]. These studies have suggested that there may be similarities between pockets formed at protein interfaces and more traditional small molecule binding sites, adding weight to the hypothesis that existing small molecule libraries may contain chemical equity that could provide initial hits for molecular glue optimisation. The development of trametiglue, which effectively stabilises the KSR-MEK1 interaction, is a good example. Here, trametiglue, which was designed as a derivative of trametinib, a MEK1 inhibitor, combines the potency

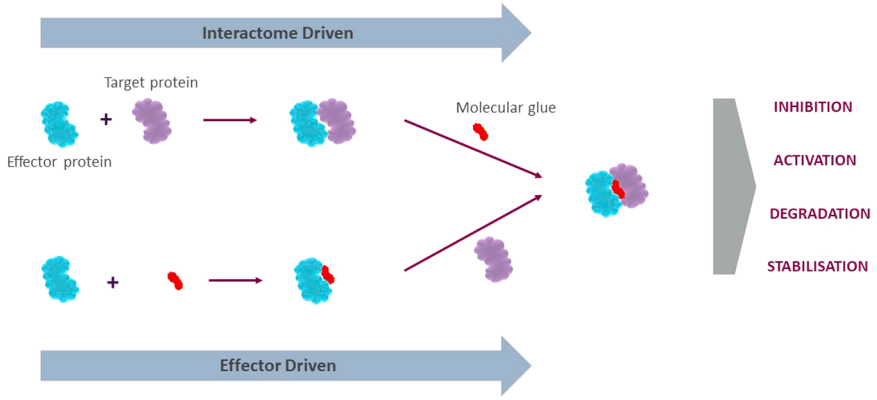

Fig. 1. Molecular glue binding mechanisms and downstream effects.
There are 2 mechanisms by which molecular glues are thought to bind. They may either stabilise existing interactions between a target and effector protein by binding to the target-effector complex (interactome driven), as shown in the top pathway; or create new interactions (effector driven) by binding first to the effector protein, as shown in the bottom pathway. This increased or induced protein proximity may have a range of downstream effects, depending upon the nature of the effector protein. For example, inhibition, degradation or even stabilisation and activation may be achieved according to the desired biological effect required for disease modulation.

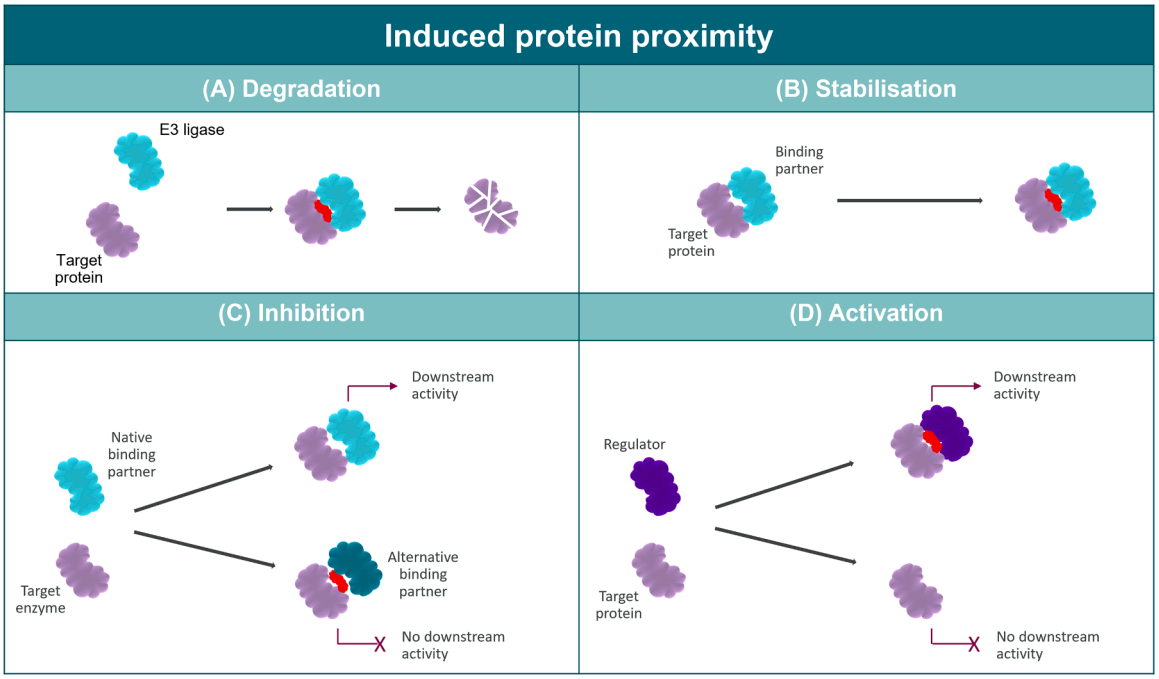

Fig. 2. Molecular glue induced protein proximity
Molecular glues often function by induced protein proximity; by inducing or enhancing interactions between two proteins. There are various biological effects which may result from this, including (A) target degradation (by inducing proximity between a target protein and an E3 ligase), (B) stabilisation of the target-effector complex, (C) inhibition of the target activity (by preventing the target protein from binding to its native binding partner required for downstream activity), or (D) activation of the target activity (by promoting the interaction of a target protein with a regulator protein which enhances target activity).

and dissociation kinetics of trametinib with the functional capability of CH5126766, another MEK inhibitor, to trap, inactive states of RAF-bound MEK [26]. For a review of protein-protein interface classification and characterisation see the review by Rui *et al.* [27].

2.2. Enzyme inhibition

Perhaps the most well-known example of molecular glue demonstrating inhibition is that of the cyclosporin A (CsA)/cyclophilin A (CYPA) inhibition of calcineurin (CN). CsA induces its biological effect – immunosuppressant activity – by forming an initial complex with the immunophilin protein, CYPA, with the complex then binding to CN and inhibiting its phosphatase activity [4]. The competitive, Ca^{2+} dependent inhibition of CN, which prevents dephosphorylation of the cytoplasmic component of the transcription factor NF-AT (Nuclear factor of activated T cells) thus prevents NF-AT translocation to the nucleus and blocks transcription of early-stage growth factors [28]. It was demonstrated that neither CsA nor CYPA alone could inhibit CN. At the same time, another natural product, and its binding partner complex (FK506-FKBP12) was also shown to inhibit CN. The FK506 binding proteins and their functions have been comprehensively reviewed [29]. It was demonstrated that FKBP12 exploited a molecular glue mechanism to inhibit the protein kinase mTOR. Binding of FKBP12 to mTOR is induced by the macrolide rapamycin [30]. Subsequently, synthetic

FKBP12-mTOR molecular glues have been discovered from a FKBP-focused, target-unbiased library screen, demonstrating induced proximity between FKBP12 and the FRB domain of mTOR [31]. Thus, inhibition of enzyme function, or downstream signalling is an important molecular glue mechanism, in addition to protein stabilisation.

2.3. Activation

Molecular glues may potentially be used to activate target proteins. For example, the molecule asukamycin has been shown to bind to the E3 ligase UBR7 covalently modifying the Cys374 site and activating the neosubstrate TP53. This increases tumour suppressor transcription activity in a UBR7-dependent manner [32]. This activation resulted in the inhibition of cancer cell growth for 250 cancer cell lines with IC_{50} values from 5 to 30 μM. Another molecular glue which activates p53, does so by a different mechanism. RO-2443, which was identified via small molecule screening induces MDMX (murine double minute X) dimerization [33]. This prevents MDMX binding to TP53 and relieves the negative regulation of the tumour suppressor and may provide an effective treatment for MDMX-overexpressing cancers by mediating the activation of TP53 apoptotic activity. Thus, molecular glues may activate proteins by removing negative regulation by gluing regulatory proteins to each other or to other binding partners.

2.4. Degradation

Protein proximity-induced target protein degradation has emerged as a promising strategy for selective protein knockdown [34] and is the category with the largest number of functional molecular glues currently. Molecular glues that induce proximity between a target and an E3 ligase have been identified from serendipitous findings, rational design, data mining or traditional screening approaches [35,36]. Close proximity between the target and the E3 ligase brings about target ubiquitination. The ubiquitylation process involves four different classes of enzyme: E1–E4 ligases [37]. The process begins when ubiquitin is covalently coupled to the E1 (ubiquitin-activating enzyme) before being transferred to the E2 (ubiquitin-conjugating enzyme). The E3 (ubiquitin–protein ligase) is responsible for transferring the ubiquitin from the E2 to the target protein. Once the first ubiquitin has been attached (monoubiquitylation), the E3 can form longer ubiquitin chains by creating ubiquitin–ubiquitin peptide bonds. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) [38], which can potentially be used for chain extension. The E4 (chain elongation factor) are a subclass of E3-like enzymes that also catalyse this process [39]. Polyubiquitinated proteins are then recognised and degraded by the large, multi-subunit protease complex, termed the proteasome [40]. Molecular glues typically act by directly modulating protein-protein interaction surfaces to introduce or increase the affinity of the interaction between the target protein and the E3 ligase. Examples include the IMiDs which enhance the interaction between cereblon (CRBN) and the target protein for degradation. The structures for these compounds binding to DDB1-CRBN E3 ligase were solved some years ago with the first molecular glue shown to function in this way being thalidomide, which was first marketed in 1957 under the trade name Contergan as a treatment for morning sickness. It was subsequently shown to be responsible for teratogenic deformities in children born after their mothers used it during pregnancy. Thalidomide was shown to interact with the E3 ligase CRBN [41], causing it to bind to, ubiquitinate and cause subsequent degradation of a number of neosubstrates. The neosubstrates for cereblon induced by thalidomide and its derivatives include Ikaros, SALL4 and CK1α [42].

One of the first rational approaches to capitalise on protein degradation enhanced by molecular glues was to undertake a cell death-phenotypic campaign to screen compounds using isogenic hyponeddylated cells coupled to multi-omics target identification [43]. The approach to study hyponeddylated cells versus neddylation-proficient cells stems from the fact that ligase activity of the ubiquitination machinery requires modification of the cullin protein with a ubiquitin-like protein called NEDD8. Neddylation results in conformational rearrangements within the cullin ring ligase, which is necessary for ubiquitin transfer to a substrate. This resulted in the identification of several compounds that caused pronounced destabilization of cyclin K.

In a targeted strategy, molecular glues were developed with a biochemical screen using fluorescence anisotropy (FA) (or fluorescence polarisation (FP)) with a pSer33/Ser37 peptide representing part of the β-catenin phosphodegron sequence (DpSGφXpS) that binds to β-TrCP. This approach led to the identification of four similar compounds, including NRX1532 which demonstrated 10-fold cooperativity between β-catenin and β-TrCP [44]. Structural studies on a more soluble analogue, NRX-1933, revealed that the compound bound at the β-catenin: β-TrCP interface with minimal impact on the conformation of either β-catenin or β-TrCP.

Of course, however potential molecular glue degraders are identified, it is critical that these compounds are profiled in assays designed to monitor degradation of the target protein. Fortunately, due to the prominence of PROTAC drug discovery over recent years, there are a range of potential approaches to monitor both ubiquitination and degradation available for application to putative molecular glues. For further information on targeted protein degradation approaches see the reviews by Zhao *et al.* [45]) and Jevtic *et al.* [46].

3. Why do we need molecular glues?

Molecular glues promise to be a ground-breaking strategy to target undruggable targets for cell biology and drug discovery.

Traditionally, small molecule drugs / chemical probes have been used successfully to modulate the activity of enzymes, that due to their function have well-defined cavities able to bind metabolites, ligands, and other small molecules in the cell. The most druggable protein families are in fact kinases and protein receptors such as GPCR or nuclear receptors.

However, it is estimated that only 10% of the coding genome is druggable by traditional small molecules inhibitors [47,48]. The remaining 90% includes proteins involved in protein complexes or DNA/RNA binding, that due to their function tend to have flat or protruding surfaces and have adapted to interact with other proteins or nucleic acids but not with small molecules. In addition, transcription factors and other scaffolding proteins tend to be intrinsically disordered, with regions that fold only in the presence of protein co-factors and therefore normally unavailable for small molecules binding.

Conceptually, PROTACs increased the number of druggable targets to include non-enzymatic proteins by adding protein degradation to protein inhibition as a therapeutic strategy. However, PROTACs still require two small molecule ligands, one recognising a pocket on an E3 ligase and the other binding the target of interest and therefore, they still require the target to contain a drug-binding pocket.

Molecular glues, on the contrary, exploit well the undruggable space, by creating or filling pockets only present when two proteins bind in the presence of the glue compound. This opens the opportunity for different types of modulation for traditionally undruggable targets, for example, by gluing the target to different types of effector protein.

Two main approaches have been used for the rational design of molecular glues; namely screening to recruit non-native effectors or to increase the affinity for known interactors.

Focusing on protein degradation, IMiDs have made it possible to drug transcription factors by recruiting them as neosubstrates to CRBN E3 ligase [42]; indisulam has made possible to drug an RNA binding protein RBM39 by recruiting it for degradation to DCAF15 E3 ligase complex [49].

As for increasing the affinity to native E3 ligases, Nurix molecular glues have been rationally designed to recruit β-TrCP to its native substrate β-catenin, a transcription factor, in the absence of phosphorylation

on the degron necessary for high affinity interaction with the E3 ligase. The molecular glue in this case sits in the pocket freed by lack of the phosphate group on β-catenin and it recreates the high affinity surface to β-TrCP [44].

CypA has been known for more than 30 years as the effector protein that cyclosporin glues to calcineurin to inhibit its activity [50]; however, only recently, CypA has emerged as an effector protein that can be used more widely to design molecular glue inhibitors to traditionally difficult to drug targets such as KRAS [51] The authors applied structural guided design to develop a molecular glue specific for the active state of KRAS; this glue recruits CYPA to KRAS blocking other oncogenic interactions and results in inhibition of KRAS- driven cell proliferation. Similarly, molecular glues were developed to glue CRAF to its native inhibitor protein 14–3–3 [52]. The molecular glue locks the CRAF/14–3–3 complex, even in the absence of the CRAF phosphorylation necessary to stabilise the complex with 14–3–3 in an inhibitory conformation.

Although the degrader approach remains one of the most exploited for target modulation via mono or bifunctional small molecules, increased knowledge of the functional interactome of undruggable targets should provide further hypotheses on how to modulate their activity. CRISPR-functional genomics has provided novel information on relevant targets/ pathways in essentiality screen, now available on hundreds of cell lines (depmap.org). However, identification and validation of proteins interacting with the target of interest to formulate hypotheses for functional molecular glue still remains difficult and lengthy. Most of those interactions are redundant and therefore effects are not always clear in CRISPR KO studies based on viability and require combinatorial CRISPR screens or more complex readouts.

Affinity purification coupled to mass spectrometry (AP-MS) approaches have been widely used to map Protein – Protein interactions, generating a wealth of information on interactors of the target of interest. More recently, proximity labeling coupled to MS (PL-MS, such as BioID) has been used to map PPIs with higher specificity (see sections below on IP and proximity labelling). Moreover, PL-MS technologies can be applied directly in cells and have the ability to identify low affinity and transient interactions. In addition, advances in proteomic methodologies such as chemoproteomics, phosphoproteomics, quantitative MS-based proteomics (SILAC, iTRAQ) are increasing our ability to characterise modulation of the human interactome and how it is rewired upon drug treatment [53].

Focused CRISPR KO screens in arrayed format coupled to target localisation or specific target-activity readouts, can then validate the functionality of the target interactome, suggesting hypothesis-directed approaches for rational discovery of molecular glues.

Below, we describe a range of assays that will facilitate the development of molecular glues exploiting those functional interactions.

4. Screening for molecular glues

Molecular glues can revolutionise our ability to modulate disease-relevant proteins, but their rational development needs a novel platform with screening approaches and chemical design focusing on target and effector and not solely on the target, as is the case with classical drug development.

Below we review several cell-free and cellular screening approaches that focus on detection of small molecules acting as molecular glues For further information regarding screening approaches see the review by Domostegui *et al.* [54].

4.1. Biochemical screening approaches

Biochemical assays that detect protein-protein interactions can identify compounds that stabilise or induce the interaction between a target protein and an effector protein partner, such as an E3 ligase, protein chaperone or scaffold protein.

Molecular glues have been identified using a range of these biochemical approaches, which are employed to monitor protein proximity or binding, either directly or indirectly. Usually, these methods require prior knowledge of the target and potential effector protein(s). Some of these methods are modifications to existing technologies, which are aligned with high-throughput experiments. Others are more bespoke approaches allowing screening for effects across numerous targets. Below we will exemplify these different approaches.

4.1.1. DEL glue screening

DNA Encoded Library (DEL) Screening is an affinity selection process that can screen potentially trillions of compounds [55,56]. Each compound within a collection is individually tagged with a unique DNA sequence which can then be amplified and serve as compound identification barcodes. Often, a split and pool synthesis approach is used to encode the library. Briefly, a starting building block is tagged with a DNA sequence, and following the addition of a second building block, the DNA tag is extended. Typically, compounds of up to three or four building blocks can be assembled, with each step yielding mixtures of thousands of different tagged building blocks leading to vast DNA encoded library sizes. DEL screening uses high protein concentration (typically in the μM range) to drive the equilibrium towards the formation of ligand-bound complexes, with the readout related to the abundance of the target-ligand complex.

DEL screening, Fig. 3A, has been applied to identify molecular glues for the target BRD9 with the E3 ligase, VHL. In this approach, the BRD9 protein was immobilised, and 2 different experimental methods were used to identify potential small molecule glues. Either the library was pre-incubated with the immobilised BRD9 (allowing formation of binary complexes), or the library was added first to the VHL, and subsequently added to the immobilised BRD9 (allowing formation of ternary complexes). To control for non-specific binding, the library was added to the immobilisation matrix in the absence of BRD9. Following 3 washes, the protein was denatured to release the binders and the DNA barcodes PCR-amplified and sequenced to identify the binding compounds. To calculate the enrichment, the counts for the binary binding were divided by the control, and the counts for the ternary binding were divided by the binary binding, yielding the ratio of ternary enrichment over binary enrichment. A higher presenter ratio suggested that the binding of the compound to BRD9 was more dependent on the presence of VHL, implying a higher degree of cooperativity. This presenter ratio was used to select small molecules with various degrees of cooperativity in their ternary complex binding with BRD9 and VHL. The screening results yielded a range of different binding behaviour which was dependent upon the nature of the linker between the VHL ligand and the triazine, BRD9-targeting core. However, all the identified enriched connectors were relatively short and cyclic in nature [57].

4.1.2. Tethered fragment screening

Tethering relies on the formation of a reversible covalent bond between a fragment and the protein of interest [58]. If the target protein contains a cysteine residue near the targeted site, it can be used directly, or site-directed mutagenesis can be used to introduce the cysteine residue at a convenient position. The protein is then incubated with a library of disulfide-containing fragments under partially reducing conditions. Fragments with inherent affinity for the protein will bind close to the cysteine and the thiol-disulfide equilibrium will favour those fragments, relative to non-binding fragments. Thus, binding fragments are effectively selected by the protein and are enriched in the detection. This allows for the detection of fragments that may otherwise have a low binding affinity.

4.1.3. Mass spectrometry detection

It has been previously shown that a natural or engineered cysteine residue in close proximity to a ligandable pocket can be used to facilitate the discovery of compounds that stabilise protein-protein interactions [59]. This disulphide tethering approach has been used to identify

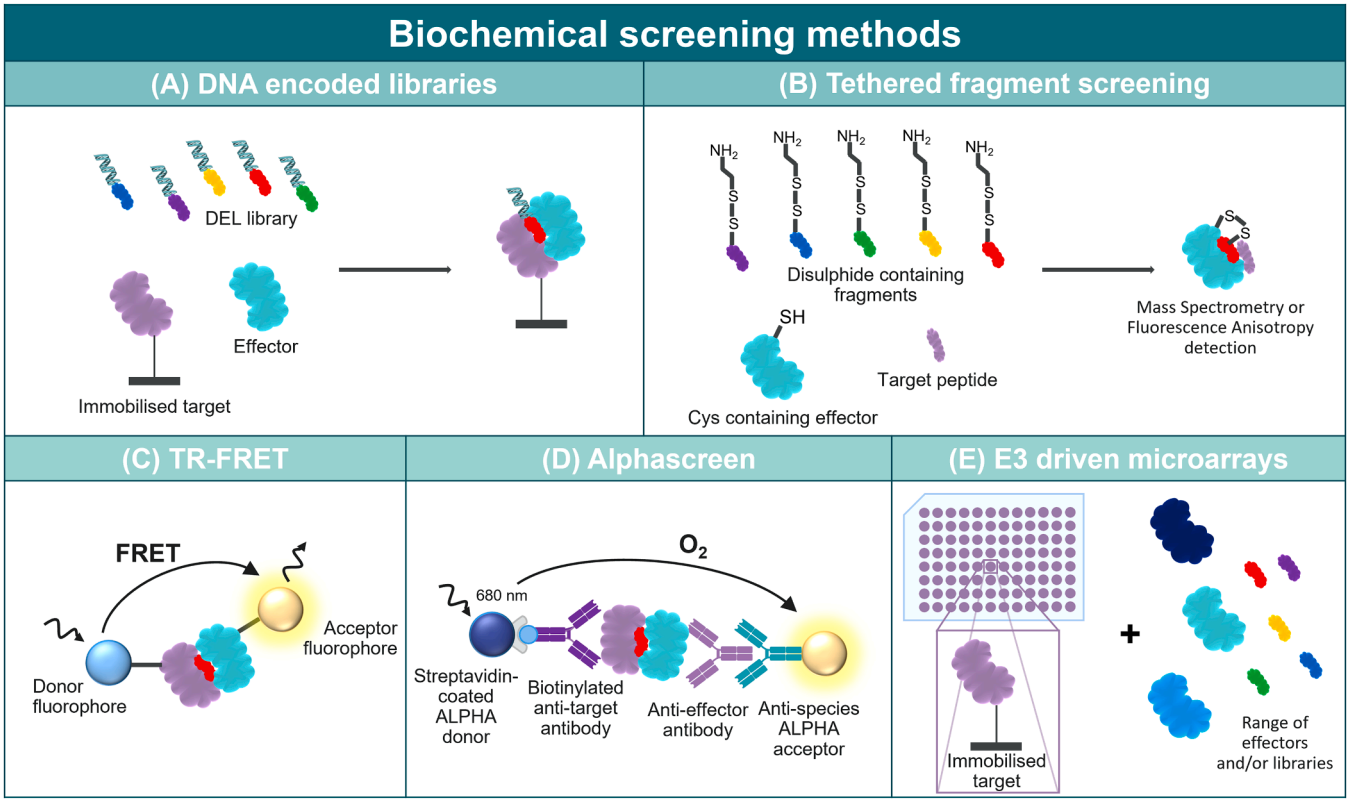

Fig. 3. Biochemical screening technologies

A range of biochemical methods have been used to screen for molecular glues. (A) DNA encoded libraries involves screening compounds tagged with a unique DNA identification barcode to find those which promote an interaction. (B) In tethered fragment screening, an effector protein with an exposed cysteine residue is incubated with a target peptide and a library of disulphide containing fragments. Fragments with inherent affinity for the protein will bind close to the cysteine and will be enriched in the detection, usually by MS or FA. (C) In TR-FRET, the target protein is tagged with a donor fluorophore and the effector with an acceptor fluorophore. Close proximity of interaction partners causes FRET from the donor to acceptor fluorophore. (D) AlphaScreen is a bead-based approach which relies on binding of two proteins of interest to specific beads. Close proximity of interaction partners causes energy transfer from one bead to the other and the production of a chemiluminescent signal. (E) In E3 driven microarrays, the target protein is immobilised to a surface and a range of effector binding partners in the presence and absence of putative glue molecules are introduced to test for any interaction.

compounds stabilising the interaction between 14−3−3 and ERα [60]. Here, a cysteine-containing isoform of 14−3−3 was incubated with a disulphide containing fragment library either in the presence or absence of a phosphopeptide from ERα. The protein-fragment conjugate then was characterised by intact protein mass spectrometry (MS).

4.1.4. *Fluorescence anisotropy detection*

In a variation to the tethering approach, a native cysteine residue found in the 14−3−3 binding motif of the Estrogen Related Receptor gamma's DNA binding domain (ERRγ DBD) was used to build a phosphopeptide probe that could be used in a FA screening approach [61]. The ERRγ-phosphopeptide was labelled with a fluorescent probe, and the fragment-induced binding of phosphopeptide to 14−3−3 was monitored by an increase in FA, Fig. 3B. In this screening method, the fluorescently labelled cysteine containing peptide was initially weakly bound to 14−3−3, demonstrated by a low FA ready (low anisotropy). Stabilization of the protein− peptide complex (increased binding of 14−3−3 to the labelled peptide) by compound binding following incubation with the disulphide library, under reducing conditions, was demonstrated by an observed increase in anisotropy [62].

Another example of the use of FA (or fluorescence polarisation) has been provided by Simonetta *et al.* [44]. These authors identified and optimized molecular glues that enhanced the decreased interaction between β-TrCP and mutant β-catenin. The β-catenin phosphodegron, including residues Ser33 and Ser37, is recognized by β-TrCP. However, when mutated, this leads to β-catenin stabilization and facilitating oncogenic transcription. The authors focused on the Ser37 as a hot-spot mutation, using a pSer33/Ser37 phosphodegron peptide with weak binding to the E3 to establish a robust high-throughput biochemical screen. Using this method, they were able to evaluate a library of 350,000 small molecules. This screen identified several compounds, including NRX-1532, which were able to increase the binding affinity between β-TrCP and the mutant β-catenin peptide. In establishing this screen, it was demonstrated that the double phosphorylated peptide (pSer33/pSer37) binds with a binding affinity of 2 nM, whereas the non-phosphorylated peptide (Ser33/Ser37) binds with notably lower affinity (>100 μM) to β-TrCP. The singly phosphorylated peptides (Ser33/pSer37 or pSer33/Ser37) demonstrated a 10,000- or 300-fold loss of affinity for β-TrCP respectively, when compared to the double phosphorylated peptide. Measurement and comparison of the binding affinities of these peptides allowed the selection of the pSer33/Ser37 peptide to facilitate the development of the screening assay. Use of the much weaker affinity peptides lacking Ser33 phosphorylation would not have permitted screen development.

4.1.5. *Time resolved Förster resonance energy transfer (TR-FRET)*

The underlying principle for FRET-based approaches is the transfer of energy between a donor and an acceptor fluorophore. When these moieties are near each other, excitation of the donor by incident light leads to transfer of energy to the acceptor. The acceptor then emits a fluorescence signal at a specific wavelength. Thus, molecular interactions between molecules can be monitored by attaching a fluorescent label to each partner and measuring the degree of energy transfer [63]. Using an immunomodulatory drug (IMiD) derivative,

ALV-02–146–03, Wang *et al.* demonstrated engagement with CRBN, before building a CRBN:Helios dimerization assay based on TR-FRET [64]. They used this approach to screen a small, focused library of compounds. This screen, following several rounds of optimization led to the design of ALV1, which has been shown to induce the CRBN-dependent degradation of Helios, Fig. 3C.

In another example, to screen for compounds stabilising the low-affinity interaction between the ubiquitin binding site of CDC34A and ubiquitin, a TR-FRET assay was developed using an N-terminal His-tagged version of CDC34A. This protein was recognized by an anti-His_6 antibody coupled to Tb^{3+} with the FRET pair formed using an N-terminal cysteine mutant of ubiquitin labelled with 5′-iodoacetamide-fluorescein. This approach yielded a screen with Z' value of 0.78, generated using the stabiliser CC0651 as a positive control and dimethyl sulfoxide (DMSO) as the negative control. This allowed the screening of a focused library which identified an isonipecotamide, which although weaker than the control CC0651, contained a biphenyl moiety, showing analogy to the dichlorobiphenyl group present in CC0651 [65].

4.1.6. AlphaScreen

AlphaScreen (ALPHA for Amplified Luminescent Proximity Homogeneous Assay) is a bead-based approach used to study interactions between molecules in a microplate format. The principle relies on binding the two proteins of interest to specific beads. When an interaction between the two molecules occurs, this results in the close proximity of the two beads, which upon laser excitation of the donor bead, allows ambient oxygen to be converted to a more excited singlet state, which then diffuses to react with the acceptor bead generating a chemiluminescent signal [66]. This methodology has been employed to measure the interaction between CDK12KD/CCNKΔC and DDB1 Here a biotinylated FLAG-Avi-DDB1 construct was used with 6HisCDK12KD/6HisCCNKDC (either WT or G731 mutants), allowing demonstration of an improved AlphaScreen signal / interaction in the presence of HQ461, with an apparent EC_{50} of 1.9 μM. The AlphaScreen assay was also used to show the competitive effect of THZ531 that inhibits the HQ461-dependent formation of the CDK12KD/CCNKΔC/DDB1 complex [67], Fig. 3D.

4.1.7. E3 driven microarrays

In an alternative approach, described by Novartis in an unpublished communication,[1] protein microarrays [68] were used to screen for compound-induced interactions between VHL and thousands of proteins, demonstrating an E3 ligase driven-target agnostic approach to molecular glue discovery. A protein microarray is a high-throughput approach that can be used to monitor the interactions of many proteins in parallel. The method consists of a support surface to which an array of capture proteins is bound. This subsequently allows the introduction of a binding partner in the presence and absence of putative glue molecules and interactions to be monitored, Fig. 3E. Several small molecules were found to stabilise the interaction between VHL and CDO1, a protein involved in the regulation of cysteine metabolism. These compounds were subsequently demonstrated to promote poly-ubiquitination and the proteasomal degradation of CDO1. In another example of the use of microarrays, a human proteome microarray was used to identify a natural product-derived molecular glue for targeting E2F2 degradation [50].

4.2. Cellular screening approaches

Historically, the discovery of molecular glues happened by serendipity rather than by applying a designed strategy [4]. Predominantly, molecular glues have been identified with cellular screens based on viability/toxicity [69] or other phenotypic screens based on reporter assays [67], and their mode of action as glues discovered only subsequently [70]. Several cellular methods have emerged in the last few years in the quest for molecular glues; we describe these approaches below (Fig. 4).

4.2.1. Viability/ toxicity assays

Viability assays measure metabolic activity in cells. The MTT (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide) assay for instance, is based on the reduction of a yellow tetrazolium salt, MTT, to purple formazan by metabolically active cells. The absorbance of formazan can be measured in a microplate reader [71]. Cell toxicity assays in contrast measure changes in membrane integrity that occur as a result of cell death. Such an assay, Cell tox green, uses a dye that is excluded from viable cells but preferentially stains the dead cells' DNA [72]. When the dye binds DNA, its fluorescent properties are substantially enhanced. Viable cells produce no appreciable increases in fluorescence. Therefore, the fluorescent signal produced by the dye binding to the dead-cell DNA is proportional to cytotoxicity. Indisulam was originally found as an anti-tumour drug using an MTT viability assay [69]. Its mechanism of action as a glue to DCAF15 and RBM39 was established in 2017 [49]. They generated indisulam resistant HCT116 clones and identified missense mutations by exome sequencing. RBM39 gene was mutated in 3 clones. Transient transfection of HCT-116 cells with either WT or mutated RBM39 followed by treatment with indisulam showed that RBM39 mutations conferred resistance to indisulam. Western blot analysis showed indisulam toxicity requires RBM39 degradation. Anti-FLAG purification from lysates of CRISPR engineered RBM39–3xFLAG HCT116 cells treated with indisulam followed by tryptic digestion and MS analysis revealed DCAF15 as the recruitment partner.

Another example is Asukamycin, which was first discovered as an antibiotic [73] before Isobe *et al.* hypothesized that it could be a molecular glue due to its multiple reactive sites [32]. They showed its antiproliferative and anti-survival properties in breast cancer cell lines and used activity-based protein profiling chemoproteomic to identify its binding targets, UBR7. Further proteomic analysis on anti-FLAG pulled-down eluate from FLAG–UBR7 expressing cells treated with asukamycin identified the tumor-suppressor p53 (TP53) as a binding partner. A different approach was used by Stabicki *et al.* to discover another Cyclin K glue, CR8 [74]. They mined databases to correlate cytotoxicity of clinical and preclinical small molecules with the mRNA expression levels of E3 ligase components across hundreds of human cancer cell lines. They then verified the dependency by running a CRISPR-mediated inactivation of the identified E3 ligase component and checking if this rescued the respective drug-induced toxicity. They identified a correlation between CR8 toxicity and mRNA level of DDB1. They ran a quantitative proteome-wide MS to check protein abundance after cells treatment with CR8 and found that Cyclin K was the only protein affected. The degradation of Cyclin K could be rescued by inhibition of the proteasome, neddylation or E1 ubiquitin-activating enzyme. They then ran an E3 ubiquitin ligase-focused CRISPR–Cas9 resistance screen and identified DDB1, CUL4B, RBX1, NEDD8, NAE1 and UBA3 as the key proteins involved. *In vitro* co-immunoprecipitation with recombinant proteins showed that addition of CR8 led to the formation of a stochiometric complex between the kinase domain of CDK12, Cyclin K and DDB1. They further established that Cyclin K degradation is part of the observed CR8 toxicity and is dependent on DDB1.

Recently, Mayor-Ruiz *et al.* established a rational screening strategy for the discovery of molecular glues degraders [43].They engineered a cell line with impaired E3 ubiquitin ligase activity (neddylation deficient) and compared the drug sensitivity of 2000 cytostatic and cytotoxic compounds between these cells and the E3-proficient parental wild type: differential viability lead to identification of compounds dependent on E3 activity and therefore potential molecular glue degraders. They prioritised 4 chemical scaffolds, dCeMM1–4, and performed a

[1] S. L. Schreiber presented at the conference "Induced Proximity-Based Drug Discovery Summit", June 2021.

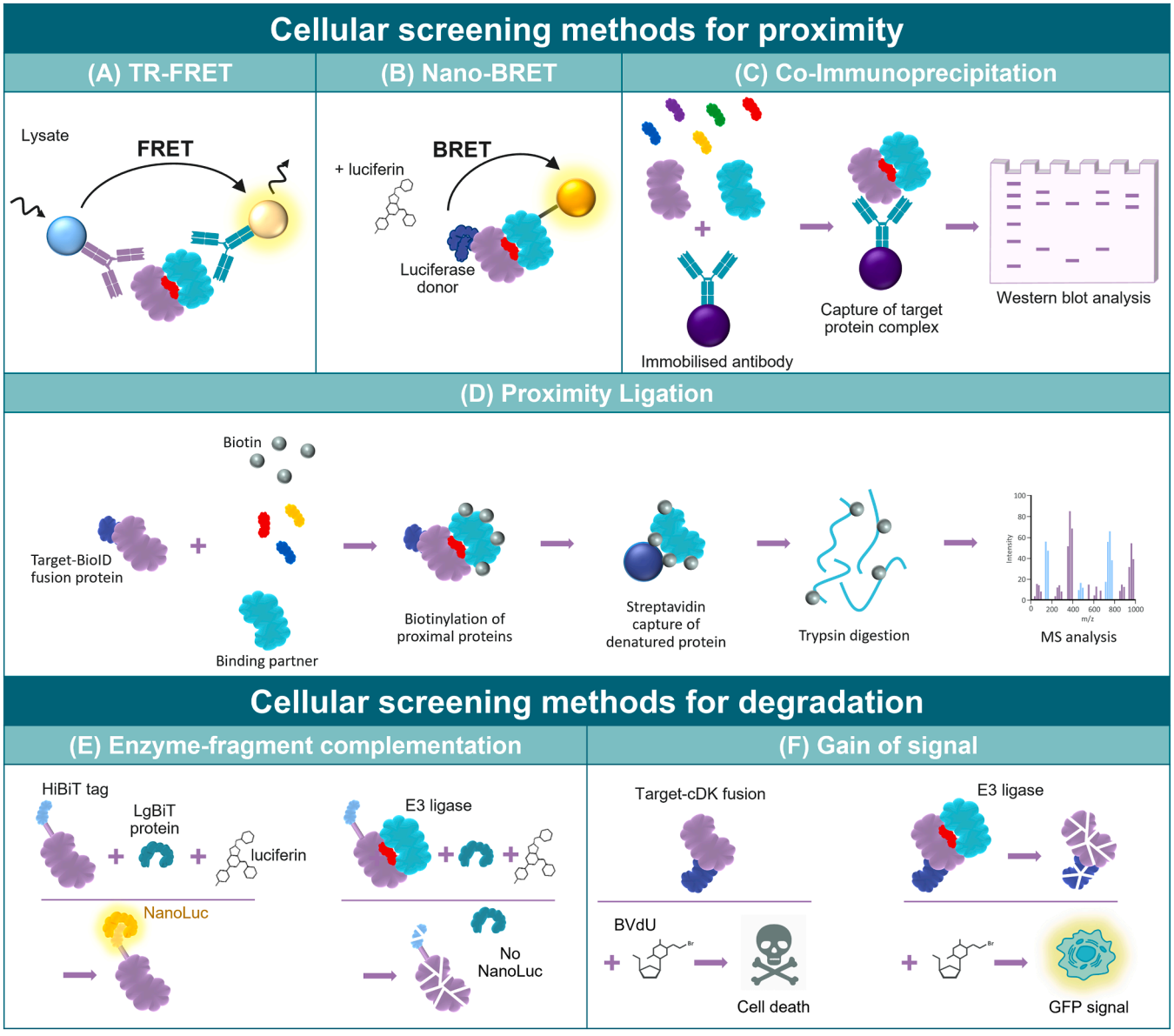

Fig. 4. Cell-based screening technologies.
A range of methods have been used to screen for molecular glue-induced proximity and degradation. (A) In TR-FRET, cells are lysed and labelled antibodies specific for each interaction partner are added. Close proximity of interaction partners causes FRET from donor to acceptor fluorophore. (B) In Nano-BRET, interacting partners are expressed as fusion proteins with either a bioluminescent protein donor, e.g., Luciferase, or a fluorescent protein acceptor. Close proximity of interaction partners causes BRET from donor to acceptor. (C) In co-immunoprecipitation, binding partners and compounds are added to the immobilised target, then complexes are immunoprecipitated and analysed by SDS-PAGE. (D) In proximity ligation assays, the POI is expressed as a fusion protein with a promiscuous biotin ligase, e.g., BioID. Upon addition of biotin, proteins proximal to the POI are biotinylated, and subsequently captured with Streptavidin beads prior to MS analysis. (E) Enzyme fragment complementation relies on tagging the POI with a small tag, e.g., HiBiT, that can interact with its complementary subunit to form Nanoluciferase. Upon degradation of the POI, no luciferase signal is generated. (F) In this gain of signal degradation assay, in GFP-expressing cells, the POI is expressed as a fusion protein with deoxycytidine kinase (dCK), which converts the non-natural nucleoside 2-bromovinyldeoxyuridine (BVdU) into a poison. Upon degradation of the POI, cell viability is retained and GFP can be measured. It should be noted that co-immunoprecipitation and proximity ligation are lower throughput screening methods.

focused CRISPR-Cas9 resistance screen using a sgRNA library covering all known cullin-RING ligases (CRL) and associated regulators. This showed the dependency of dCeMM1 efficacy with DCAF15. Quantitative proteomics on cells treated with dCeMM1 revealed that RBM39 was destabilized exclusively. Finally, they showed that CRISPR-induced frameshift mutations in DCAF15 rendered cells insensitive to dCeMM1 and rescued RBM39 degradation establishing dCeMM1 as a RBM39 molecular glue degrader. Using a similar strategy with dCeMM2–4 they showed those to induce cyclin K degradation by enhancing the CDK12-cyclin K interaction with DDB1.

GSPT1/2 degraders were identified by screening a focused library of Thalidomide derivatives in five patient-derived cell lines using cell viability assay, CellTiter-Glo [75]. They identified several potent hits (EC50 < 1 μM), some active in all five cell lines while others showed selectivity, hinting to different mechanisms of action. They confirmed a CRBN-dependent mechanism of action by running ligand competition experiments in the presence of high concentrations of lenalidomide and by running cell viability assay in CRBN knockout cells. They showed degradation of neosubstrate GSPT1 but not IKZF1 by immunoblotting for some hits and induction of apoptosis in a Caspase-Glo assay correlating with the antiproliferative potencies. Multiplexed MS-based proteomics analysis showed that one hit, compound 6 (SJ6986) selectively

reduced the abundance of GSPT1 and GSPT2 over ~9000 proteins upon treatment in MV4–11 cells, an acute leukaemia cell line. This is the first GSPT1 degrader reported with bioavailability.

CC-885, a novel CRBN modulator was discovered by screening a library of ligand analogues in a proliferation assay in a panel of human cancer cell lines [76]. By CRISPR knockout in 293HEK cells as well as AML cell lines NB-4, MOLM-13 and OCI-AML2 they showed that the anti-proliferative effects of CC-885 were CRBN-dependent. They generated a Flag and Hemagglutinin (HA) tagged-CRBN HEK293 stable cell line and performed immunoprecipitation followed by MS and identified GSPT1 as a CC-885-dependent CRBN substrate. They showed that CC-885 treatment of HEK293 cells resulted in CRBN-dependent ubiquitination and subsequent proteasomal degradation of GSPT1. By crystallisation studies they showed that CC-885 creates an interaction hotspot on the CRBN surface for direct protein–protein interactions with the substrate, GSPT1, resulting in a gain-of-function neomorphic activity.

More recently, King, *et al.* utilised a phenotypic anti-proliferation screen coupled with chemoproteomics in the identification of the covalent molecular glue EN450 which promotes degradation of NFKB1 [77]. The authors screened a library of 750 cysteine-reactive covalent ligands for anti-proliferation activity using Hoescht staining as a measure of cell viability.

4.2.2. Reporter gene assays

Reporter gene assays measure gene expression by linking a reporter gene whose product can be easily detected and quantified to the regulatory sequence of a transcription factor of interest. The reporter gene then acts as a surrogate for the activity of the transcription factor. Common reporter genes include beta-galactosidase [78], luciferase [79], beta-lactamase [80], alkaline phosphatase [81], and GFP (green fluorescent protein) [82]. Absorbance, fluorescence, or luminescence detection methods are used typically to measure expressed reporter gene protein, Fig. 4E. HQ461 was identified as a hit from a NRF2 high throughput screen using a reporter gene assay in A549 cells [67]. A pooled genome-wide CRISPR-Cas9 knockout screening on A549-HQ461 resistant cells showed that HQ461 toxicity requires DDB1, RBX1 and other regulators of the ubiquitin proteasomal degradation system. Following the same strategy as previously published for indisulam, they generated HQ461 resistant HCT116 clones and identified missense mutations in CDK12 by whole exome sequencing. Transient transfection of HCT-116 cells with either WT or mutated CDK12 followed by treatment with HQ461 showed that CDK12 mutations conferred resistance to HQ461. Western Blot studies revealed that HQ461 induces degradation of Cyclin K, and this can be inhibited in the presence of either a proteosome or a neddylation inhibitor. CRISPR-Cas9 knock-in of FLAG in endogenous CDK12 locus in A549 cells and immunoprecipitation from lysates of cells treated with HQ461 showed CDK12 associated with Cyclin K in an HQ461 independent manner but HQ461 mediated recruitment of the CDK12-Cyclin K complex to DDB1. Mutant CDK12 failed to recruit DDB1. Further pulldown established that HQ461 functions as a glue between CDK12/Cyclin K and DDB1.

4.2.3. TR-FRET assays

Although TR- FRET assays have been mainly used to screen for PPI inhibitors [63] they have been successfully used for molecular glues discovery, Fig. 4A. Tang *et al.* published a cell lysate TR-FRET assay to screen for modulators of SMAD4-SMAD3 interactions [83]. By using cell lysates, they avoided the need for purified protein components. They were able to miniaturise it to a 1536-well format and showed that their platform could be adapted for the discovery of both small-molecule PPI inducers and inhibitors. In 2022, Payne *et al.* developed an endogenous cellular TR-FRET assay measuring both target engagement and degradation to profile PROTACs of BRD4 [84]. It relies on the use of an antibody to BRD4, a labelled anti-species nanobody (nano-secondary) as donor and fluorescently labelled BRD4 inhibitor JQ1 tracer as acceptor. This assay uses unmodified cell lines and could be deployed to other proteins of interest for which a specific antibody is available and a tracer, even non-specific, can be identified [83]. They were able to miniaturise it to a 1536-well format and showed that their platform could be adapted for the discovery of both small-molecule PPI inducers and inhibitors.

4.2.4. NanoBRET assays

NanoBRET (Nanoluciferase Bioluminescence Resonance Energy Transfer) is a detection technique used to measure binding events, signaling pathways or receptor trafficking in live cells [85]. In BRET, the energy transfer takes place between a luciferase donor and a fluorophore acceptor, Fig. 4B. In 2023, Nowak *et al.* used a NanoBRET CRBN occupancy assay to study the pharmacology of CRBN-based degraders [86]. The assay measures the dose-dependent reduction in BRET signal following displacement of a fluorescent CRBN tracer from NanoLuc-tagged CRBN in live cells. They screened 14 thalidomide derivatives to establish structure-activity relationships. In particular, they focused on 2 neosubstrates, IKZF1, a transcription factor and GSPT1, a translation termination factor. They also deployed degradation assays for both proteins to quantify the degradation achieved by each compound. Their data provide direction for the design of degraders devoid of GSPT1 activity thereby avoiding broad cytotoxicity.

4.2.5. Protein degradation: enzyme fragment complementation (EFC) assays

EFC is based on two recombinant β-galactosidase (β-gal) enzyme fragments that function as an enzyme acceptor (EA) and an enzyme donor (ED). Separately, the fragments are inactive, but when combined, they form an active β-gal enzyme that hydrolyzes its substrate to produce a chemiluminescence signal [87]. These assays were developed by DiscoverX years ago and have various applications, one being an adaptation as PathHunter degradation assays, Fig. 4E. In this setting, the protein of interest is tagged with the small peptide fragment while the large β-gal fragment is included in the detection reagent added to the lysis buffer at the end of the incubation time.

In 2020, Hansen *et al.* developed an Aiolos and GSPT1 degradation assay to search for CRBN modulators (CELMoDs) for the treatment of relapsed and refractory multiple myeloma [88]. They performed a phenotypic screen (cell viability) using a lenalidomide-resistant H929 cell line followed by Aiolos protein degradation screen. They selected compounds showing *in vitro* selectivity ratio between the viability in PBMCs versus the resistant cells and explored the SAR around a specific compound hit series. They optimised the degradation efficiency of this series defined as the level of protein remaining over a time course of measurement. When plotting the level of protein remaining as a percent of control versus the compound concentration, the minimum protein remaining (Ymin) depicts the depth of degradation of the compound. The minimum protein remaining is reached when degradation and protein synthesis have reached equilibrium. Comparing Ymin across compounds evaluated in the same conditions allows their ranking on degradation efficiency. This method led to the identification of CC-92, 480 as the first CELMoD specifically designed for high efficiency and rapid protein degradation kinetics to enter clinical development.

PathHunter was used by Bonazzi *et al.* in the discovery of a molecular glue promoting degradation of the zinc finger transcription factor IKZF2 [89]. Zinc finger transcription factors are challenging targets because they are largely unstructured and do not have ligand binding sites. However, closely related TFs (IKZF1 and IKZF3) have been targeted by IMiDs, which bind to CRBN E3 ligase and then recruit IKZF1/3, inducing degradation [90,91]. With this knowledge, Bonazzi *et al.* used the IKZF1 molecular glue degrader pomalidomide as a starting point for SAR. Pomalidomide-bound CRBN binds to a glycine β-hairpin in zinc finger 2 of IKZF1. The zinc finger β-hairpins of IKZF1 and IKZF2 differ by a single amino acid, and this mediates the selectivity of the pomalidomide for IKZF1. Therefore, the authors aimed to find a compound that can

accommodate the histidine in IKZF2 zinc finger 2. They performed SAR using a PathHunter cellular degradation assay with ProLabel-tagged IKZF2 or IKZF1 to identify compounds that promote degradation of IKZF2 and not IKZF1. They confirmed that degradation was dependent on CRBN using a CRBN knout-out cell line and reported compound DKY709 as a selective molecular glue degrader of IKZF2.

NanoBiT and HiBiT systems are additional examples of enzyme-fragment complementation technology that rely on the tagging of a protein of interest (POI) with a small subunit of an enzyme that can interact with its complementary subunit to form a luminescent complex, Fig. 4E. Similar to PathHunter, NanoBiT and HiBiT systems are distinguished by the small peptide tag used to create the POI-fusion, usually only 11 amino acids [92,93]. The smaller size of the peptide tag is attractive both because it is amenable to CRISPR-Cas9 genome editing of endogenous proteins, and because it is less likely to interfere with the normal biological function of the POI within the cell. The method was first developed using the engineered luciferase NanoLuc, a 19 kDa enzyme derived from a deep-sea luminous shrimp [92]. The NanoBiT reporter system uses an 11 amino acid peptide (SmBiT) and an 18 kDa polypeptide (LgBiT), each fused to a target protein. The two subunits weakly associate (Kd $>$ 100 μM), such that their complementation is dictated by the interaction of the target proteins. The same group later developed the HiBiT reporter system, which utilises a high affinity (Kd = 700 pM for LgBiT) 11-amino acid peptide tag enabling luminescence quantification [93]. In this system, the HiBiT-tagged POI can be quantified following cell lysis and addition of LgBiT and the NanoLuc substrate furimazine. The group showed that luminescence signal correlated linearly with protein levels in cells, and that signal could be detected at concentrations as low as 1 amol, corresponding to around 10 molecules per cell [92,93].

HiBiT technology has been used in molecular glue discovery, for example Shergalis, Marin *et al.* used a HiBiT-BRD4 degrader assay to identify the ligase involved in BRD4 degradation upon treatment with the molecular glue compound "1a" [94–97]. Using a CRISPR/Cas9 knockout library of 1158 genes in the ubiquitin proteosome system, they found that silencing of DCAF-16, a substrate recognition component of the Cul4 ligase complex, rescued BRD4-HiBiT signal upon treatment with 1a.

Hanzl *et al.* developed a live-cell ligase tracing screen to identify a novel DCAF15 dependent molecular glue degrader [98]. They screened in a target-agnostic, ligase-specific manner, and identified dRRM-1 as a distinct degrader of splicing factors RBM39 and RBM23. The authors assembled a compound library of 10,000 sulphonamides, including 8000 aryl sulphonamides, leveraging the known molecular glue space for DCAF15, and used HEK-293 *DCAF15*$^{-/-}$ cells over-expressing HiBiT-tagged DCAF15 to screen the library for degraders.

4.2.6. Protein degradation: gain of signal assays

One limitation of the degradation assays where activity is measured by a decrease in signal is that the screening output is contaminated with compounds inducing cytotoxicity. In 2021, Koduri *et al.* engineered a gain of signal assay to look for degraders of IKZF1 [99]. They fused a modified version of deoxycytidine kinase (dCK), an enzyme that converts the non-natural nucleoside 2-bromovinyldeoxyuridine (BVdU) into a poison, to IKZF1 in 293 cells, Fig. 4F. Thus, protein degradation prevents formation of the toxin and gives a positive selection readout of resistance. A chemical screen of previously uncharacterised IMiD-like molecules from the literature in the presence of BvdU lead to the identification of MI-2–61 and MI-2–197 as molecular glue degraders.

4.3. MAPPIT

The mammalian protein-protein interaction trap (MAPPIT) is a two-hybrid system used for the study of PPI based on the JAK-STAT signal transduction pathway. The bait and prey proteins (target of interest and effector protein of choice) are linked to deficient cytokine receptor chimeras. When a PPI occurs between the specific bait and prey chimeras, the activity is restored and the JAK-STAT signaling results in reporter gene expression controlled by a STAT3-responsive promoter [100]. This system has been expanded to include various adaptation such as reverse MAPPIT to identify modulators of PPIs [101,102], MASPIT (mammalian small molecule-protein interaction trap) to analyze interactions between compounds and protein [103], array MAPPIT for high throughput screening of arrayed proteins [104]. A similar method called MaMTH for mammalian-membrane two-hybrid assay has been developed by Petschnigg *et al.* [105]. In this assay a membrane bait protein is tagged with the C-terminal half of ubiquitin (Cub) and a chimeric transcription factor (TF), and a cytosolic or membrane-bound prey is tagged with the N-terminal half of ubiquitin (Nub). Upon interaction of bait and prey, pseudo ubiquitin is formed, which is recognized by cytosolic deubiquitinating enzymes, resulting in cleavage of the TF and expression of a luciferase reporter gene.

4.4. Tango and split TEV assays

Another promising approach is the use of split TEV or Tango assays. In 2006, Wehr *et al.* developed the Split-TEV technique to monitor PPI in mammalian cells [106]. The method is based on functional TEV-protease fragment complementation and subsequent proteolytic activation of reporters, hence combining the advantages of split enzyme and reporter gene–mediated assays. They showed this method could be used to study receptors dimerization, receptor signaling, inducible cytosolic PPIs or receptor tyrosine kinases (RTKs) activation. They also showed that split-TEV technology could be used to measure the interaction of FKBP and FRB in the cytosol when induced by the molecular glue rapamycin. They used a 'proteolysis-only' split-TEV reporter assay, where the interaction of FKBP and FRB led to TEV activity, causing the release of luciferase that can turnover luciferin to produce light. In this assay, increasing concentrations of rapamycin led to an increase in luminescence units [106]. Barnea *et al.* modified the Split-TEV method in 2008 to study three classes of receptors, namely G protein-coupled receptors, RTK and steroid hormone receptors and called it Tango [107]. They found that regulated localization within the cell was sufficient to discriminate between free and associated TEV- and transcription factor-fused partners and therefore splitting the TEV protease was not required. So, in their assay, a transcription factor is bound to a membrane receptor via a linker containing a cleavage site for a specific viral protease, TEV. Activation of the receptor recruits a signaling protein fused to the protease, interaction between the two partners results in the cleavage and release of the transcription factor that translocates to the nucleus where it activates a luciferase reporter gene. Tango assays were also used by Yan *et al.* to study membrane protein interactions [108]. These methodologies can be adapted and could be used to screen for molecular glues.

4.5. Lower throughput assays for molecular glue characterisation

Below we describe two methods, the more traditional IP, and the recent proximity labelling technology, which can be used for lower throughput characterisation of molecular glues interaction in cells.

4.5.1. IP or affinity purification assays

In co-immunoprecipitation (co-IP) assays, the target protein is bound by a specific antibody immobilized to a support [109]. Following the addition of small molecules of interest, immunoprecipitation of the target protein is undertaken and the presence of the immunoprecipitated protein and their binding partners detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, Fig. 4C. If the small molecule promotes the interaction between the two proteins, an increase in the co-IP signal is detected and indicates successful stabilisation of the protein-protein interaction by the compound as a potential molecular glue. Thalidomide, the first ever

molecular glue, originally discovered by CIBA in 1952 and then developed by Chemie Grünenthal in 1957 as a sedative and now used for i its anti-inflammatory and anti-tumour properties, was validated as a molecular glue only in 2010 by Ito *et al.* [41]. They performed affinity purification from cell lysates with thalidomide magnetic beads and identified CRBN and DDB1as proteins interacting with thalidomide. Using purified proteins, they showed that thalidomide binds to CRBN and interacts indirectly with DDB1 due to its interaction with CRBN. In 2014 several groups showed that thalidomide analogue lenalidomide, increased the activity of CRBN to ubiquitinate and degrade IKAROS protein family members [90,91,110]. The crystal structure of thalidomide in complex with CRBN-DDB1 established its action as a molecular glue [111].

4.5.2. *Proximity ligation assays*

BioID (Proximity-dependent biotin identification) is a method for proximity-labelling that relies on the *E. coli* biotin ligase BirA, which is mutated at R118G [112]. The POI is expressed in cells as a BioID fusion protein, and upon addition of biotin, biotinoyl-5′AMP is released, causing the promiscuous biotinylation of lysine residues of proteins within a distance of 10 nm [113]. Proteins are then precipitated with streptavidin-coated beads and analysed using MS, Fig. 4D. However, the labelling kinetics of BioID are slow, thus sufficient time is needed for biotin-labelling (18–24 h). This limits its application for studying transient, short-lived PPIs. Branon, Bosch *et al.* engineered two highly efficient, promiscuous mutants of BirA named TurboID and the smaller miniTurbo, enabling proximity labelling in just 10 min [114]. However, both TurboID and miniTurbo have exhibited toxicity in mammalian cells. AirID (ancestral BirA for proximity-dependent biotin identification) is another enzyme used in proximity labelling and is advantageous to TurboID due to its reduced toxicity, but it requires longer incubation-time for labelling [115]. UltraID is the latest addition to the proximity ligation enzyme arsenal; at 19.7 kDa, it is the smallest enzyme engineered thus far, and exhibits highly efficient biotinylation without the high levels of background labelling observed with TurboID [116].

Proximity ligation assays have been used in the study of molecular glues. For example, Yamanaka, Horiuchi *et al.* used AirID to analyse PPIs driven by molecular glues and PROTACs [117]. They used an AirID-CRBN fusion and detected an increase in biotinylation of SALL4 and IKZF1 upon treatment with the known molecular glue, pomalidomide.

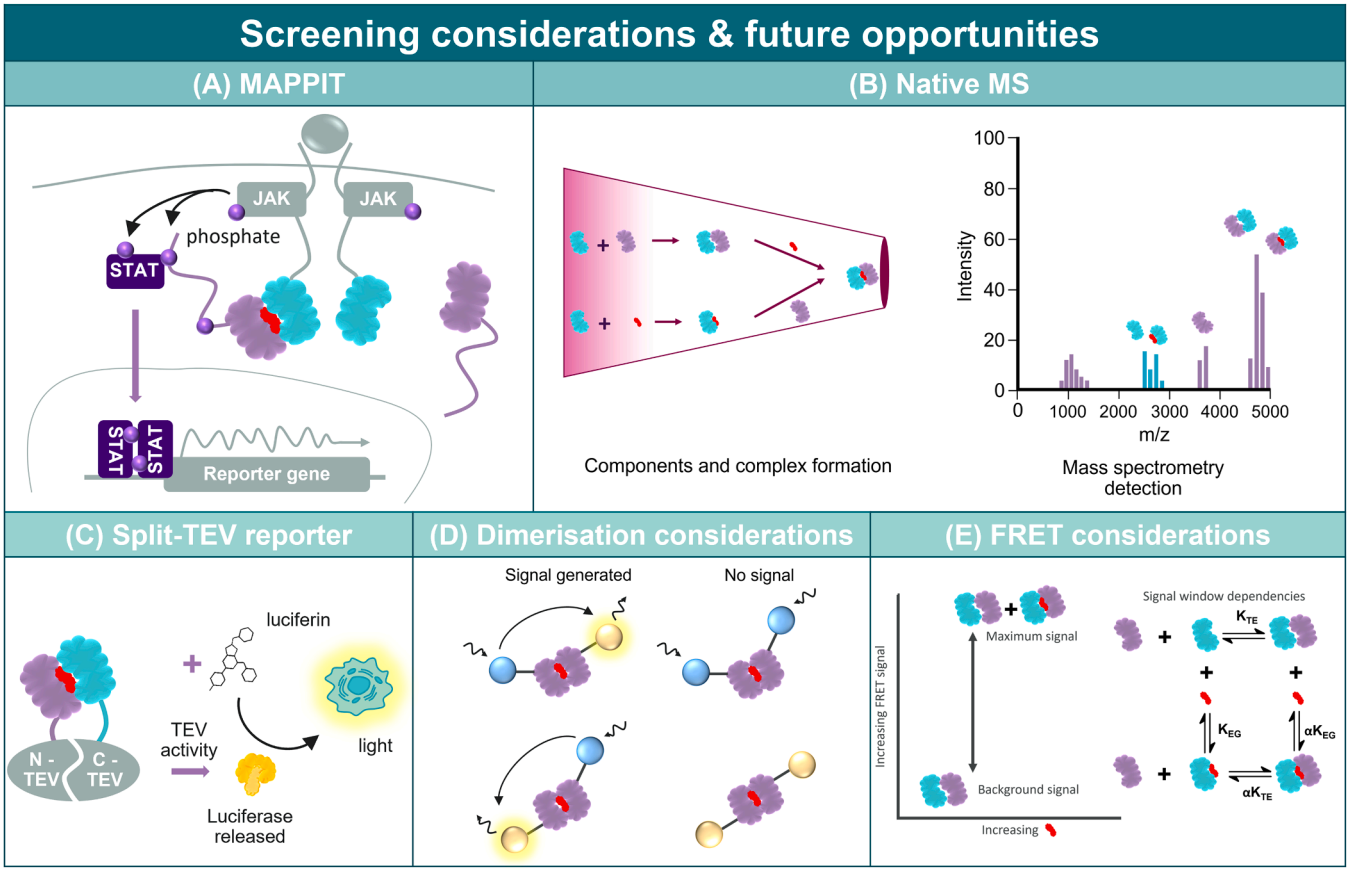

Fig. 5. Screening considerations and future opportunities.
Summary of some of the considerations when screening for molecular glues, and some examples of screening technologies that could be exploited for molecular glue screening in the future. (A) In MAPPIT, bait and prey proteins are linked to deficient cytokine receptor chimeras. When molecular glues induce proximity of bait and prey, chimera activity is restored and the JAK-STAT pathway is activated, leading to reporter gene expression. (B) In native MS, target proteins can be incubated with various effector proteins and putative glues, and the unbound proteins and/or resulting complex can be measured directly in using MS. (C) In the Split-TEV reporter system, each interaction partner is labelled with either N-terminal TEV or C-terminal TEV, upon molecular glue-induced proximity of interaction partners, TEV activity causes the release of luciferase, producing light in the presence of substrate. (D) Dimerization considerations. In FRET assays designed to identify molecular glues that induce dimerization, consideration of the complexes that may contribute to the signal will need to be given. In this example, where different batches of protein are separately labelled with donor and acceptor, only 50% of the glue induced complexes will yield a signal. (E) The signal window (difference between background signal and maximum signal) is dependent upon the concentrations of the interacting proteins, the affinity between target and effector (KTE), the affinity between glue and effector (KEG) and the cooperativity factor, α.

5. Future outlook

Biochemical approaches are potentially well suited for the identification and characterization of proximity inducing molecular glues, and their use is expected to increase in the rational screening for molecular glues. However, the downstream optimisation and validation remains challenging due to their diverse mechanisms of action and potentially complex interactions within cellular systems, and this will require a combination of approaches that will be mentioned briefly below.

We have described a range of technologies that can be used to create advanced biochemical and cellular assays for the systematic screening and validation of potential molecular glues. However, the construction of these assays needs to be carefully considered to ensure that detection of the change in protein-protein complex concentration can be adequately measured and controlled in the assay. For example, FRET-based assays may be used to investigate the ability of potential molecular glues to increase the affinity between a target and an effector protein. The signal to background in this type of assay is dependent upon the difference in concentration between the binary complex of the 2 proteins and the ternary complex including the molecular glue. This difference is dictated by the concentrations of the two partners, the intrinsic affinity between the two and the degree of cooperativity introduced by the molecular glue (Fig. 5E). Molecular glues that stabilise dimers of target protein or of a regulatory protein [33] therefore preventing normal function or activating the target may be constructed by utilising differently labelled protein batches. However, due to the potential for differently labelled protein molecules to interact, only half of the potential complexes will be detected (Fig. 5D).

The use of native MS may overcome the issues presented by FRET based methods since this is a sensitive, and label-free approach that can be used to measure the stoichiometry of macromolecular assemblies, Fig. 5B. This method uses nano electrospray ionization (nESI) in buffer conditions that maintain the structures of the analytes as they enter the gas phase [118]. It has been used to successfully identify and characterise molecular glue interactions between DCAF15 and RMB39 [119]. It has also been used to study covalent molecular glues combining the method with biophysical and structural techniques [120].

Biochemical screens to identify molecular glues that function by preventing the interaction between a target and its natural binding partner(s) by establishing a new interaction between the target and a ubiquitously available blocking protein can be configured using several formats. The choice of blocking protein should be guided by the abundance and location relative to the target as well the recruitment to the target having the desired competitive effect and little cellular consequence resulting from the decrease in free concentration of the blocking protein. This approach has been taken for targeting mutant KRAS [51], where remodelling of cyclophilin A to generate a new surface that binds to active KRAS has been achieved. The screen uses CPYA binders containing a covalent warhead offering the ability to target reactive cysteines at the binding interface of the ternary complex. Subsequent compound design and optimisation potentially allows the removal of the covalent warhead to yield non-covalent, reversible molecular glues. A similar approach has been taken for 14–3–3-based molecular glues [121]. Hence, careful consideration as to which blocking partner should be selected before running the hit finding activity, to provide the best chance of introducing new interacting surfaces with the target of interest and to increase the probability of success.

High-throughput screening platforms subsequently provide an efficient approach for identifying potential molecular glues from large compound libraries. Whichever method is selected, assays should be designed to measure the impact of test compounds on the protein-protein interaction of interest. Orthogonal methods, such as surface plasmon resonance (SPR) should be positioned to quantitatively assess changes in PPIs induced by compounds, following identification of actives in primary screening. Of course, enhancing the relevance of HTS actives and those orthogonally validated hits requires cell-based assays for the assessment of molecular glue activity within a cellular context.

As with traditional drug discovery, structural information is critical for understanding the molecular mechanisms underlying PPI modulation by molecular glues. Where proteins can be expressed and purified X-ray crystallography or cryo-electron microscopy (cryo-EM) should be exploited to explore how pockets may be filled to improve affinity or cooperativity and selectivity. Additionally nuclear magnetic resonance (NMR) spectroscopy may be used to elucidate the binding mode of potential molecular glues with their binding partners.

Alongside considerations of the screening methodology and the protein reagents to be glued, the decision around what libraries to screen in biochemical assays is equally important. This should be intricately linked to the mechanisms by which molecular glues typically operate. The modification of protein surfaces by occupying pockets on one protein or at the protein-protein interface is a predominant mechanism [27]. Thus, although many of the serendipitously identified molecular glues have been large molecules, often macrocycles, it would seem prudent to screen small fragments or small peptides which may provide the complementarity required to effectively provide new and cooperative surfaces leading to enhanced interaction. Recently, combining covalent library screening and chemoproteomics proved a successful approach for targeted protein degradation via molecular glue [122].

To effectively screen for and characterize molecular glues, a combination of innovative biochemical assays and computational approaches is highly valuable. In silico techniques can play a critical role in molecular glue discovery. Molecular docking, molecular dynamics simulations, and free energy calculations can predict the binding interactions between compounds and target proteins. The application of virtual screening can then be used to select a list of potential molecular glues to prioritise those with the highest probability of success for experimental screening.

Phenotypic approaches have been crucial for the discovery of molecular glues, in particular for glue degraders. While the most appropriate screening approach to deploy depends on the specific target of interest and the wanted mode of action, emerging cellular approaches should allow rapid expansion in the field of molecular glues discovery, especially for the detection of weak and transient interactions.

One such approach is the rapid evolution of protein–protein interaction glues (rePPI-G) . Dewey *et al.* developed a new continuous evolution platform for generating PPI inducers based on PACE (phage assisted continuous evolution) and using the RNAP technology (proximity dependent split T7 RNA polymerase) [123]. They previously showed that T7 RNA Polymerase could be split into N- and C-terminal components that spontaneously assemble to form a functional RNAP enzyme. Like enzyme fragment complementation, they engineered an activity-responsive RNAP where assembly of the functional enzyme is dependent on the induction of interaction between fused target proteins, each tagged with either the N-terminal or C-terminal part of RNAP. They linked induced PPI to the expression of gIII, a required phage gene for bacteriophage replication. Their technology can be deployed in mammalian cells [124].

Another promising approach is the cellular thermal shift assay (CETSA). First described in 2013, CETSA is a technique used to measure target engagement in a cellular context, and relies on the biophysical principle of ligand-induced thermal stabilisation of target proteins [125]. CETSA has been widely applied to the field of drug discovery, particularly in target identification and hit validation, and more recently in a high-throughput manner (CETSA-HT) for hit identification [126]. When combined with quantitative MS-based proteomics, CETSA-MS can be used to profile the interactome of a compound as well as the downstream effects on a global proteomic scale. This technique has been used by Chernoockin *et al.* to profile thalidomide, pomalidomide and lenalidomide in K562 cells [127]. CETSA-MS identified clear temperature- and concentration-dependent stabilisation of CRBN, indicating target-engagement, alongside a concentration-dependent, temperature-independent reduction in ZFP91, IKZF1, and RNF166 soluble

protein amount, which taken together indicates compound-induced protein degradation via CRBN. This work highlights how CETSA-MS can be used to profile molecular glues, although currently its application in screening is limited due to the low-throughput nature of MS.

Further down the screening cascade, understanding of the impact of molecular glues on cellular networks is vital for developing structure-activity relationship (SAR) studies that can guide the optimization process and translational studies. Omics technologies such as proteomics, transcriptomics, and interactomics allow the understanding of global changes induced by molecular glue treatment. Network analysis approaches can then identify key nodes and pathways influenced by the formation of new protein complexes, elucidating potential therapeutic mechanisms.

Thus, full validation of candidate molecular glues requires a combination of functional assays. These include the primary screening assay, additional biochemical or biophysical assays to confirm new or altered PPIs, cellular phenotypic assays, and studies to assess stability, pharmacokinetics, and toxicity. Additionally, advanced imaging techniques, including super-resolution microscopy, can provide insights into the spatial distribution of newly formed protein complexes within cells.

The application of advanced biochemical assays, combined with computational strategies or cell-based screens and systems-level analysis, holds great promise for the efficient identification and characterization of molecular glues. These approaches can expedite the discovery of innovative therapeutic agents and advance our understanding of complex cellular processes governed by PPIs. As our understanding of molecular glues and the assay technology to screen for them continues to evolve, the effective integration of multidisciplinary approaches will drive breakthroughs in molecular glue research and drug development. A number of reviews covering the current and future application of molecular glue discovery are available, for example Dewey *et al.* [123], Oleinikovas *et al.* [128], and Fang *et al.* [129].

6. Conclusion

The molecular glue field has seen a huge increase in interest recently and the applicability of the approach has been effectively demonstrated. There are molecular glue degraders which are successfully marketed drugs (the IMiDs, thalidomide (Thalomid) and its analogs, lenalidomide (Revlimid) and pomalidomide (Pomalyst)) and the current efforts are focused on the underlying principles important in rationally designing and screening for new molecular glues. The generation of initial probe molecules that may not have the attributes to become molecular glue drugs but which have the desired mechanism of action can be used tohelp in understanding the ways protein surfaces can be modified by small molecules, will be useful tools in developing our understanding of molecular glue structure-activity relationships. Here, we have reviewed the methods that have been successfully used to screen libraries for molecular glue activity, both in isolated protein assays and in cell-based screens. This has demonstrated that the field is moving from serendipitous discovery to focused and deliberate hit finding campaigns, energised by the findings that glue-based mechanisms are more prevalent than may have been expected. The application of different screening methodologies has enabled glue-based projects versus difficult or intractable targets, where traditional approaches have often failed. And of course, the potential for combination of target proteins with different effectors is large. Considering only the 600 - 700 E3 ligases, which may potentially allow targeted protein degradation, already provides a significant opportunity, without including other mechanisms for target modulation. Methods to identify which of these opportunities provides the highest probability of success are required. Thus, approaches to understand the target protein interactome, and identify which proteins already have some level of affinity for the target are key. Understanding, the degree of cooperative enhancement in affinity that may be achieved and the requirements for initial interaction between target and effector proteins will be important in deciding which interactions are the most fruitful in pursuing. Computational methods will become increasingly important as the databases of protein-protein interactions and their modifications by small molecules grow. This will provide the required substrate for machine learning models that will help to predict pockets or surfaces that can be targeted for molecular glue intervention. Ultimately, computer-aided design for molecular glues themselves will be the goal for AI-driven methods.

However, experimental screening approaches will continue to be required, at least for now, to allow these data sets to be delivered and we believe that the combination of both isolated protein and cell-based methods will continue to be desirable. Phenotypic or target proximal cell-based assays, where monitoring full length proteins in their natural environment, may be optimal in driving molecular glue discovery. This is especially relevant for targets which require the cellular environment to be targetable (for example those proteins that show a large degree of disorder or require the presence of other components in large complexes). However, where feasible isolated protein assays will facilitate faster characterisation of glue mechanisms, especially where high-information content biophysical and structural methods can be combined to understand the interactions facilitating ternary complex formation. We have already seen that utilising cell-based assays as primary screens, where protein reagents are limiting, avoiding the need to isolate protein for primary screening, can be a successful strategy. Often these cell-based screens can be successfully followed up with lower scale biochemical and/or biophysical assays, which require far less protein than a biochemical primary screen. This type of cascade provides a deeper understanding of glue behaviour and will be advantageous for developing the guidelines for molecular glue behaviour.

Although target un-biased, or agnostic, discovery of molecular glues may play a role in revealing disease specific opportunities for intractable targets, we believe that utilising methods such as high-throughput global proteomics to understand the target interactome ahead of establishing screening will be more successful. This will undoubtedly require assays that are able to measure weak and transient interactions between target and effector. To this point, gain of signal assays will be particularly important, especially where the result of a glue-interaction may be the degradation of the target protein. Effector unbiased approaches may have a larger role to play. Here, the opportunity may be to screen a target protein versus many potential effector proteins to understand which may offer the highest potential to establishing a glue-based mechanism. This approach may help to establish a picture of the degrons recognised by different E3 ligases, leading to subsequently improved targeted screening in the future.

To facilitate molecular glue design, medicinal chemists will need to embrace a range of different strategies that will help to understand developing SARs. This will include the use of traditional structure-based drug design (SBDD) methods including X-ray crystallography, in silico modelling and computational docking. It will also extend to the use of cryo-electron microscopy, which may well be better suited to the determination of large ternary complexes. This will help to provide a detailed understanding of the opportunities at the protein-protein interface as well as insights into how molecular glues stabilise the ternary complex, whilst avoiding off target complexes with the effector protein. Thinking more broadly about how pockets may be filled, or created and filled, by molecular glues at these interfaces will be required, which will necessitate medicinal chemists to embrace alternative ideas, distinct from traditional pocket filling on a single protein. It will also be important to use both biochemical and cell-based assays to derive SARs in the absence of structural information. Whilst this approach may be slower, the use of efficiency metrics based on both potency and degradation capability (for molecular glue degraders) will help to guide chemistry. Ultimately, detailed knowledge of the mechanistic details of glue stabilisation will provide insights into the medicinal chemistry features and optimisation strategies that will be required to deliver novel molecular glues to the clinic. These approaches may require exploration of a range of different chemical entities, including

fragments, which being smaller than traditional small molecules may bridge protein interfaces more effectively. Small peptides, having potential complementarity, and containing the same chemical 'currency' as both protein binding partners may also provide effective molecular glue start points. Additionally, several known molecular glues have macrocyclic structures and so, the medicinal chemist will need to be open to exploring not only traditional small molecules, but both smaller and larger entities that may bring about ternary complex stabilisation. Thus, integrated hit identification and undertaking a number of parallel screening approaches, combined with chemical assessment of a range of different chemical equity may well be required in order to derive the knowledge to underpin successful molecular glue identification, design and optimisation.

What is required is a suite of methods that can be effectively combined to provide the necessary information to design and optimise screening cascades that will deliver initial hits that can be chemically optimised into useful probes. These can then be used to provide some general guidelines to aid rational glue design. We will then be able to supplement current diversity-based screening collections with chemical matter that has known glue propensity, and therefore increase the likelihood of success.

In conclusion, the discovery that small molecules with more drug-like properties than PROTACs can efficiently induce protein proximity and modulate target protein function and concentration has provided the impetus to focus efforts on rational design and discovery. We are at an exciting stage of this journey into the field of molecular glue drug discovery and are fortunate that technological advances in assay methodologies, biophysical and structural studies and AI-driven machine learning approaches can now all be combined to help deliver a greater understanding of the molecular properties of small molecules that facilitate new and enhanced protein-protein interactions. We may be witnessing a revolution in drug discovery that may open a wide range of new disease-targeting opportunities.

Declaration of Competing Interest

All authors are employees of AstraZeneca. G. A. Holdgate is on the editorial board for SLAS Discovery.

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