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New approaches to target RNA binding proteins

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Abstract

RNA binding proteins (RBPs) are a large and diverse class of proteins that regulate all aspects of RNA biology. As RBP dysregulation has been implicated in a number of human disorders, including cancers and neurodegenerative disease, small molecule chemical probes that target individual RBPs represent useful tools for deciphering RBP function and guiding the production of new therapeutics. While RBPs are often thought of as tough-to-drug, the discovery of a number of small molecules that target RBPs has spurred considerable recent interest in new strategies for RBP chemical probe discovery. Here we review current and emerging technologies for high throughput RBP-small molecule screening that we expect will help unlock the full therapeutic potential of this exciting protein class.

Keywords

RNA binding proteins (RBPs); High throughput screening (HTS); Chemical probes; Chemoproteomics

Introduction

RNA binding proteins (RBPs) are a large and diverse protein class (1000+ members) [1], which act as essential modulators of RNA function. RBPs regulate RNA splicing,

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Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data
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as well as initiation, elongation, and termination of translation. RBPs can also inhibit translation, modulate RNA subcellular localization, and regulate RNA degradation. RNA-protein interactions (RPIs) play essential roles in stem cell maintenance, differentiation, and somatic cell reprogramming [2,3]. Dysregulated RPIs have been linked to cancers [4,5], cardiovascular diseases [6], and neurodegenerative disorders [7]. For example, misfolding and aggregation of RBPs in membraneless organelles, such as stress granules, has been associated with Alzheimer's disease [8], Parkinson's disease [9], and amyotrophic lateral sclerosis (ALS) [10].

Chemical probes are attractive tools to study and pharmacologically manipulate RBPs. Complementary to biologics, such as antibodies and peptides, small molecules offer several advantages, including cell permeability, oral availability, and cost-effectiveness. As demonstrated by the wild success of kinase inhibitors as both probes and drugs, chemical probes can be designed to inactivate individual members of highly homologous protein domains (e.g. RNA-binding domains, such as the RNA-recognition motif (RRM), hnRNP K homology (KH) domain, and the zinc-finger domain). Complicating matters, the absence of defined small molecule binding sites and the highly disordered structure of many RBPs has generally limited their tractability for small molecule probe development. This perception of 'undruggability' has begun to change, and recent advances in small molecule screens and RPI identification technologies have revealed the 'druggable' potential of this protein class. A number of chemical probes have been shown to function by targeting select RBPs (Table 1), including spliceosomal proteins [11–19], ribosomal proteins [20], HuR [21–23], EIF4 family proteins [24–26], toll-like receptors [27,28], stress granule-associated proteins [29,30], and microRNA-binding proteins [31–34]. For a comprehensive overview of RBP probes, we would direct the readers to the following review [35]. Here, we review the techniques that have advanced these efforts and highlight future opportunities and technical innovations that are well suited to enable RBP small molecule probe development.

RBP-RNA high-throughput small molecule screens

Conventional biophysical assays, including fluorescence polarization (FP) and Föter resonance energy transfer (FRET), are compatible with RBP small molecule screening. For FRET, a pair of donor/acceptor fluorophores with overlapping spectra are individually appended to the RNA and RBP. Small-molecule binding is reported by decreased emission of the acceptor fluorophore (Figure 1a). In a similar fashion, for FP, the polarized light emission of a fluorescently tagged RNA is monitored upon binding to an RBP. Decreased polarization corresponds to the increased tumbling speed of unbound RNA, indicative of a small molecule blockade of the RBP-RNA interaction (Figure 1b). These techniques have enabled RBP probe discovery for components of the spliceosome, the protein Lin28, HuR, MSUT2, MUSASHI family proteins, and EIF4 family proteins [23,31–33,35,40,49,52,55]. Another proximity-based fluorescent assay, AlphaScreen® (ALPHA for Amplified Luminescent Proximity Homogeneous Assay; Figure 1c), has also been applied to screen for RPI inhibitors, most recently identifying an inhibitor of the tauopathy-inducing MSUT2-poly(A)RNA interaction [55]. In a similar format, a recent turn-off fluorescence screen takes advantage of the enhanced fluorescence observed when RBPs bind to RNAs appended to certain fluorophores (Figure 1d). Screening of the interaction between Lin28

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and preE-NA identified both known and novel inhibitors. Extension of this assay to the RBP Roquin—implicated in the degradation of multiple immune-relevant mRNAs, such as tumor necrosis factor-α (TNF-α) mRNA—identified the well-characterized RPI inhibitor ATA as a Roquin binder [53]. While conventional fluorescent assays sometimes suffer from low specificity and sensitivity, the use of red-shifted dyes in this 'enhanced fluorescence' assay can prove useful in reducing the likelihood of spectroscopic interference from library members.

The catalytic enzyme-linked click chemistry assay (cat-ELCCA; Figure 2a) represents an exciting alternative for RBP screening, offering the potential for improved sensitivity compared with fluorescence-based assays. Cat-ELCCA combines copper-catalyzed azide—alkyne cycloaddition (CuACC) bioorthogonal chemistry and enzyme-catalyzed signal amplification to identify small molecule modulators of RBPs [54,56,57]. While the cat-ELCCA concept was developed using the RNA-processing protein Dicer, the application of cat-ELCCA to screen the oncogenic pre-let7 miRNA-Lin28 interaction identified several potent hits, including CCG-233094 and CCG-234459 [57]. Advantages of the cat-ELCCA screening platform include robustness (Z' score > 0.5), sensitivity as a result of signal amplification, lack of interference from fluorescent compounds, and as with FRET and FP, generalizability to a range of RNA-RBP interactions [54,57].

Cell-based screening methods

A key limitation of the aforementioned assays is their general incompatibility with cell-based screens, due, for example, to modest assay sensitivity that requires RNA and protein concentrations in excess of those feasible in cells and to low assay specificity in more complex biological mixtures caused by nonspecific binding of a reporter RNA to other RBPs. In-cell RBP screening has been achieved using a split luciferase engineered from NanoLuc (NanoLuc Binary Technology; NanoBiT) [58], in which the split luciferase's subunits, SmBiT (13 kDa) and LgBiT (18 kDa), are conjugated to the biomolecules of interest, such that a luminescent signal is detected upon their interaction. Validation of this system was first achieved in vitro by assaying the interaction between Lin28 and the pre-let7 miRNA (Figure 2b). When trans-cyclooctene (TCO)-function-alized pre-let7 RNA is mixed with the fusion proteins SmBiT-HaloTag and Lin28-LgBiT, together with a tetrazine-functionalized chloroalkane ligand, Lin28-pre-let7 binding promotes assembly of catalytically competent luciferase. Pretreatment with the previously identified Lin28 inhibitor CCG-233094 abolished the luciferase signal. Extension of this platform to the cell-based assay, termed RNA interaction with Protein-mediated Complementation Assay, or RiPCA [59], enabled live cell detection of Lin28B interactions with a number of pre-miRNAs, suggesting RiPCA will be compatible with future cell-based small molecule screens. Unlike in vitro approaches (e.g. cat-ELCCA), these cell-based assays do not differentiate between indirect and direct interactors.

Cell-based screens have also been developed to identify small molecule modulators of RNA splicing. Because dysregulated splicing has been implicated in many cancers and neurodevelopmental disorders, components of the spliceosome, particularly SF3B1, have been the focus of considerable interest as potential drug targets. In fact, the natural

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product pladienolide B [41], its derivative E7107 [16], its synthetic analog H3B-8800 [17], spliceostatin A [18], and Sudemycin D6 (SD6) [19] all function by acting on the spliceosome. The activity of the spliceosome modulator Sudemycin D6 (SD6) was validated in cells using a luciferase-MDMD2 fusion construct containing a premature stop codon in exon 4. Luciferase expression and the bioluminescent signal is only detected upon exon skipping [60]. Using this Triple Exon Skipping Luciferase Reporter (TESLR) assay, three antitumor agents were established as splicing modulators, and two novel antagonists of SF3B1 were also identified [47]. Comparable to the luciferase-based assays, two color exon skipping assays have also identified novel small molecules that target cellular splicing [61]. Whether these compounds function by binding directly to a putative RBP or indirectly, for example, by altering signaling cascades, remains to be seen.

RPI assays and their potential applications to small molecule screening

In looking beyond small molecule screens, many additional technologies have recently emerged that enable the high throughput identification of RBP-RNA interactions. While these methods are not specifically tailored to small molecule screening applications, given their widespread adoption and flexible design, we anticipate that these tools will facilitate RBP probe development efforts. For example, a number of mass spectrometry-based proteomic methods have been developed to identify RBPs. We would direct readers to the following reviews for more comprehensive surveys of all available technologies [62,63]. Many methods, including conventional crosslinking (cCL) [64] and photoactivatable-ribonucleoside enhanced crosslinking (PAR-CL), rely on oligo(dT) resin for RNA-RBP enrichment, and thus, only capture RBPs bound to polyadenylated mRNAs. However, several new methods that rely on metabolic labeling with unnatural nucleosides have emerged to capture RBPs bound to all types of RNAs, including, for example, ncRNAs, pre-mRNAs, and circRNAs [65,66]. In the click chemistry-assisted RNA interactome capture (CARIC) method [66], cellular RNAs are metabolically labeled with two analogs of the nucleotide uridine (5-ethynylurdine [EU] and 4-thiouridine [4SU]). The photoactive 4SU improves the efficiency of UV-crosslinking of RBPs to RNA, and the EU is used for unbiased enrichment of proteins bound to all types of RNAs using bioorthogonal labeling chemistries (e.g. CuAAC, strain-promoted click chemistry, and IEDDA labeling reactions). We expect that these methods will soon take advantage of new nucleoside probes that offer decreased toxicity and cell-specific labeling [67]. When combined with proteomic studies that employ isotopic labels, these methods should prove amenable to competitive small molecule screens for the identification of RPI modulators. However, complicating their application to small molecule screening, these approaches typically do not report the RBP-RNA contact sites. As a result, such methods may fail to capture compound-dependent changes in specific RNA-protein interactions for proteins that contain multiple RNA binding domains, with only one labeled by a lead compound, or for those where small molecule binding blocks interactions with only a subset of cognate RNAs. Proteins in these categories may show modest to no changes in enrichment upon compound-labeling.

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Photoactivatable RNA probes

Photocrosslinkable activity-based probes are poised to enable RBP small molecule screening and probe discovery. Synthetic diazirine-containing RNA photoprobes enabled the chemoproteomic identification of RBPs that bind to N6-methyladenosine (m6A) modified RNA [68]. In this method, the m6A-containing synthetic probe is photocrosslinked to its protein interactors, and chemoproteomic analysis is used to enrich and identify cross-linked proteins. Given the widespread adoption of photoaffinity probes for competitive activity-based protein profiling (ABPP) and small molecule screening [69,70], we anticipate that when integrated into competitive ABPP small molecule screening workflows, the m6A photo probes should prove useful for identifying small molecules targeting readers and writers of epitranscriptomic modifications. More broadly, photoaffinity probes targeting additional RNA modifications (e.g. 7-methylguanosine (m^7G) modification) will likely also prove compatible with ABPP and chemoproteomic studies.

Proximity-based labeling

Proximity-based biotinylation strategies, while not yet widely adopted for small molecule screening applications, represent another intriguing option for in situ RBP probe discovery campaigns. When fused with the MS2 coat protein (MCP), the APEX engineered peroxidase, which catalyzes promiscuous biotinylation of proximal proteins, can be applied to identify proteins located proximal to MS2-labeled transcripts [71]. Similarly, APEX fusion to a catalytically inactive Cas13 (dCas13) initiates biotinylation of proteins proximal to an RNA that is complementary to the Cas13 gRNA. When these methods were applied to uncover proteins proximal to the telomerase RNA, the MCP- and dCas13-APEX labeling strategies functioned comparably, with substantial overlap observed for both strategies [71]. We expect that these methods should readily extend to assaying compound-dependent changes to RBP-RNA interactions. It is worthwhile to note, however, that such proximity-based labeling assays will likely be subject to off-target effects as a result of endogenous biotinylation and nonspecific APEX-mediated biotinylation of distal proteins. The use of properly controlled experiments (e.g. untargeted APEX as a negative control) and statistical filtering of quantitative proteomic data will mitigate the identification of false positives.

Ligandable RBPs identified by global chemoproteomic studies

Mass spectrometry-based chemoproteomic studies, using both irreversible and reversible probes (Figure 3), have generated proteome-wide maps of protein ligandability [69,72–75] —ligandability in this context refers to proteins and protein domains found to bind to small drug-like molecules, which in some instances can serve as leads for more potent chemical probes or even drugs. Our prior screen of cysteine-reactive chemical probes using the chemoproteomic method isotopic tandem orthogonal proteolysis—activity-based protein profiling (isoTOP-ABPP), revealed that an astonishing 18% of ligandable cysteine residues are found in proteins annotated as RBPs (See Table S1 for examples, which includes 139 total cysteines and 7 cysteines located within known RNA interaction sites) [72]. Consistent with these findings, RBPs are also known to be rich in cysteine residues, including redox-sensitive residues and the numerous cysteines found in RNA-binding zinc fingers. For example, oxidative stress regulates the RNA binding activity of RBP Iron regulatory protein

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2 (IRP2) in a cysteine dependent manner [76]. The Parkinson's associated protein PARK7 (also known as DJ-1) has a conserved redox-sensitive cysteine (Cys106) that has been implicated in Park7 mRNA binding. However, as Park7 lacks a canonical RNA binding motif, it remains to be determined whether Cys106 contributes directly to RNA interactions or whether this residue modulates RNA binding indirectly, by, for example, modulating other protein—protein interactions [77]. Whether compound-labeling at the many identified cysteine residues will impact RBP function remains an open question.

Chemoproteomic studies that assay lysine- and tyrosine-reactive compounds have similarly revealed an enrichment for reactive and ligandable residues in oligonucleotide-binding proteins, many of which are RBPs. Global analysis of lysine ligandability using the method isoTOP-ABPP and a lysine-reactive sulfotetrafluorophenyl (STP)-ester probe, revealed that over 26% of liganded lysines are found in annotated RBPs (Table S1, which includes 32 total lysines and 4 lysines located within known RNA interaction sites) [73]. These results are perhaps not surprising, as RNA binding domains are known to be enriched for positively charged residues, including lysines and arginines. Sulfur—triazole exchange chemistry was used to identify ligandable tyrosines in the human proteome. 22% of ligandable tyrosines were found in RBPs (Table S1, which includes 82 total tyrosines and 13 tyrosines located within known RNA interaction sites) [74]. As with irreversible probes, global chemoproteomic analysis of the protein targets of reversible inhibitors uncovered a substantial number of ligandable RBPs. In enrichment studies, 461 RBPs (Table S1) were found to interact with members of a fully functionalized fragment (FFF) library, which consists of compounds that feature both photocrosslinkable diazirine moieties and alkyne enrichment handles [69]. In more focused studies, chemoproteomic methodology has also validated that previously identified [78] 2,4-Diaminoquinazoline-containing scaffolds bind to the mRNA decapping scavenger enzyme DcpS, yielding both reversible photoprobes and irreversible sulfonyl-fluoride-containing inhibitors that react with an active site tyrosine residue [79]. Given the enrichment for tyrosine residues in RNA binding sites, this work, together with the aforementioned tyrosine-directed chemoproteomic study, highlights future opportunities to target RBPs with tyrosine reactive probes, containing electrophilic warheads such as sulfonyl fluoride, fluorosulfonate, and the recently reported sulfuramidimidoyl fluorides [80].

Conclusions and future prospects

RBPs function in a wide range of biological processes, both in the context of translation and other regulatory networks [2,3]. RBP dysregulation, including aberrant expression, altered RNA interactions, and aggregation and phase separation, has been implicated in many diseases, including neurodegenerative disorders [7], cardiovascular disorders [6], and cancers [4,5]. Consequently, RBPs are an attractive protein class to chemically target and functionally manipulate with chemical probes. As highlighted here and recently reviewed comprehensively [35], a number of small molecules, including some compounds in clinical trials, have been identified that function by binding to RBPs. Despite these considerable advances, RBPs are often still categorized as tough-to-drug. A central reason for this seeming intractability is the absence of suitable high throughput assays for RBP small molecule probe discovery.

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As we have discussed here, a number of recent assays have emerged that address this challenge and are poised to facilitate future RBP probe discovery campaigns. Conventional screening methods (e.g. FRET and FP) can be readily adopted to RBP screens. As many RBPs function in large complexes, screens of recombinant proteins may fail to yield compounds that are active in cells. Cell-based methods, such as RiPCA and established fluorescent and luminescent reporters of RNA splicing, address this limitation and enable small molecule screens for a subset of RBPs, including splicing factors and microRNA-binding proteins.

In looking beyond these assays, numerous other technologies have emerged that are well suited to RBP probe discovery efforts, such as established proteomic methods for RBP identification, including proximity-based labeling strategies and photo-crosslinking and capture methods. The ubiquity of RBPs in several global chemoproteomic profiling studies further points to the general tractability of RBPs. Particularly intriguing is the number of RBPs labeled by FFFs, which points to the potential utility for fragment-based screens in targeting RBPs. Computational methods, such as in silico screening, will likely also prove useful for RBP probe discovery, as demonstrated by the recent identification of RBP probes targeting the stress granule-associated protein TDP-43 [29,30]. We anticipate that such in silico screens will likely prove most enabling when combined with secondary screens of potential hits by, for example, using one or more of the screening assays detailed above.

To unlock the full potential of RBP probes, a number of challenges still need to be addressed. As many RBPs still remain functionally unannotated, deconvolution of the functional impact of probe binding for these proteins may prove nontrivial. Resources such as accurate predictions of RBP-RNA contact sites [81] and the multiomic integration of genetic predictions of pathogenicity with chemoproteomics data [82], should facilitate prioritization of RBP-small molecule pairs for downstream functional validation and medicinal chemistry. Such meta-analyses will likely prove particularly impactful for prioritizing ligandable RBPs identified in global chemoproteomic screens.

Taken together, the application of innovative screening technologies to RBPs should yield chemical probes that can function both as useful tools to annotate RBP function and serve as leads for future drug development efforts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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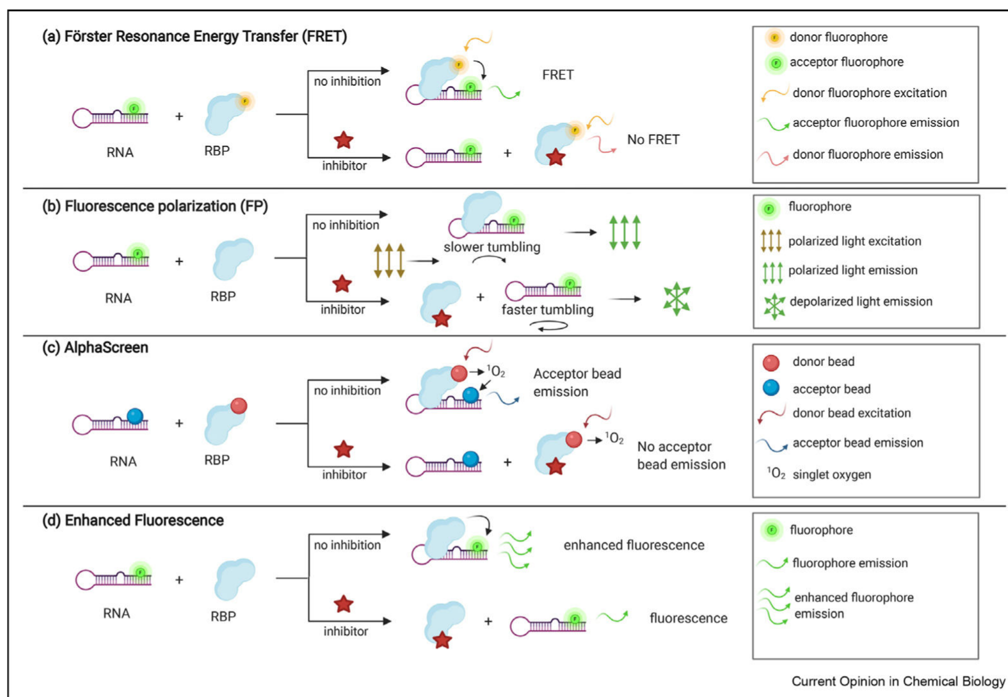

Figure 1. Fluorescence-based assays for RPI inhibitor screening.

(**a**) Förster Resonance Energy Transfer (FRET). Both RNA and RBP are appended to fluorophores with overlapping spectra, such that FRET emission is detected upon their interaction and abolished upon inhibition of their interaction. (**b**) Fluorescence polarization (FP). The RNA (or RBP) is appended to a fluorophore that is excited by plane-polarized light. If RBP binding is inhibited, the RNA will tumble faster in solution, increasing the detection of perpendicularly polarized light. (**c**) AlphaScreen®. RBP and RNA are appended to donor and acceptor beads, respectively. Excitation of donor beads leads to conversion of oxygen to singlet oxygen, which reacts to generate a chemiluminescent signal with the acceptor bead that then excites a fluorophore within the same bead, resulting in a fluorescent emission. No emission is detected upon small-molecule inhibition. (**d**) Enhanced fluorescence. The RNA is appended to a fluorophore whose fluorescence is enhanced upon RBP binding. Weak fluorescence indicates inhibition of interaction

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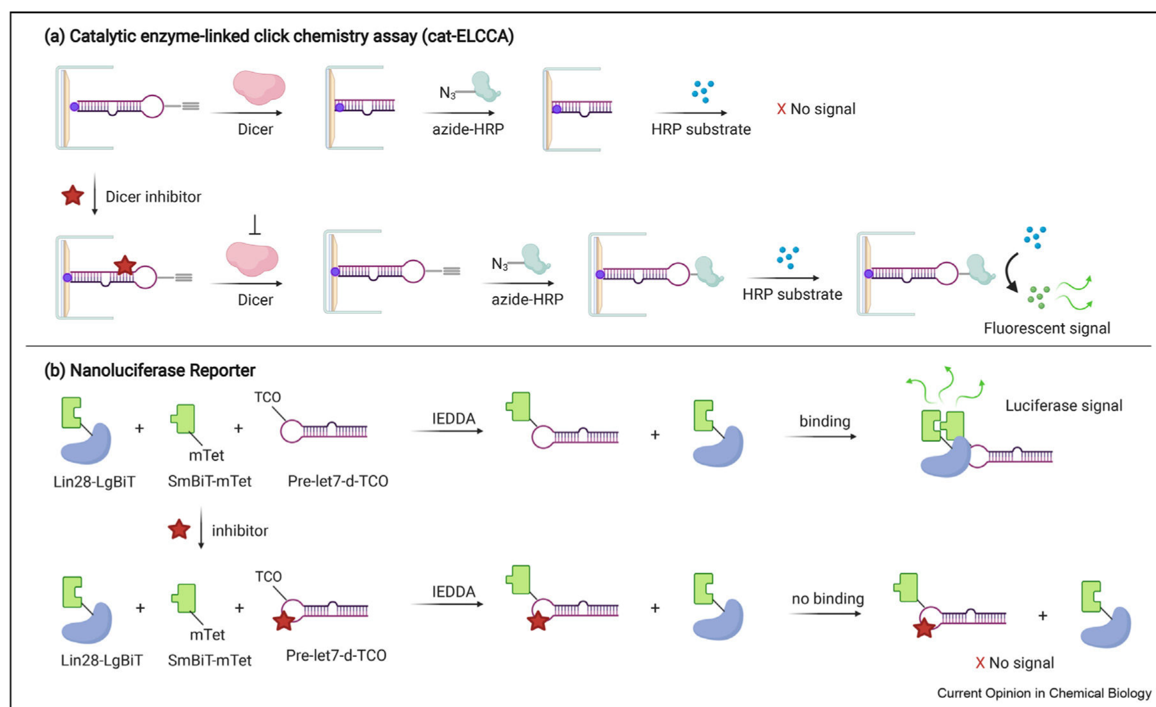

Figure 2. Bioorthogonal chemistry-based assays for RPI inhibitor screening.

(**a**) Catalytic enzyme-linked click chemistry assay (cat-ELCCA). Inhibition of Dicer-mediated RNA processing is reported by CuAAC conjugation of alkyne-functionalized RNA to azide-HRP. The use of inverse electron demand Diels–Alder chemistry (IEDDA) can further increase the sensitivity and reproducibility of cat-ELCCA. (**b**) Nanoluciferase reporter assay. The combination of TCO-functionalized RNA with mTet-functionalized SmBiT affords a SmBiT-RNA fusion. Binding to a LgBiT-RBP fusion produces a detectable signal by luciferase formation. Inhibition of RNA-RBP binding abolishes such signal

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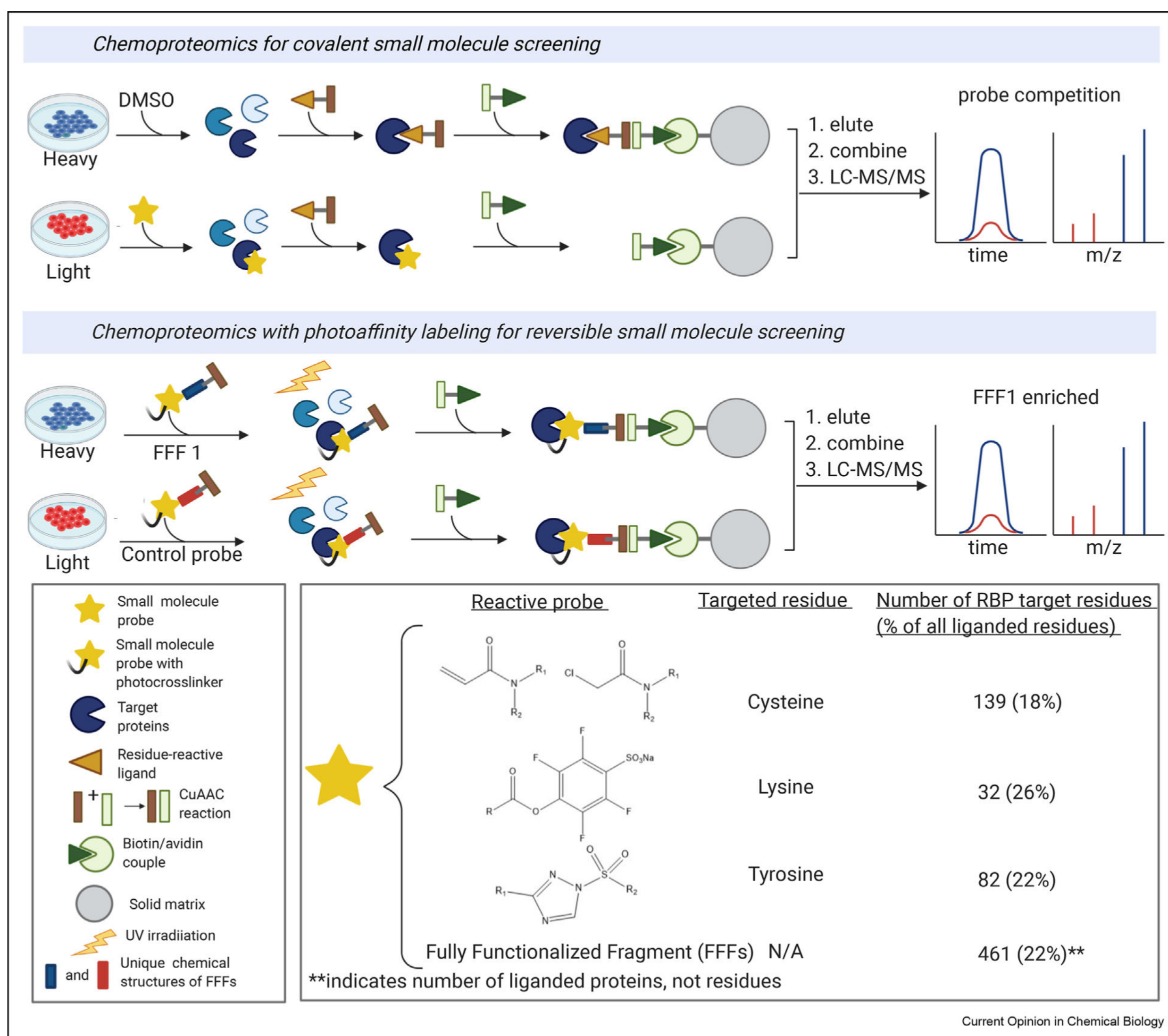

Figure 3. Detection of druggable RBPs through chemoproteomics.

A reactive ligand featuring a bioorthogonal handle is introduced to cells or cell lysates to react either covalently (top scheme) or reversibly (bottom scheme) with protein targets. Reversible probes harbor a photocrosslinkable moiety for covalent linkage to protein targets upon UV irradiation. Probe-labeled targets are enriched by conjugation to biotin via CuAAC and then identified by LC-MS/MS.

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Table 1

Small molecules targeting RBPs.

Target/Compounds	Molecular Function	Method of identification	Refs.
Spliceosome			
SMN-C1, -C2, -C3, -C5, NVS-SM1, NVS-SM2	Increases affinity between U1 snRNP and SMN2 exon 7 5′ splice site	HTS, firefly luciferase	[11,36,37]
RG7800, Risdiplam	Modifies alternative splicing of SMN2	HTS, firefly luciferase and lead optimization	[12,14,38]
Branaplam	Stabilizes interaction between the spliceosome and SMN2 pre-mRNA	HTS phenotypic screen and lead optimization	[13]
spiro[indol-3′2-pyrrolidin]-2(1H)-one	Inhibits helicase activity of Brr2	HTS ATPase screen and lead optimization	[39]
Topotecan	Blocks the interaction between U4 RNA and NHP2L1	Time-resolved FRET assay	[40]
SF3B complex			
Pladienolide B, E7107, H3B-8800	Competitively blocks pre-mRNA binding by stalling SF3B complex in open conformation	ABPP and medicinal chemistry	[16,17,41–43]
FR901464, Spliceostatin A	Initiates cell cycle arrest and inhibits splicing to afford antitumor effects	Natural product	[18,44]
Sudemycins	Similar to FR901464/Spliceostatin A	Rational design and synthesis	[19,45,46]
Sudemycinol C and E, milciclib, PF-3758309, and PF-562271	Modulates/inhibits splicing and inhibits a number of splicing factor kinases	Triple Exon Skipping Luciferase Reporter (TESLR) assay	[47]
RBM39			
Indisulam/E7820	Degrades RBM39 by targeting it to E3 ligase	Forward genetics strategy	[15,48]
HuR			
Dehydromutactin, MS-444, and okicenone	Blocks HuR dimerization that inhibits RNA binding	HTS confocal fluctuation spectroscopic assay	[21]
KH-3	Blocks HuR and FOXQ1 RNA interaction	HTS fluorescence polarization (FP) assay	[23]
EIF4E			
Arylsulfonyl fluoride	Inactivates EIF4E by labeling noncatalytic Lys162	Covalent docking	[24]
Bn7GxP-based PROTACs	Degrades EIF4E by targeting it to E3 ligase	Rational design and synthesis	[26]
4EGI-1	Binds to EIF4E to block EIF4E/EIF4G complex formation	HTS FP assay	[49]
EIF4A			
Hippuristanol	Blocks EIF4A binding to RNA	Forward chemical genetics	[50]
MUSASHI			
Ro 08-2750	Inhibits MS1 RNA-binding activity	HTS FP assay	[51,52]
TLR			
CU-115, CU72	Blocks RNA binding	Cell-based HTS and lead optimization	[27]
CU-CPT17e	Activates TLRs to trigger immune response and promote apoptosis	Cell-based HTS	[28]

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Target/Compounds	Molecular Function	Method of identification	Refs.
LIN28			
Compound 1632	Blocks Lin28 and pre-let7 RNA interaction	HTS FRET assay	[31]
TPEN, LI71	Blocks Lin28 and pre-let7 RNA interaction	HTS FP	[32]
	Blocks Lin28 and pre-let7 RNA interaction	HTS enhanced fluorescence assay	[53]
KCB170522, luteolin, rhynchophylline, and tenuifolin			
CCG-233094, CCG-234459	Blocks Lin28 and pre-let7 RNA interaction	Catalytic-enzyme linked click chemistry assay (cat-ELCCA)	[54]
MSUT2			
Duloxetine, saquinavir, clofazimine, DIDS	Blocks MSUT2 binding to poly(A) RNA	HTS FP assay and HTS AlphaScreen	[55]
TDP-43			
rTRD01	Binds RRM domains of TDP-43 to inhibit association with disease-linked *c9orf72* RNA	*In silico* docking	[29]
nTRD22	Binds N-terminal domain of TDP-43 to allosterically decrease RNA binding	*In silico* docking	[30]
Ribosome			
Ribosome-targeting antibiotics	Inhibits translation	Various	[20]