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High-throughput approaches to profile RNA-protein interactions

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Abstract

RNA-protein interactions play a critical role in post-transcriptional gene regulation. Characterizing these interactions in their native context has been challenging, however advances in RNA sequencing and mass spectrometry-based proteomics combined with innovative chemical biological tools have heralded the development of robust strategies for performing biochemistry on a cellular scale. Herein, we review recent advances in the development and application of proteomic and transcriptomic approaches to profile cellular RNA-protein interactions, focusing on sequencing-based strategies and proteomic analysis of RNA-binding proteins (RBPs), as well as approaches to address the role of RNA modifications in protein-RNA binding events.

Introduction

Post-transcriptional gene regulation plays an important role in biological processes. In large part, the underlying molecular mechanisms are mediated by physical interactions between RNA transcripts and a large complement of RNA-binding proteins (RBPs)[1] that regulate RNA splicing, stability, nuclear export, and translation, among other properties. Therefore, characterizing the RNA-binding preferences of RBPs and mapping the RBP proteome can reveal fundamental insights into the biological function of RNA-protein binding events. In addition, recent studies have implicated RNA modifications (the "epitranscriptome")[2] as regulators of RNA-protein complexes, adding an additional dimension of complexity to these interaction networks. While early studies of RNA-protein interactions were limited to studies of individual complexes[3], technological advances in RNA sequencing and mass spectrometry-based proteomics have enabled transcriptome and proteome wide analysis of these processes. Notably, UV-mediated photocrosslinking of protein-RNA complexes, which can be performed on intact biological samples[4,5], has played a major role in the development of approaches to study cellular RBP-RNA interactions in a high-throughput fashion. In addition, chemical biology approaches such as metabolic labeling with artificial nucleotides, bioorthogonal chemistry and protein engineering have been applied to aid in the elucidation of the protein-RNA interactome. In this review, we highlight recent methodological

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developments in the high-throughput characterization of cellular RNA-protein interactions and discuss future directions for this area of research.

Approaches to footprint RNA-protein binding sites

A key step in understanding the molecular mechanisms underlying the biological function of RBPs is to identify the collection of RNA transcripts with which they physically associate; further, the RNA-binding site or "footprint" of the RBP can provide additional insight into its biological activity. Early efforts towards this end relied upon *in vitro* selection/SELEX[6,7] to characterize the inherent sequence-binding preferences of RBPs using libraries of random-sequence RNA. This strategy efficiently identifies high-affinity binding motifs, which can then be used to mine for related sequences in the transcriptome. *In vitro* selection is operationally straightforward and results can be analyzed using low-throughput Sanger sequencing; however, these experiments are not always directly relevant to physiological RNA-protein interactions, and therefore, most approaches have focused on the direct characterization of protein-RNA complexes isolated from living systems.

The first widely accepted strategy to characterize RNA-protein interactions *en masse* was reported by Darnell and co-workers in 2008[8]. Known as HITS-CLIP (also CLIP-seq) (Figure 1a), this approach combined several key methods for the isolation of native RNA-protein complexes[4,5,9,10] by crosslinking and immunoprecipitation (CLIP) with high-throughput RNA sequencing (a new technology at that time). Central to the method is UV-induced photocrosslinking, which can be performed on intact cells and crosslinks RNA-protein interactions with much greater efficiency than protein-protein species (thereby favoring crosslinking of RNA with only directly associated RBPs). Once RNA-protein crosslinks have been generated, even low affinity protein-RNA interactions can be isolated under stringent immunoprecipitation conditions, and the associated RNA identified by reverse-transcription and sequencing. RNA is typically subject to partial enzymatic digestion before and after immunoprecipitation in order to map the RBP footprint with greater precision. HITS-CLIP was the forerunner to a number of UV-photocrosslinking based CLIP methods designed to map the transcriptome-wide RNA binding of a single RBP. These include CRAC[11], PAR-CLIP[12], iCLIP[13], and eCLIP[14], as well as improved HITS-CLIP methodology[15] (Figure 1a). The improvements reported in these modified CLIP methods have generally fallen into the following categories: 1) enhanced photocrosslinking facilitated by metabolic labeling of cellular RNA with 4-thiouridine (4-SU) or 6-thioguanosine (6-SG)[12], 2) improved immunoprecipitation and isolation conditions[11], and 3) modifications to cDNA library generation and bioinformatic analysis designed to map RBP footprints with single nucleotide resolution[13–15] (often relying upon identification of crosslinking-induced mutations, deletions, or truncations). Currently, these improved CLIP strategies represent the state-of-art in mapping RBP footprints transcriptome-wide and have been applied to study numerous RBPs.

While UV crosslinking is a general strategy that can be applied to almost any RBP, the unique catalytic mechanism of RNA 5-methylcytidine (m^5C) methyltransferases[16] enables their crosslinking to substrate RNA through chemical means. Two crosslinking-based approaches have been reported for mapping m^5C methyltransferase substrates – Aza-IP[17]

and miCLIP[18] (Figure 1b). In the Aza-IP strategy, RNA-protein crosslinking is mediated by 5-azacytidine, which can be incorporated transcriptome-wide through metabolic labeling; for the miCLIP approach, a mutant methyltransferase is utilized which remains bound to its substrate cytidine residue. After chemical crosslinking, immunoprecipitation and RNA sequencing analysis is performed using strategies similar to those employed in UV-based CLIP workflows. While UV CLIP approaches can also be applied to m^5C methyltransferases, mechanism-based crosslinking reports on RNA transcripts that are subject to enzymatic methylation, as opposed to simple binding.

Non-crosslinking strategies have also seen application in RBP substrate mapping. Rosbash and co-workers have developed the TRIBE approach[19] (Figure 1c, right), based on the ability of adenosine deaminase enzymes (ADARs) to catalyze adenosine to inosine (A:I) editing when brought into close proximity to an RNA transcript (such as through fusion to the RBP of interest). In this strategy, no purification of RNA-protein species is required since inosine is predominantly converted to C during reverse transcription and its presence can be identified directly by sequencing and bioinformatic analysis. An enhanced version of TRIBE (named HyperTRIBE[20]), which uses a more active ADAR mutant has been recently described. An alternative non-crosslinking based strategy was developed by Wickens and co-workers[21]. Their approach, named “RNA tagging”, relies upon the fusion of *C. elegans* poly(U)polymerase PUP-2 to the RBP of interest resulting in the deposition of polyU tails on RBP-associated RNAs (Figure 1c, left). PolyU-tagged RNAs can then be selectively enriched during cDNA library generation. The major advantage of both the TRIBE and RNA tagging approach is that these strategies do not require isolation of RNA-protein complexes. However, they both involve fusing an enzyme to the RBP of interest, which may affect RNA-binding, and are unlikely to provide binding data at nucleotide resolution.

Approaches to profile the RNA-binding proteome

CLIP-based sequencing approaches provide information on the binding behavior of a single RBP, but fail to report on the RNA-binding proteome. To discover new RBPs and characterize their behavior on a proteome-wide level, it is necessary to apply proteomics for RBP analysis. This is generally more challenging than RNA sequencing-based analysis due to the lower throughput and lack of amplification, but in the last decade multiple strategies for proteomic characterization of the cellular RBP complement have emerged. These approaches have built upon advances in RNA-protein crosslinking initially developed for CLIP experiments and are described below.

The first strategy to isolate RBPs from cells, known as “interactome capture” was developed independently by the Hentze[22] and Landthaler[23] labs (Figure 2a). In their respective approaches, RBPs are first UV crosslinked to RNA and polyadenylated RNA-protein complexes are enriched from cells by hybridization to oligo-dT beads. After enzymatic digestion of RNA, mass spectrometry-based proteomics is used to characterize the identities of the isolated RBPs. Since nucleic acid hybridization is tolerant to high salt and ionic detergents, oligo-dT enrichment can be performed under stringent, denaturing conditions, allowing the clean isolation of RNA-crosslinked proteins from free proteins in the cellular milieu. The interactome capture method has been widely used to study RBPs interacting

with mRNA[22–29], and can be employed with 254 nm UV crosslinking or longer wavelength irradiation combined with 4-SU-labeled RNA. In the original reports[22,23], ~800 RBPs were identified in HeLa and HEK293 cells, many of which were novel RBPs. An alternative strategy that is not restricted to polyadenylated RNA is the RICK[30]/CARIC[31] approach that can be applied to profile RBPs associated with RNA transcripts irrespective of their polyadenylation status (Figure 2b). This methodology relies upon 5-ethynyluridine (5-EU) labeling of RNA and click chemistry with biotin-azide in order to enrich RBPs crosslinked to nascent transcripts, and provides orthogonal results to oligo-dT-based enrichment.

Further efforts to profile RBPs have focused on approaches that afford greater generality and are not restricted to the analysis of RBPs that associate with polyadenylated RNA, as well as those that provide insight into protein domains and amino acid residues involved in RNA binding[29,32,33]. One such approach, RBR-ID[32], was developed by Bonasio, Garcia and colleagues (Figure 2c). In their strategy, RBP binding regions are identified by analysis of crosslinked tryptic peptides. Rather than seeking to measure the specific crosslinked peptide-oligonucleotide species, RBR-ID takes advantage of the concomitant decrease in signal intensity for the parent (non-crosslinked) tryptic peptide. Using this strategy on the nuclear proteome of embryonic stem cells resulted in the identification of ~800 RBP binding regions at peptide level resolution, including several previously unknown RNA-binding domains in chromatin regulators. Moreover, the RBR-ID strategy does not require RNA enrichment and therefore provides an unbiased snapshot of the RNA-binding proteome.

While the above approaches for RBP analysis largely rely upon specific recognition of nucleic acid sequences or affinity reagents for the isolation of crosslinked RBP-RNA complexes, the physicochemical properties of covalent protein-RNA conjugates can be directly exploited in order to enrich these species from free RNA and free protein (Figure 2d). Indeed, the differential partitioning of RNA from DNA and protein upon extraction with acidic guanidinium thiocyanate/phenol/chloroform (i.e. “Trizol”) is a common technique for RNA isolation from cellular samples. With the rationale that covalent RNA-protein complexes may exhibit hybrid phase separation properties distinct from either individual component, several groups decided to investigate whether these crosslinked species could be enriched by isolation of the interphase layer, which is typically discarded. In three independent reports published this year[34–36], phase separation was described as an effective strategy to isolate RNA-crosslinked RBPs. In two approaches, named XRNAX[35] and OOPS[36] (Figure 2d), the standard Trizol formulation was used; the XRNAX method also incorporates an additional silica-based purification of crosslinked peptide-RNA fragments prior to mass spectrometry analysis. The third method, PTex[34], utilizes a modified phenol-toluol organic layer, and sequential extractions at neutral and acidic pH (Figure 2d). Application of all 3 approaches to mammalian cells consistently identified greater numbers of RBPs than were found by oligo-dT based interactome capture, reflecting the more general nature of these strategies for isolating crosslinked RNA-RBP complexes. In addition, OOPS and PTex were applied to study bacterial RBPs, which are not accessible through oligo-dT interactome capture since bacterial RNA lacks polyadenylation. Tollervey and co-workers[37] have also developed an approach based on silica-bead purification (named “TRAPP”) in order to enrich RNA-RBP complexes from cells that is not restricted to polyA-RNA interactome analysis (Figure 2e).

Approaches to study RNA-modification-associated proteins

CLIP and RNA interactome capture strategies are not readily adaptable to study RNA modification-dependent RBPs (or “readers”) since modification levels are typically low and we lack approaches to bias photocrosslinking events in the proximity of modification sites in cells. Therefore, strategies for identifying readers have largely relied upon affinity pulldown with synthetic biotinylated oligonucleotides containing the modified base of interest within a particular sequence context. When combined with quantitative proteomics, this approach has been used successfully to identify readers of N^6-methyladenosine (m^6A)[38–42] and 5-methylcytidine (m^5C)[43,44]. Since RBP readers often bind to modified oligonucleotides with low affinity, additional steps may be necessary in order to efficiently capture these proteins. Notably, Vermeulen and co-workers[38] utilized a synthetic oligonucleotide containing four tandem repeats of the GG(m^6A)CU consensus sequence and SILAC-based quantitative proteomics to profile the m^6A interactome in mammalian cells (Figure 3a). Our lab has developed a photocrosslinking-based chemical proteomics approach[39] relying upon synthetic oligonucleotides modified with a diazirine-containing uridine residue flanking the modification of interest (Figure 3b). For the purpose of discovering new readers, this strategy combines the advantages of photocrosslinking (e.g. stabilization of low-affinity binders and stringent purification conditions) with the versatility of chemical synthesis, enabling the interrogation of any synthetically accessible nucleotide. Both our approach and traditional affinity pulldown strategies rely upon a comparative analysis between enrichment with modified and unmodified oligonucleotides, and therefore reveal both positive and negative effects of modified nucleotides on RNA-protein interaction affinity. In the case of m^6A, for example, the stress granule protein G3BP1 has been characterized as a protein that is repelled by the m^6A modification[38,39].

As a complementary method for RNA modification analysis, we have developed an *in vitro* selection platform for interrogating the sequence binding preferences of epitranscriptomic reader proteins (Figure 3c)[45]. Our strategy relies on the chemical synthesis of a site-specifically modified random-sequence RNA library, affinity selection, and next-generation RNA sequencing. We applied this approach to profile the binding preferences of YTH-domain proteins, the major class of m^6A readers, revealing distinct biochemical preferences for m^6A-modified sequence motifs. This strategy should be readily generalizable to study other RNA modification-protein interactions.

Conclusion and future outlook

The discovery and characterization of new gene regulatory mechanisms operating on the transcriptomic and post-transcriptomic level have spurred increased interest in RNA-centric biology. These advances in our basic understanding of cellular biology have coincided with (and in part were made possible by) transformative technological advances in RNA and DNA sequencing and mass spectrometry-based proteomics. Taking advantage of these technologies, researchers have developed robust approaches for mapping RNA-protein interactions in cellular populations, thereby laying the groundwork for a holistic understanding of the physical associations between the proteome and transcriptome. Moving forward, several areas present promising directions for further study and methodological

development. First, the principles underlying the trafficking and subcellular localization of RNA are still poorly understood, and would benefit from the application and development of tools to study these dynamic processes at subcellular resolution – towards this end, RNA-focused proximity labeling strategies[46–50] have emerged as a promising strategy to address these questions. Second, the effect of RNA post-transcriptional modifications on RNA-protein interactions and cellular processes is still largely unexplored, and we lack general approaches to probe and study modifications on individual transcripts in living cells. Thirdly, transcriptomic and proteomic analyses need to be applied to single cells[51], rather than in bulk, in order to capture the heterogeneity present among cellular populations. Finally, it is important to consider that the approaches described in this review are largely observational and serve as tools for hypothesis generation. Therefore, with the explosion in the number and size of available transcriptomic and proteomic datasets, an important goal is to develop robust strategies for selecting high-confidence interactions that are of functional significance and warrant further biological examination.

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References

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1. Gerstberger S, Hafner M & Tuschl T A census of human RNA-binding proteins. Nat Rev Genet 15, 829–845, doi:10.1038/nrg3813 (2014). [PubMed: 25365966]
2. Roundtree IA, Evans ME, Pan T & He C Dynamic RNA Modifications in Gene Expression Regulation. Cell 169, 1187–1200, doi:10.1016/j.cell.2017.05.045 (2017). [PubMed: 28622506]
3. Schoemaker HJ & Schimmel PR Photo-induced joining of a transfer RNA with its cognate aminoacyl-transfer RNA synthetase. J Mol Biol 84, 503–513, doi:10.1016/0022-2836(74)90112-0 (1974). [PubMed: 4840999]
4. Mayrand S, Setyono B, Greenberg JR & Pederson T Structure of nuclear ribonucleoprotein: identification of proteins in contact with poly(A)+ heterogeneous nuclear RNA in living HeLa cells. J Cell Biol 90, 380–384, doi:10.1083/jcb.90.2.380 (1981). [PubMed: 6169730]
5. Mayrand S & Pederson T Nuclear ribonucleoprotein particles probed in living cells. Proc Natl Acad Sci U S A 78, 2208–2212, doi:10.1073/pnas.78.4.2208 (1981). [PubMed: 6941279]
6. Ellington AD & Szostak JW In vitro selection of RNA molecules that bind specific ligands. Nature 346, 818–822, doi:10.1038/346818a0 (1990). [PubMed: 1697402]
7. Tuerk C & Gold L Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249, 505–510 (1990). [PubMed: 2200121]
8. Licatalosi DD et al. HITS-CLIP yields genome-wide insights into brain alternative RNA processing. Nature 456, 464–469, doi:10.1038/nature07488 (2008). [PubMed: 18978773]
9. Dreyfuss G, Choi YD & Adam SA Characterization of heterogeneous nuclear RNA-protein complexes in vivo with monoclonal antibodies. Mol Cell Biol 4, 1104–1114, doi:10.1128/mcb.4.6.1104 (1984). [PubMed: 6204191]
10. Ule J et al. CLIP identifies Nova-regulated RNA networks in the brain. Science 302, 1212–1215, doi:10.1126/science.1090095 (2003). [PubMed: 14615540]
11. Granneman S, Kudla G, Petfalski E & Tollervey D Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high-throughput analysis of cDNAs. Proc Natl Acad Sci U S A 106, 9613–9618, doi:10.1073/pnas.0901997106 (2009). [PubMed: 19482942]

12. Hafner M et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 141, 129–141, doi:10.1016/j.cell.2010.03.009 (2010). [PubMed: 20371350]
13. Konig J et al. iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution. Nat Struct Mol Biol 17, 909–915, doi:10.1038/nsmb.1838 (2010). [PubMed: 20601959]
14. Van Nostrand EL et al. Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). Nat Methods 13, 508–514, doi:10.1038/nmeth.3810 (2016). [PubMed: 27018577]
15. Zhang C & Darnell RB Mapping in vivo protein-RNA interactions at single-nucleotide resolution from HITS-CLIP data. Nat Biotechnol 29, 607–614, doi:10.1038/nbt.1873 (2011). [PubMed: 21633356]
16. Bohnsack KE, Hobartner C & Bohnsack MT Eukaryotic 5-methylcytosine (m(5)C) RNA Methyltransferases: Mechanisms, Cellular Functions, and Links to Disease. Genes (Basel) 10, doi:10.3390/genes10020102 (2019).
17. Khoddami V & Cairns BR Identification of direct targets and modified bases of RNA cytosine methyltransferases. Nat Biotechnol 31, 458–464, doi:10.1038/nbt.2566 (2013). [PubMed: 23604283]
18. Hussain S et al. NSun2-mediated cytosine-5 methylation of vault noncoding RNA determines its processing into regulatory small RNAs. Cell Rep 4, 255–261, doi:10.1016/j.celrep.2013.06.029 (2013). [PubMed: 23871666]
19. McMahon AC et al. TRIBE: Hijacking an RNA-Editing Enzyme to Identify Cell-Specific Targets of RNA-Binding Proteins. Cell 165, 742–753, doi:10.1016/j.cell.2016.03.007 (2016). [PubMed: 27040499] •• This paper describes an enzymatic strategy for identifying RBP-associated transcripts using ADAR-mediated A:I editing.
20. Xu W, Rahman R & Rosbash M Mechanistic implications of enhanced editing by a HyperTRIBE RNA-binding protein. RNA 24, 173–182, doi:10.1261/rna.064691.117 (2018). [PubMed: 29127211]
21. Lapointe CP, Wilinski D, Saunders HA & Wickens M Protein-RNA networks revealed through covalent RNA marks. Nat Methods 12, 1163–1170, doi:10.1038/nmeth.3651 (2015). [PubMed: 26524240]
22. Castello A et al. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. Cell 149, 1393–1406, doi:10.1016/j.cell.2012.04.031 (2012). [PubMed: 22658674]
23. Baltz AG et al. The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. Mol Cell 46, 674–690, doi:10.1016/j.molcel.2012.05.021 (2012). [PubMed: 22681889]
24. Beckmann BM et al. The RNA-binding proteomes from yeast to man harbor conserved enigmRBPs. Nat Commun 6, 10127, doi:10.1038/ncomms10127 (2015). [PubMed: 26632259]
25. Conrad T et al. Serial interactome capture of the human cell nucleus. Nat Commun 7, 11212, doi:10.1038/ncomms11212 (2016). [PubMed: 27040163]
26. Perez-Perri JI et al. Discovery of RNA-binding proteins and characterization of their dynamic responses by enhanced RNA interactome capture. Nat Commun 9, 4408, doi:10.1038/s41467-018-06557-8 (2018). [PubMed: 30352994] • This report describes an improved strategy for oligo-dT interactome capture using a modified LNA containing probe.
27. Reichel M et al. In Planta Determination of the mRNA-Binding Proteome of Arabidopsis Etiolated Seedlings. Plant Cell 28, 2435–2452, doi:10.1105/tpc.16.00562 (2016). [PubMed: 27729395]
28. Sysoev VO et al. Global changes of the RNA-bound proteome during the maternal-to-zygotic transition in Drosophila. Nat Commun 7, 12128, doi:10.1038/ncomms12128 (2016). [PubMed: 27378189]
29. Castello A et al. Comprehensive Identification of RNA-Binding Domains in Human Cells. Mol Cell 63, 696–710, doi:10.1016/j.molcel.2016.06.029 (2016). [PubMed: 27453046]
30. Bao X et al. Capturing the interactome of newly transcribed RNA. Nat Methods 15, 213–220, doi:10.1038/nmeth.4595 (2018). [PubMed: 29431736] • This paper describes an approach for profiling RBPs associated with nascent RNAs based upon metabolic labeling with 5-EU and click chemistry enrichment.

31. Huang R, Han M, Meng L & Chen X Transcriptome-wide discovery of coding and noncoding RNA-binding proteins. Proc Natl Acad Sci U S A 115, E3879–E3887, doi:10.1073/pnas.1718406115 (2018). [PubMed: 29636419]
32. He C et al. High-Resolution Mapping of RNA-Binding Regions in the Nuclear Proteome of Embryonic Stem Cells. Mol Cell 64, 416–430, doi:10.1016/j.molcel.2016.09.034 (2016). [PubMed: 27768875] •• This method provides a general strategy for mapping RNA-binding regions based upon the decrease in signal intensity of tryptic peptides resulting from RNA photocrosslinking.
33. Kramer K et al. Photo-cross-linking and high-resolution mass spectrometry for assignment of RNA-binding sites in RNA-binding proteins. Nat Methods 11, 1064–1070, doi:10.1038/nmeth.3092 (2014). [PubMed: 25173706]
34. Urdaneta EC et al. Purification of cross-linked RNA-protein complexes by phenoltoluol extraction. Nat Commun 10, 990, doi:10.1038/s41467-019-08942-3 (2019). [PubMed: 30824702] • This report describes a phase separation-based strategy for isolating crosslinked RNA-protein complexes using a modified phenol-toluol organic phase.
35. Trendel J et al. The Human RNA-Binding Proteome and Its Dynamics during Translational Arrest. Cell 176, 391–403 e319, doi:10.1016/j.cell.2018.11.004 (2019). [PubMed: 30528433] •• This paper describes a phase separation-based approach for enriching crosslinked RNA-protein species using Trizol and silica-based based purification.
36. Queiroz RML et al. Comprehensive identification of RNA-protein interactions in any organism using orthogonal organic phase separation (OOPS). Nat Biotechnol 37, 169–178, doi:10.1038/s41587-018-0001-2 (2019). [PubMed: 30607034] • This paper describes a phase separation-based strategy for isolating crosslinked RNA-protein complexes from the interphase layer of Trizol extracted cellular lysate.
37. Shchepachev V et al. Defining the RNA interactome by total RNA-associated protein purification. Mol Syst Biol 15, e8689, doi:10.15252/msb.20188689 (2019). [PubMed: 30962360]
38. Edupuganti RR et al. N(6)-methyladenosine (m(6)A) recruits and repels proteins to regulate mRNA homeostasis. Nat Struct Mol Biol 24, 870–878, doi:10.1038/nsmb.3462 (2017). [PubMed: 28869609] •• This report describes an affinity pulldown with synthetic, m6A-modified oligonucleotides providing a comprehensive view of the m6A interactome in mammalian cells.
39. Arguello AE, DeLiberto AN & Kleiner RE RNA Chemical Proteomics Reveals the N(6)-Methyladenosine (m(6)A)-Regulated Protein-RNA Interactome. J Am Chem Soc 139, 17249–17252, doi:10.1021/jacs.7b09213 (2017). [PubMed: 29140688] •• This paper presents a photocrosslinking-based pulldown approach with synthetic oligonucleotides and applies this strategy to profile the m6A interactome in HeLa cells.
40. Dominissini D et al. Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. Nature 485, 201–206, doi:10.1038/nature11112 (2012). [PubMed: 22575960]
41. Huang H et al. Recognition of RNA N(6)-methyladenosine by IGF2BP proteins enhances mRNA stability and translation. Nat Cell Biol 20, 285–295, doi:10.1038/s41556-018-0045-z (2018). [PubMed: 29476152]
42. Alarcon CR et al. HNRNPA2B1 Is a Mediator of m(6)A-Dependent Nuclear RNA Processing Events. Cell 162, 1299–1308, doi:10.1016/j.cell.2015.08.011 (2015). [PubMed: 26321680]
43. Yang X et al. 5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase and ALYREF as an m(5)C reader. Cell Res 27, 606–625, doi:10.1038/cr.2017.55 (2017). [PubMed: 28418038]
44. Sajini AA et al. Loss of 5-methylcytosine alters the biogenesis of vault-derived small RNAs to coordinate epidermal differentiation. Nat Commun 10, 2550, doi:10.1038/s41467-019-10020-7 (2019). [PubMed: 31186410]
45. Arguello AE, Leach RW & Kleiner RE In Vitro Selection with a Site-Specifically Modified RNA Library Reveals the Binding Preferences of N(6)-Methyladenosine Reader Proteins. Biochemistry, doi:10.1021/acs.biochem.9b00485 (2019).• An *in vitro* selection strategy with a chemically synthesized m6A-modified random sequence library to profile the sequence binding preferences of RNA modification reader proteins.

46. Fazal FM et al. Atlas of Subcellular RNA Localization Revealed by APEX-Seq. Cell 178, 473–490 e426, doi:10.1016/j.cell.2019.05.027 (2019). [PubMed: 31230715] •• Application of the APEX proximity labeling strategy in order to sequence RNA transcripts in a spatially selective manner.

47. Padron A, Iwasaki S & Ingolia NT Proximity RNA labeling by APEX-Seq Reveals the Organization of Translation Initiation Complexes and Repressive RNA Granules. bioRxiv, (2018).

48. Benhalevy D, Anastasakis DG & Hafner M Proximity-CLIP provides a snapshot of protein-occupied RNA elements in subcellular compartments. Nat Methods 15, 1074–1082, doi:10.1038/s41592-018-0220-y (2018). [PubMed: 30478324]

49. Kaewsapsak P, Shechner DM, Mallard W, Rinn JL & Ting AY Live-cell mapping of organelle-associated RNAs via proximity biotinylation combined with protein-RNA crosslinking. Elife 6, doi:10.7554/eLife.29224 (2017).

50. Ramanathan M et al. RNA-protein interaction detection in living cells. Nat Methods 15, 207–212, doi:10.1038/nmeth.4601 (2018). [PubMed: 29400715]

51. Eberwine J, Sul JY, Bartfai T & Kim J The promise of single-cell sequencing. Nat Methods 11, 25–27 (2014). [PubMed: 24524134]

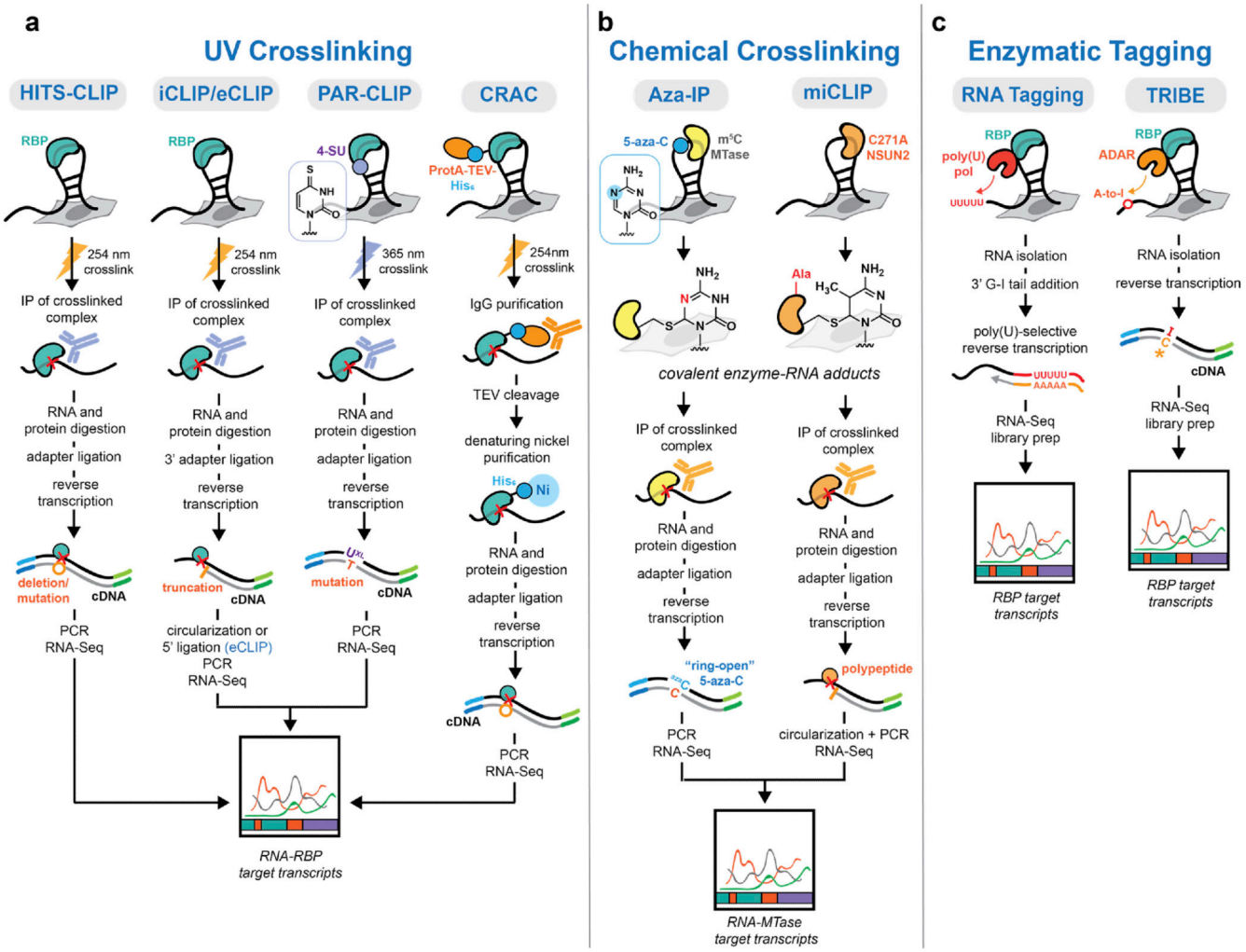

Figure 1.

Methods to identify RNA-protein binding sites. **(a)** Comparison of UV crosslinking-based CLIP methods. RBP footprints are identified by UV crosslinking, immunoprecipitation, and sequencing of covalently linked RNA. Variations on the prototypical HITS-CLIP protocol have been developed including modified photocrosslinking (PAR-CLIP), cDNA library generation (iCLIP/eCLIP), and immunoprecipitation (CRAC). **(b)** Chemical crosslinking approaches take advantage of the catalytic mechanism of RNA m^5C methyltransferases, which involves formation of a covalent adduct with RNA substrates. In the Aza-IP strategy, metabolic incorporation of 5-azacytidine generates stalled covalent adducts at sites of modification. In miCLIP, a mutant RNA m^5C methyltransferase lacking the ability to release itself from RNA substrates generates covalently bound complexes. **(c)** Non-crosslinking methods to map RBP substrates. In RNA Tagging, 3' poly(U) tails are deposited on RBP-associated RNA by a poly(U) polymerase, PUP-2, fused to the RBP of interest. Following RNA isolation, target transcripts are enriched by reverse transcription with a primer specific for uridylated RNAs. TRIBE utilizes an RBP-ADAR construct to label associated RNA transcripts through adenosine to inosine (A:I) editing, which can then identified by RNA sequencing analysis (I pairs to predominantly to C).

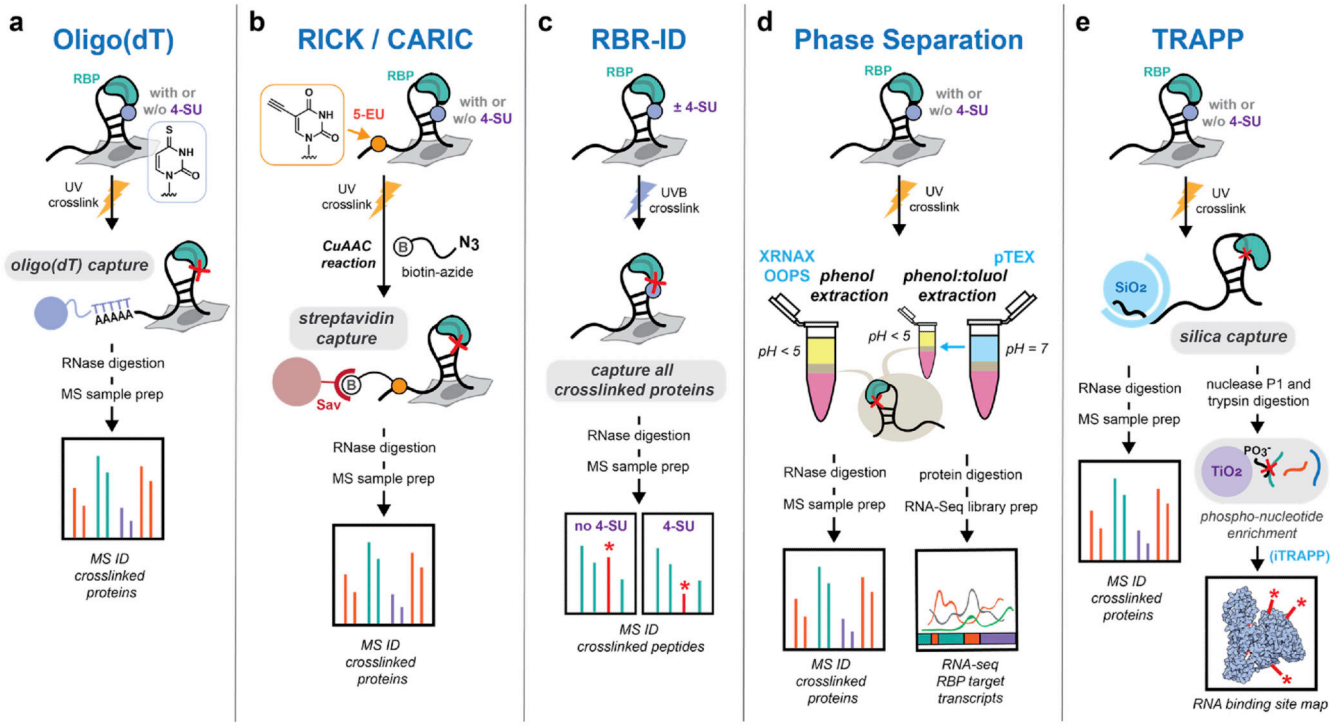

Figure 2.

Methods to profile RNA-binding proteins. **(a)** RBPs that bind to polyadenylated RNA are captured by hybridization of UV-crosslinked protein-RNA to oligo-dT beads. The isolated complexes are digested with RNAse to release proteins for MS analysis. Variations of this method utilizing photocrosslinkable nucleosides such as 4-thiouridine (4-SU) can improve the efficiency of crosslinking. **(b)** RICK/CARIC approaches rely on labeling of nascent RNA transcripts with 5-ethynyluridine (5-EU). After UV treatment, labeled transcripts with crosslinked RBPs can be modified by click chemistry with biotin-azide to enable affinity purification. **(c)** In RBR-ID, RNA binding regions in RBPs are identified by differences in MS signal intensity resulting from UV crosslinking. **(d)** Phase separation techniques use aqueous-organic partitioning to purify RNA-protein complexes based on their unique physicochemical properties. The XRNAX and OOPS methods use acidic guanidinium thiocyanate/phenol/chloroform (Trizol) to capture UV-crosslinked RNA-protein complexes in the interphase between the aqueous and organic layers. In PTex, a preliminary neutral phenol-toluol extraction removes DNA and lipids which would otherwise contaminate the Trizol interphase. **(e)** TRAPP relies on the strong interaction between nucleic acids and silica beads to purify RNA-bound proteins under strongly denaturing conditions. The method can be further applied to map peptide-RNA crosslinks with amino acid resolution by titanium dioxide (TiO_2) enrichment of peptide-nucleotide species for tandem MS analysis.

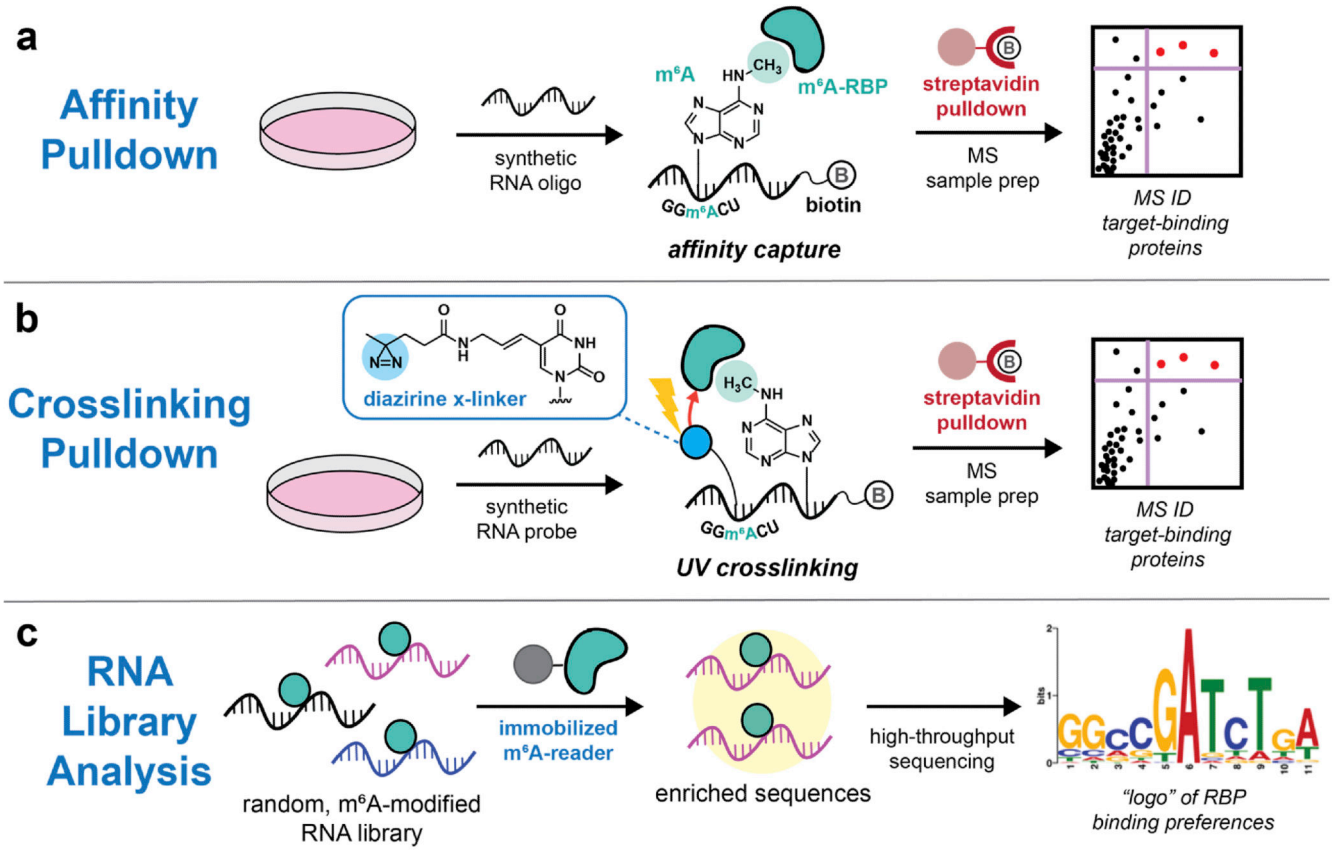

Figure 3.

Methods to study RNA modification-dependent RBPs. **(a)** Affinity pulldown using synthetic modified oligonucleotides. RBP-oligonucleotide complexes are captured by streptavidin and RBPs are identified by mass spectrometry-based proteomics. **(b)** Photocrosslinking pulldown to identify RNA modification readers. To enhance capture of low-affinity binders and facilitate stringent affinity purification, a photocrosslinkable nucleotide can be incorporated near the site of modification in a synthetic oligonucleotide. **(c)** *In vitro* selection of a site-specifically modified RNA library followed by next-generation RNA sequencing enables the identification of the sequence binding preferences of m^6A-binding RBPs.