

Review

Rethinking RNA-binding proteins: Riboregulation challenges prevailing views

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SUMMARY

RNA-binding proteins (RBPs) are best known as effectors along the entire gene expression pathway and as constituents of RNA-protein machines such as the ribosome and the spliceosome. Around 1,000 RBPs account for these functions in mammalian cells. The total number of RBPs has recently more than tripled to include many “well-known” proteins such as metabolic enzymes or membrane proteins, sparking debate about the biological relevance of their RNA binding. We examine the experimental basis underlying the dramatic expansion of the RBPome, consider arguments that challenge its relevance, and discuss recent data that describe new RBP and RNA functions. We suggest that the scope of interplay between RNA and proteins is underexplored and that riboregulation of proteins represents an emerging theme in cell biology and translational medicine.

INTRODUCTION

RNA-binding proteins (RBPs) enjoy recognition for their critical roles in gene expression and as structural elements of RNA-protein machines. Many of these nearly 1,000 mammalian RBPs bind RNA with a high degree of specificity by recognition of short RNA sequences and/or structural motifs, often mediated by one or several of their specialized globular RNA-binding domains (RBDs).^{1–4} Decades of intensive research have yielded a solid understanding of the underlying biochemistry, functions, and structural aspects of RNA-protein interactions. In many cases, we can explain how RBP-binding specificity and affinity for RNA jointly foster the desired biological outcomes and how biological cues regulate RNA-protein interactions.^{5–8}

When system-wide proteomic approaches effectively tripled their count,^{3,9–14} the dramatic growth in the number of (candidate) RBPs disturbed this satisfactory setting. Confusingly, among the newly identified proteins, many so-called non-canonical RBPs were identified, which lack recognizable RBDs, the key determinants of RNA-binding specificity of previously identified RBPs. Even more of a surprise, these new RBPs have not emerged from the dark matter of uncharacterized polypeptides in search of a function but mostly represent the exact opposite: well-studied proteins with validated cell biological functions such as metabolic enzymes, signaling molecules, adapters, or membrane channels without any discernible connection to RNA biology.^{9,15,16} Their unexpected, apparent RNA binding raised concerns rather than provided looked-for answers, and for most of these new RBPs, there simply did not seem to be a place for a meaningful role of RNA binding.

With the discovery of riboregulation, this puzzling situation recently changed profoundly for some of these non-canonical RBPs, uncovering new RNA functions on the way. This review will build on textbook features of classical RBPs and further aims to broaden the perspective of biologically meaningful RNA-protein interactions.

CLASSICAL RBPs—THEIR FEATURES AND FUNCTIONS

RBPs play directive roles along the entire gene expression pathway, including RNA transcription, splicing, modification, transport, localization, stability, and translation of RNAs.^{17,18} They rarely do so as soloists but commonly in ensembles of ribonucleoprotein (RNP) complexes.¹⁸ As such, RBPs also mediate interactions between RNA and other proteins, contributing to the assembly of sometimes highly complex RNPs, such as, e.g., the spliceosome or the ribosome.

RBP-RNA interactions—How partners find each other

RNA-protein interactions are dynamic and influenced by a multitude of factors, including the concentration of interaction partners, their affinity, RNA modifications, and protein post-translational modifications (PTMs).^{18–20} Specific interactions are frequently achieved by the combinatorial use of different RBDs, as illustrated below with the fused in sarcoma (FUS) protein.^{1,21} These determinants collectively ensure controlled interactions between RBPs and their RNA targets. Despite their diversity, all forms of RBP-RNA binding are governed by fundamental biophysical principles, including hydrogen bonding, hydrophobic interactions, and stacking interactions, whose number and combinations determine RNA-binding



affinity and specificity, as reviewed by Corley et al.¹, Jankowsky et al.⁵, and Helder et al.²² Binding affinity measures how strongly an RBP binds to RNA, i.e., how rapidly the interaction is established and for how long it lasts. Specificity, on the other hand, refers to the selectivity of an RBP for its targets compared with other RNAs in general. Specificity and affinity are frequently, but not always, linked. Highly specific RBPs such as the histone stem-loop-binding protein (SLBP) bind to their biologically relevant binding sites with orders of magnitude higher affinity (nanomolar range) than to other RNAs (micromolar range or weaker).²³ As one might guess, sequence specificity of RNA binding often hinges on whether the RBD largely interacts with the RNA bases or the RNA backbone. RBPs that bind with low sequence specificity (being able to bind to many different RNAs), like helicases, primarily interact with the RNA backbone, whereas sequence-specific RNA binders, such as the Pumilio and FBF (PUF) domain proteins, interact predominantly with RNA bases.¹ The affinity and specificity of a given RBP need to evolve in tune with their function(s) in the physiological context. A high binding affinity does not ensure high specificity. Conversely, relatively low-affinity interactions can be highly specific.

The RNA-binding activity of many RBPs is architecturally rooted in globular RBDs that have evolved to recognize specific features of the RNA sequence, the RNA backbone, and/or structure. RBDs typically include α -helices and/or β -sheets.¹ They have been identified and characterized by biochemical and biophysical approaches as well as structurally. Computational prediction tools for RBDs²⁴ have also proven useful for the identification of RBPs, recognizing additional “classical RBPs” by the presence of RBD motifs. Over 30 different RBDs have been described, often co-occurring as multiples within RBPs, and their molecular interactions with RNA have been extensively studied and reviewed.¹ Each RBD displays prototypic structural features and biophysical properties that facilitate its role in RNA binding, yet RBDs also share some common characteristics based on the biophysical principles of intermolecular interactions. Modular combinations of RBDs often determine the RNA-binding specificity and the affinity between RBPs and their bound RNAs.¹⁷ The RNA recognition motif (RRM), the heterogeneous nuclear ribonucleoprotein (hnRNP) K-homology (KH) domain, and helicase domains are representatives of the larger family of RBDs and illustrate diverse RNA-binding strategies. Most RRMs bind with moderate, KH domains typically with high, and helicases—as ATP-driven remodelers—with low sequence or structure specificity to RNA. Intrinsically disordered regions (IDRs) have also recently emerged as common RNA interactors.¹⁵ By highlighting these examples, we set the stage for the discussion of non-canonical RBPs.

The RRM

The RRM stands out as one of the most prevalent and extensively studied RBDs. It is found in approximately 1% of the human proteome, and it has been documented in over 500 structures, including hnRNPs and splicing factors as prominent examples.¹ RRMs typically span 90–100 amino acids and are characterized by two conserved motifs, RNP1 and RNP2, which are essential for RNA binding. Structurally, RRMs are composed of an antiparallel β -sheet flanked by two α -helices. The β -sheet

surface interacts with 2–8 nucleotides of single-stranded RNA (ssRNA), often achieving nanomolar binding affinities.^{25,26} RRMs exhibit remarkable versatility and can bind a broad spectrum of RNA sequences and structures, thereby playing crucial roles in diverse RNA processing events.^{1,27,28} The combination of multiple RRMs often contributes to achieving high RNA-binding affinity and specificity, as seen for hnRNP A1. HnRNP A1, involved in pre-mRNA processing, uses both of its two RRM domains to simultaneously bind its specific RNA target in exon 7 of the survival of motorneuron 2 (SMN2) pre-mRNA.²⁹ Apart from RNA binding, RRMs can participate in protein-protein interactions. A prominent example is the U2AF homology motif (UHM), originally described in the splicing factor U2AF65 and found thereafter in many other proteins.^{30–32}

KH domain

KH domains bind ssRNA or single-stranded DNA (ssDNA), recognizing 4-nucleotide sequence motifs.³³ They occur in several hnRNPs and proteins regulating translation. Comprising ~70 amino acids, the KH domain forms a triple-stranded β -sheet flanked by three α -helices in eukaryotes.³³ RNA binding occurs via a hydrophobic pocket featuring a conserved “GXXG” motif.²⁵ The presence of glycine residues in the GXXG motif increases the structural flexibility of the KH domain, allowing it to bind ssRNA as well as ssDNA.³⁴ Interactions of single KH domains with RNA are generally not very strong, with binding affinities in the micromolar range.²⁵ However, the binding affinity of KH domain-containing RBPs is usually enhanced by the presence of multiple KH domains or by co-occurrence with other RBDs.

Helicase domain

Helicase domains bind nucleic acids and ATP, utilizing the energy of ATP hydrolysis to unwind inter- or intramolecular double-stranded regions of DNA or RNA.³⁵ They can be sub-classified into superfamilies, with the DEAD-box helicases (named after the presence of Asp-Glu-Ala-Asp) as a prominent example¹ involved in, e.g., translation, splicing, and ribosome biogenesis. Helicase domains are relatively large and span 350–400 amino acids, including multiple subdomains that each harbor an ATP-binding catalytic core and an RNA-binding region. Helicase domains contact multiple nucleotides simultaneously, with affinities often in the nanomolar range.¹ Since helicases unwind nucleic acid structures, they bind in a sequence-independent way with hydrogen bonds formed between the helicase domains and the phosphate-sugar backbone of DNA or RNA.

IDRs

Despite their lack of the organized structure of globular protein domains, IDRs have emerged as commonly employed RNA-binding modules.^{3,6} The frequency at which IDRs appear to contribute to RNA binding came as a surprise when first recognized,¹⁵ but their lack of pre-formed structure and their ability to adopt “induced fits” make them perfectly suited to RNA binding.³⁶ IDRs critically contribute to the formation of phase-separated membrane-less organelles.^{37,38} RNA-binding IDRs often include arginine/serine (RS) or arginine/glycine (RGG box) repeats or harbor arginine-/lysine-rich patches (R/K basic

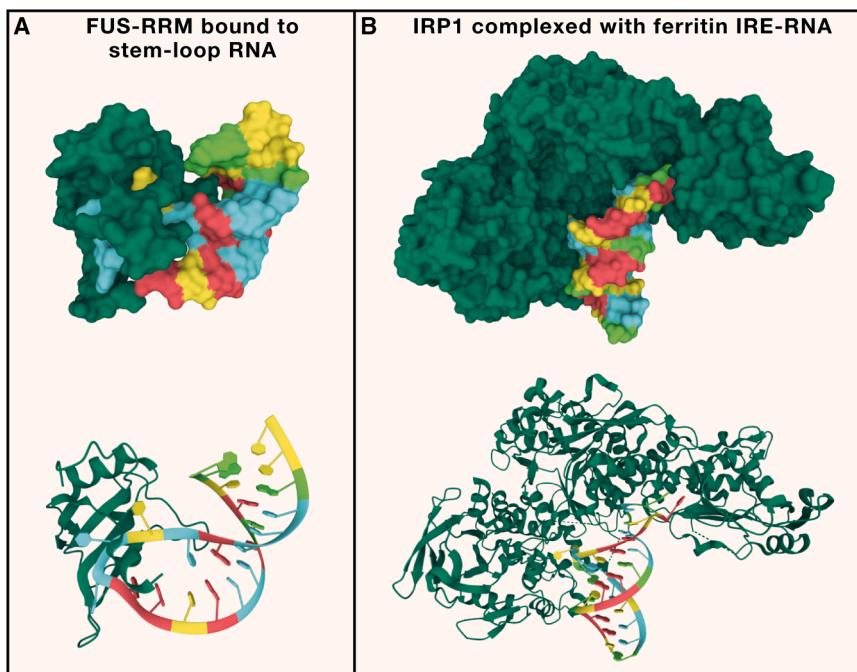


Figure 1. Structures of FUS RRM and IRP1 in complex with their respective RNA targets

(A) Structure of FUS RRM bound to stem-loop RNA (visualized using NAKB [<https://nakb.org/>]; PDB: 6GBM; see Loughlin et al.²¹).
(B) Structure of IRP1 complexed with ferritin IRE-RNA (visualized using NAKB [<https://nakb.org/>]; PDB: 3SNP; see Walden et al.⁴⁶).

patches).^{6,39,40} They generally exhibit low RNA sequence specificity, relying instead on electrostatic interactions between the (positively charged) amino acid repeats and the (negatively charged) phosphodiester backbone of RNA.^{39,40} On the other hand, emerging studies suggest that IDRs can sometimes exhibit sequence specificity in RNA binding. The RGG box of fragile X mental retardation protein (FMRP) binds to the base of a synthetic G-quadruplex through shape complementarity and base-specific contacts with several nucleotides.^{41,42} This strategy is likely shared by other RBPs harboring RGG motifs, for example, FUS (see below).⁴³ Upon RNA binding, IDRs typically undergo a disorder-to-order transition, adopting a more ordered structure.⁶ Recent studies, however, suggest that some IDRs tend to form transient, fuzzy complexes rather than undergo conformational transitions, and while this has been observed in protein-protein interactions,⁴⁴ further research is needed to clarify their role in protein-RNA binding.

IDRs can mediate RNA binding independently and are perfectly suited to binding RNA in combination with globular RBDs within an RBP.^{6,15} Indeed, IDRs frequently co-occur with globular RBDs. An illustrative example is FUS, which binds pre-mRNAs and couples transcription and splicing.⁴⁵ FUS not only harbors an RRM (Figure 1A) and a zinc finger (ZnF) domain but also an RGG box repeat-containing IDR that is involved in RNA binding.^{6,21} FUS thus represents a paradigm of a modular RBP that combines globular RBDs with an IDR to establish binding specificity and affinity in support of its functions.

Classical RBPs can be defined by the presence of well-characterized RBDs (Figure 1A) and functional annotations that link them to RNA biology. By contrast, non-canonical RBPs lack RBDs, and their primary functional annotations relate to other aspects of cell biology (Table 1). While these distinctions provide a

useful framework, it can be difficult to apply retrospectively, as historical data may not clearly indicate whether an RBP fits the classical definition. In some cases, proteins initially categorized as non-canonical have later been found to contain RBDs. Some non-canonical RBPs have been found historically early—in “classical times” of posttranscriptional gene expression—blurring the boundaries between the two groups. Some long-known and well-characterized RBPs, such as the iron regulatory protein 1 (IRP1, see below), do not possess conventional RBDs and were first recognized as metabolic enzymes,

thus placing them into the category of non-canonical RBPs. IRP1 binds highly specific RNA elements known as iron-responsive elements (IREs) by contacts along the entire ~100 kDa protein (Figure 1B).⁴⁶ This situation differs from another well-characterized RBP, SLBP. SLBP regulates histone mRNA 3' end formation by binding the 3' stem loop through a unique 73-amino-acid RBD distinct from previously known RBDs.⁴⁷ Thus, we consider SLBP to represent a classical RBP (with an RBD found late), whereas IRP1 appears to represent a well-understood example of a non-canonical RBP that was found historically early.

A PLETHORA OF NEW RBP CANDIDATES FROM SYSTEMATIC DISCOVERY

Our knowledge of RBPs was gained in a cumulative fashion using largely biochemical and genetic approaches, as well as collective high-throughput efforts featured in encyclopedia of DNA elements (ENCODE) (<https://www.encodeproject.org/>). During the past decade, comprehensive “omics” methods for the systematic discovery of proteins bound to RNA *in cellulo* led to the discovery of many hundreds of new candidate RBPs.^{3,9} A large fraction of these represented rather unexpected candidates, lacking annotated RBDs and recognized functions related to RNA biology. Instead, these proteins were described with primary roles in, e.g., metabolism, cell signaling, or cellular transport.

To date, dozens of such omics studies are integrated into publicly accessible databases such as RBPbase (<https://apps.embl.de/rbpbase/>), RBP to Gene Ontology (RBP2GO) (<https://rbp2go.dkfz.de/>), and eukaryotic RBP database (EuRBPDB) (<http://eurbpdb.gzsys.org.cn/>), which list thousands of proteins

Table 1. Comparison of classical and non-canonical RBPs

| Features | Classical RBP | Non-canonical RBP |
|---------------------------------|--|---|
| Primary method of discovery | Cumulatively identified over decades by biochemical, genetic, and computational means | Only a few “historical” examples, mostly identified since the introduction of RIC-like methods |
| Primary function | (Post-)transcriptional RNA metabolism, component of RNP machines | Primarily known for non-RNA-related functions such as enzymatic functions, transmembrane transport, or signaling |
| RNA-binding surface(s) | One or several globular RNA-binding domains (RBDs), such as, e.g., RRM, KH, zinc finger, etc.; can also harbor intrinsically disordered regions (IDRs) | No classical RBDs, frequently intrinsically disordered regions (IDRs); can involve contacts along much of the protein |
| Binding specificity | Often sequence- and/or structure-specific, determined by the nature and combination of RBDs present | Less well studied; to be determined |
| Binding affinity | Typically in the pico- or nanomolar range and determined by the combination of multiple RBDs plus, in some cases, IDRs | Variable; often contributed by IDRs |
| <i>In cellulo</i> RNA occupancy | Typically high | Often intermediate to low due to transient (regulatory) interactions |
| Structural information | Many structures of classical RBPs bound to RNA and of RNP machines are available | Structural information for non-canonical RBPs bound to RNA is only beginning to emerge |

found to bind RNA across mammalian and non-mammalian species.^{48,49} Taken together, these studies suggest that a surprising 10%–20% of cellular proteomes can bind RNA, raising the question of their underlying biological functions. To critically assess the experimental basis that gave rise to these surprisingly large numbers, we briefly summarize the technical approaches that yielded their identification. As a common feature, these approaches all share *in vivo/in cellulo* ultraviolet light (UV)-mediated covalent crosslinking between ssRNA and proteins that are directly bound (at “zero distance”), thus capturing RNA-protein interactions^{50,51} (Figure 2). Importantly, and unlike chemical crosslinking with, e.g., formaldehyde, UV crosslinking has little tendency to promote protein-protein crosslinks,^{52–54} which means that RNA-crosslinked proteins are highly enriched for direct RNA binders. On the other hand, UV crosslinking covalently fixes even transient chance interactions, and therefore outcomes of UV-crosslinking experiments have to be interpreted carefully against this background, especially when abundant cellular proteins are implicated as RNA binders. Finally, crosslinking approaches merely select for the biophysical property of RNA binding of a protein. Consequently, the resulting lists of RBPs must be interpreted as indicative of RNA binding alone, without any implication (positive or negative) regarding the functional significance of it.

RNA interactome capture

Two studies from 2012 identified ~800 proteins bound to polyadenylated RNAs in human cells, greatly expanding the mRNA-bound proteome^{10,11} (Figure 2A). Using oligo(dT) beads, polyadenylated RNAs were isolated together with their UV-crosslinked proteins. Rigorous washing was applied to remove non-crosslinked proteins before release by RNase treatment and identification by mass spectrometry (MS). Following these initial reports, UV crosslinking and oligo(dT) capture (RNA interactome capture [RIC]) have been applied to numerous cell lines

and model organisms, leading to an increasing identification of non-canonical candidate RBPs.^{16,55–60} The replacement of oligo(dT) by LNA-modified oligo(dT) beads (enhanced RIC [eRIC]) allowed for even more stringent washing of captured RNA-protein complexes, resulting in the removal of contaminants that arise from nucleic acid–nucleic acid interactions and increase the signal-to-noise ratio.⁶¹ In addition to the analysis of cultured cells, *ex vivo* eRIC was developed to survey the RBPs of whole organs or biopsy specimens.⁶² It confirmed earlier observations regarding non-canonical RBPs from cultured cells and further added to their number. Importantly, this study reported that most crosslinked proteins matched their expected molecular masses, ruling out major protein-protein crosslinking and supporting their assignment as direct RBPs.⁶²

Silica-based approaches

To enable global assessments of RBPs bound to all RNA species independent of their polyadenylation status, silica-based approaches such as total RNA-associated protein purification (TRAPP) and 2C (complex capture) were developed.^{14,63} These exploit the capacity of silica columns or beads to efficiently retain RNA based on its charge, to co-capture UV-crosslinked proteins in this way, and to identify these in the eluates by mass spectrometry (MS) (Figure 2B).^{14,64} Since binding and elution conditions can be adjusted to favor the retention of short RNAs, silica columns have also been used to characterize the RBPome bound to small RNAs.⁶⁴ Typically, analyses include negative/specificity controls of samples without prior UV crosslinking and/or pre-treated by RNase to assure that silica matrix retention is based on genuine RNA binding.

Methods based on differential solubility

As an alternative to silica matrix binding, the differential solubility of crosslinked RNA-protein complexes in organic solvents has been utilized to allow for the unbiased recovery of RBPs using

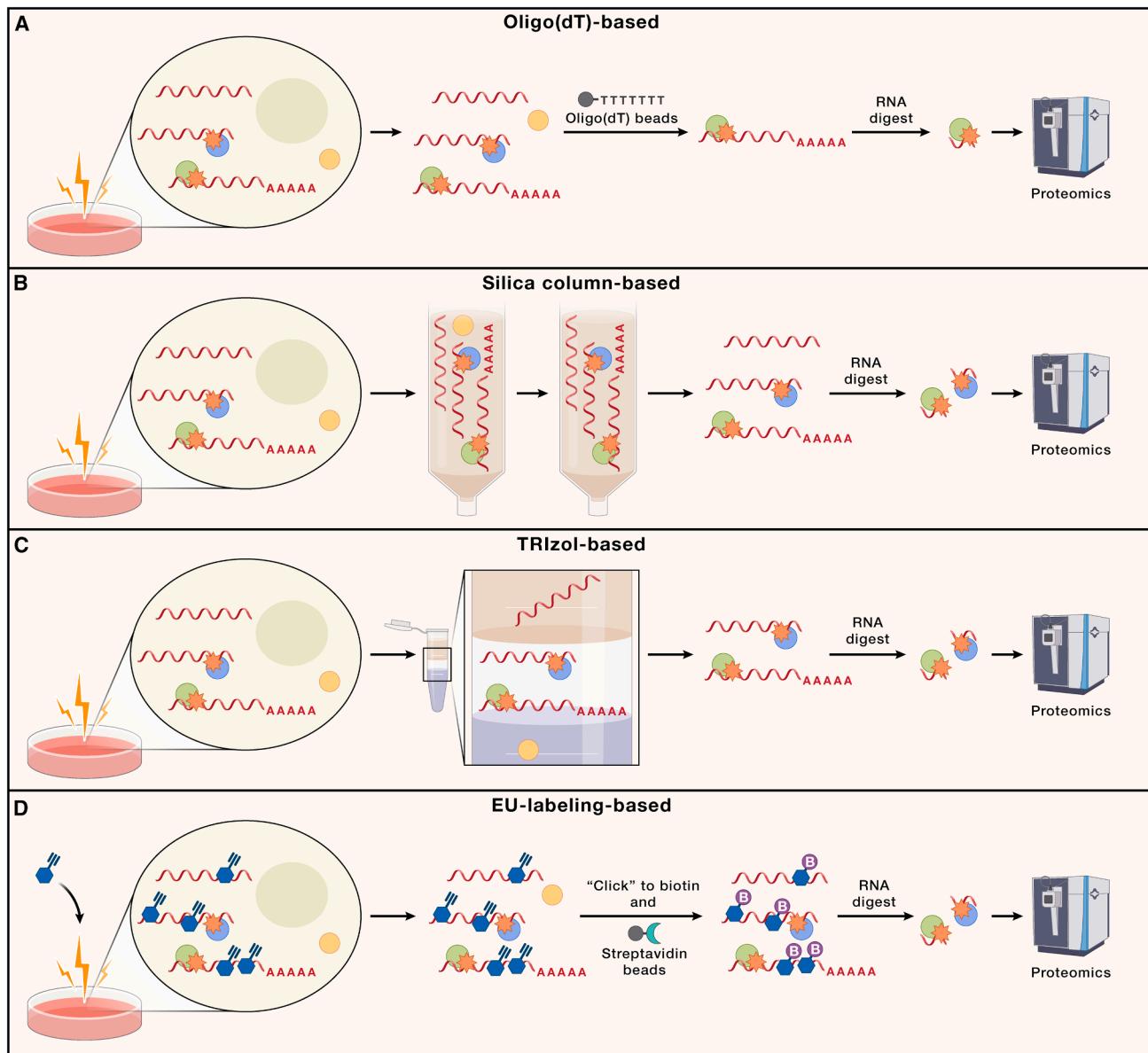


Figure 2. Global analyses of RNA-bound proteomes uncover hundreds of new candidate RBPs

- (A) RIC analysis is based on the pull-down of polyadenylated RNA using Oligo(dT) beads after UV-based RNA-protein crosslinking.
- (B) Silica columns or beads are used to pull down all RNA species, co-purifying crosslinked RNA-protein complexes.
- (C) TRIzol-based extraction partitions crosslinked RNA-protein complexes to the interphase. Alternatively, acid guanidinium thiocyanate-phenol-chloroform or phenol-toluol extractions paired with acidic phenol extraction can be performed. Following RNA-protein complex purification, RNA is usually digested, and the RNA-bound proteome is analyzed by MS.
- (D) EU labeling of nascent RNA precedes UV-crosslinking and cell lysis. EU-labeled RNAs are biotinylated using click-iT chemistry, and streptavidin beads are used for RNA pull-down. The click-iT chemistry can be performed directly after cell lysis, or cells are fixed and permeabilized after crosslinking and the click-iT reaction is performed prior to cell lysis.

methods referred to as orthogonal organic phase separation (OOPS), crosslinked RNA extraction (XRNAX), or phenol-toluol extraction (PTex) (Figure 2C)^{12,13,65}. Crosslinked RNA-protein complexes typically partition to the interphase of classical acidic guanidinium-thiocyanate-phenol-chloroform or TRIzol extraction buffers.^{12,13} Alternatively, phenol-toluol extractions paired with acidic phenol extraction can be performed,⁶⁵ followed by MS.

RNA labeling-based analyses

To assess RBP binding to newly synthesized transcripts, the uridine analogs 4-thiouridine⁶⁶ or 5-ethynyluridine (EU) can be used to (pulse-)label RNA prior to UV crosslinking and cell lysis (Figure 2D).^{67,68} EU contains an alkyne reactive group that can be “clicked” to a biotin azide using click-iT chemistry. Thus, newly transcribed RNAs are biotinylated, and streptavidin-based

pull-down enables analysis of all RNA species, not limited to polyadenylated RNA. In addition, by timing the pulse labeling phase and adapting the length of a chase period, changes and regulation of RBP binding during RNA biogenesis and decay can be analyzed.^{66–68}

CHARACTERISTICS OF NON-CANONICAL (CANDIDATE) RBPs

Since non-canonical RBPs lack the RBDs that characterize classical RBPs, experimental methods were needed to map their RNA-binding regions. These methods mostly follow RIC or silica-based protocols and then aim to pinpoint the region(s) of the protein to which the RNA crosslink has occurred using MS.^{15,69,70} Interestingly, a substantial fraction of non-canonical RBPs interacts with RNA via other well-characterized globular domains such as those annotated as, e.g., catalytic cores, cofactor-binding domains, regions of protein-protein interactions, or DNA-binding domains.^{15,69,70} Strikingly, however, nearly half of all the RNA-binding peptides identified in one study map to IDRs, with many RBPs harboring an IDR as the sole detectable RNA-binding region.¹⁵ The RNA-interacting regions were found to be enriched for positively charged and aromatic residues, suggesting that the structural flexibility of IDRs in combination with electrostatic and stacking interactions contributes to RNA binding. In addition, numerous metabolic enzymes that bind nucleotide cofactors such as NAD⁺, NADPH, FAD⁺, or coenzyme-A have recurrently been identified as RBPs by different methods and across different model systems, with the di-nucleotide cofactor-binding Rossmann fold emerging as a structure that frequently binds RNA.^{11,12,15,16,62,71} Furthermore, a previously uncharacterized domain similar to the arginine-rich motif of the HIV transcriptional activator Tat has recently been found in many transcription factors that also bind RNA.⁷² Taken together, some globular domains previously not implicated in RNA binding have been identified to serve as RNA-binding modules of newly identified RBPs. While RBDs represent domains that have evolved to bind RNA, RNAs may potentially have evolved to bind to many of the proteins that lack RBDs, akin to aptamers that can be evolved *in vitro* to bind to surfaces that themselves did not evolve to bind RNA.^{73–75} Insights from validated, non-canonical RBPs

INSIGHTS FROM VALIDATED, NON-CANONICAL RBPs

Some non-canonical RBPs were discovered as physiologically relevant *trans*-acting factors involved in posttranscriptional regulation long before omics methods were applied. For example, searching for the protein-binding partners of *cis*-regulatory elements of individual mRNAs by biochemical or genetic methods, several enzymes of intermediary metabolism were identified to “moonlight” as RBPs binding to specific RNAs. Already in the early 1990s, cytosolic aconitase (which interconverts between citrate and isocitrate) was recognized to be identical to IRP1, playing a dual role as a metabolic enzyme and as a posttranscriptional regulator of mRNAs that encode proteins involved in iron metabolism.⁷⁶ IRP1 binds to iron-responsive elements (IREs), ~30-nucleotide-long stem-loop structures with

distinct sequence motifs, which occur within the untranslated regions (UTRs) of their target mRNAs, such as the 5' UTR of ferritin or the 3' UTR of transferrin receptor mRNAs, and regulates their translation or stability, respectively.^{77–79} The cellular iron levels operate a dynamic switch between the two activities of the protein. Under iron-replete conditions, a 4Fe–4S iron-sulfur cluster is assembled, which allows IRP1 to function as an enzyme but precludes RNA binding. On the contrary, the iron-sulfur cluster disassembles in iron deficiency, allowing the apo-protein to bind to IREs and exert posttranscriptional control of its target mRNAs.^{46,80} The two activities of the protein are thus mutually exclusive and represent an elegant example of how an enzyme can co-opt additional moonlighting roles in RNA biology in a context-dependent manner.

Another example of a moonlighting metabolic enzyme is the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate using NAD⁺ as a cofactor. Beyond its enzymatic role, GAPDH binds to different RNA targets, including mRNAs, tRNAs, rRNAs, and viral RNAs.^{81,82} GAPDH has been reported to bind to AU-rich elements (AREs) within the 3' UTR of target mRNAs, regulating their stability and translation.^{81,83,84} Competition experiments show that RNA binding by GAPDH is sensitive to the cofactor NAD⁺, suggesting involvement of the Rossmann fold (that binds NAD⁺) in RNA binding.^{83,85} Indeed, other enzymes that bind mono- or di-nucleotide cofactors or substrates, such as lactate dehydrogenase (LDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), alcohol dehydrogenase (ADH), thymidylate synthase (TS), or dihydrofolate reductase (DHFR), have also been implicated as RBPs.^{85–93} While the dehydrogenases bind NAD⁺ as a cofactor, TS and DHFR bind to their nucleotide substrates, deoxyuridine monophosphate (dUMP) and dihydrofolate, respectively.^{88–90} Nucleotide cofactors or substrates compete with RNA binding in several of these examples, suggesting possible feedback regulation and giving rise to the idea that the di-nucleotide-binding domains might have evolved to bind longer stretches of ribonucleotides.⁹¹ This intricate interplay between RNA, enzymes, and metabolites gave rise to the RNA-enzyme-metabolite (REM) hypothesis, which posits that these three entities are part of a complex regulatory network where they mutually influence each other.⁹⁴

In addition to metabolic enzymes, other proteins involved in various cellular processes have been identified to moonlight as RBPs. For example, the cell cycle regulator cyclin A2 binds to the 3' UTR of the meiotic recombination 11 (Mre11) mRNA and enhances its translation, presumably facilitating the recruitment of the translation initiation factor eIF4A2.⁹⁵ The cyclin A2-mediated increase in Mre11 expression helps to repair DNA lesions caused by replication errors, complementing its roles in the initiation and progression of DNA synthesis during S phase.⁹⁵

The mammalian protein chaperone Hsp70 also binds to AU-rich sequences within the 3' UTRs of several mRNAs and regulates their stability despite lacking any canonical RBD.^{96,97} Furthermore, Ssa2p, a major cytoplasmic Hsp70 in *Saccharomyces cerevisiae*, directly binds to tRNAs and facilitates their nuclear import under nutrient starvation.⁹⁸ A recent meta-analysis of RNA interactome datasets revealed that about half of all

chaperones in human cells (151 of 332 chaperone proteins) have been implicated to bind directly to RNA, pointing to widespread interactions between protein chaperones and RNA, although the functional significance of these interactions remains to be explored.⁹⁹

The emergence of riboregulation

Some proteins of the innate immune system, including protein kinase R (PKR), retinoic acid-inducible gene-I (RIG-I), or Toll-like receptor (TLR) 3, TLR7, and TLR8, share an interesting characteristic: they are activated by (pathogen-derived) RNAs.^{100–103} While this aspect has been appreciated as a critical feature of their mechanisms of innate immune system activation, the underlying common principle has received little attention from an RNA biology perspective. Unlike most of the classical RBPs, these RBPs of the innate immune system are regulated by RNA instead of regulating RNA fate themselves. They represent early examples of riboregulation and RNA-mediated control of protein function, together with a few others spanning distinct aspects of cell biology.

The recent surge in the number of non-canonical RBPs with well-known functions unrelated to RNA biology motivated explorations of whether RNA may exert riboregulatory functions far more broadly than the innate immune system, with a profound harvest already (Figure 3).

Riboregulation of protein-protein interactions

Starvation signals such as serum and amino acid deprivation trigger the homo-oligomerization of the selective autophagy receptor p62/sequestosome-1, promoting autophagy.¹⁰⁴ p62 had been identified as a non-canonical RBP,¹⁵ and prior research had not indicated a possible function of RNA binding. The small non-coding vault RNA1-1 (vtRNA1-1) was identified to bind to the N-terminal PB1 domain of p62, which is critical for p62 oligomerization, disrupting interactions between p62 monomers and inhibiting autophagy. Furthermore, starvation induces a rapid decrease of vtRNA1-1 levels and p62 binding, liberating the monomers for oligomerization.¹⁰⁵ Therefore, a specific, small non-coding RNA riboregulates autophagy by controlling a critical protein-protein interaction in a signal-responsive way.^{105,106}

tRNA-derived fragments (tRFs), once regarded as mere degradation products of tRNAs, also have been implicated as regulators of protein-protein interactions in cells. For instance, pseudouridylated mini tRFs containing a 5' terminal oligo-guanine (mTOG-Ψ) bind polyadenylate-binding protein cytoplasmic 1 (PABPC1) and directly interfere with the binding and recruitment of its translational co-activator PABPC1-interacting protein 1 (PAIP1), leading to repression of translation of cancer-associated transcripts.¹⁰⁷ By contrast, the cysteine tRNA fragment 5'-tRFCys, which is upregulated during breast cancer metastatic progression, appears to facilitate the oligomerization of nucleolin, allowing it to bind to the 5' UTR of target transcripts and to form a protective ribonucleoprotein complex that shields transcripts from degradation.¹⁰⁸ Collectively, these findings highlight the immense potential of (small non-coding) RNA fragments, whose roles were poorly defined in the past, to control fundamental biological processes.

In addition to small non-coding RNAs, circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) can also directly regulate protein-protein interactions. Examples include circACC1, a circRNA transcribed from the human acetyl-CoA carboxylase 1 (ACC1) locus, which positively controls AMP-activated protein kinase (AMPK), a sensor of cellular energy status.¹⁰⁹ The AMPK holoenzyme complex is a tri-complex of the catalytic α subunit and regulatory β and γ subunits. The circACC1 RNA, whose expression is induced under serum-deprived conditions, specifically binds to the β and γ subunits of AMPK and promotes the assembly and activation of the AMPK holoenzyme complex, which in turn facilitates metabolic adaptation in serum-starved cells.¹⁰⁹ Interestingly, another study reported that the lncRNA NBR2, which is induced by AMPK, binds AMPK and stimulates its activity, forming a feed-forward loop under energy-stress conditions.¹¹⁰ This example highlights the possibility of multilayer riboregulatory effects of RNA on protein complexes in cells.

lncRNA IDH1-AS1 binds to the enzyme isocitrate dehydrogenase 1 (IDH1), which converts isocitrate to α-KG. Like the circACC1-AMPK complex, the lncRNA IDH1-AS1 does not merely promote dimerization of IDH1 but forms a stable ternary complex with the enzyme, which in turn promotes enzyme activation.¹¹¹ lncRNAs can also drive key cellular processes by inhibiting protein-protein interactions. For example, lnc-DC, an lncRNA specifically expressed during dendritic cell (DC) differentiation, binds directly to the C-terminal region of signal transducer and activator of transcription 3 (STAT3) in the cytoplasm and prevents its association with the Src homology region 2 domain-containing phosphatase-1 (SHP1). This inhibition blocks SHP1-mediated dephosphorylation of a key tyrosine residue within the C-terminal region, thereby enhancing STAT3 phosphorylation and activation.¹¹² Additionally, lncRNAs have been shown to stabilize proteins by inhibiting their association with ubiquitin ligases. For instance, the lncRNA hFAST, a cytosolic lncRNA localized in human embryonic stem cells, activates Wnt signaling by inhibiting the interaction of phosphorylated β-catenin with the E3 ubiquitin ligase β-TrCP, thereby preventing its degradation.¹¹³ Similarly, the AKT/STAT3-induced lncRNA vimentin-associated lncRNA (VAL, LINC01546) promotes metastasis of lung adenocarcinoma by directly binding to vimentin and preventing the recruitment of the TRIM16 E3 ligase, thereby abrogating vimentin polyubiquitination and degradation.¹¹⁴

Non-coding RNAs have thus emerged as RNA effectors that can regulate protein assemblies, especially under stress, in cell differentiation or disease (Figure 3A).

Direct riboregulation of enzymatic activities

In addition to altering enzymatic complexes, RNA can also directly regulate the activity of enzymes (Figure 3B). Enolase 1 (ENO1), which catalyzes the penultimate step in glycolysis, is riboregulated during the differentiation of mouse embryonic stem cells (mESCs).¹¹⁵ RNA appears to bind close to the catalytic center of the enzyme, inhibiting its activity and competing with the enzyme's substrates for binding. The RNA-binding activity of ENO1 is regulated post-translationally during differentiation by lysine acetylation, providing a mechanism for regulated RNA binding. Unlike most of the examples discussed above, ENO1 can bind thousands of distinct RNA sites across the

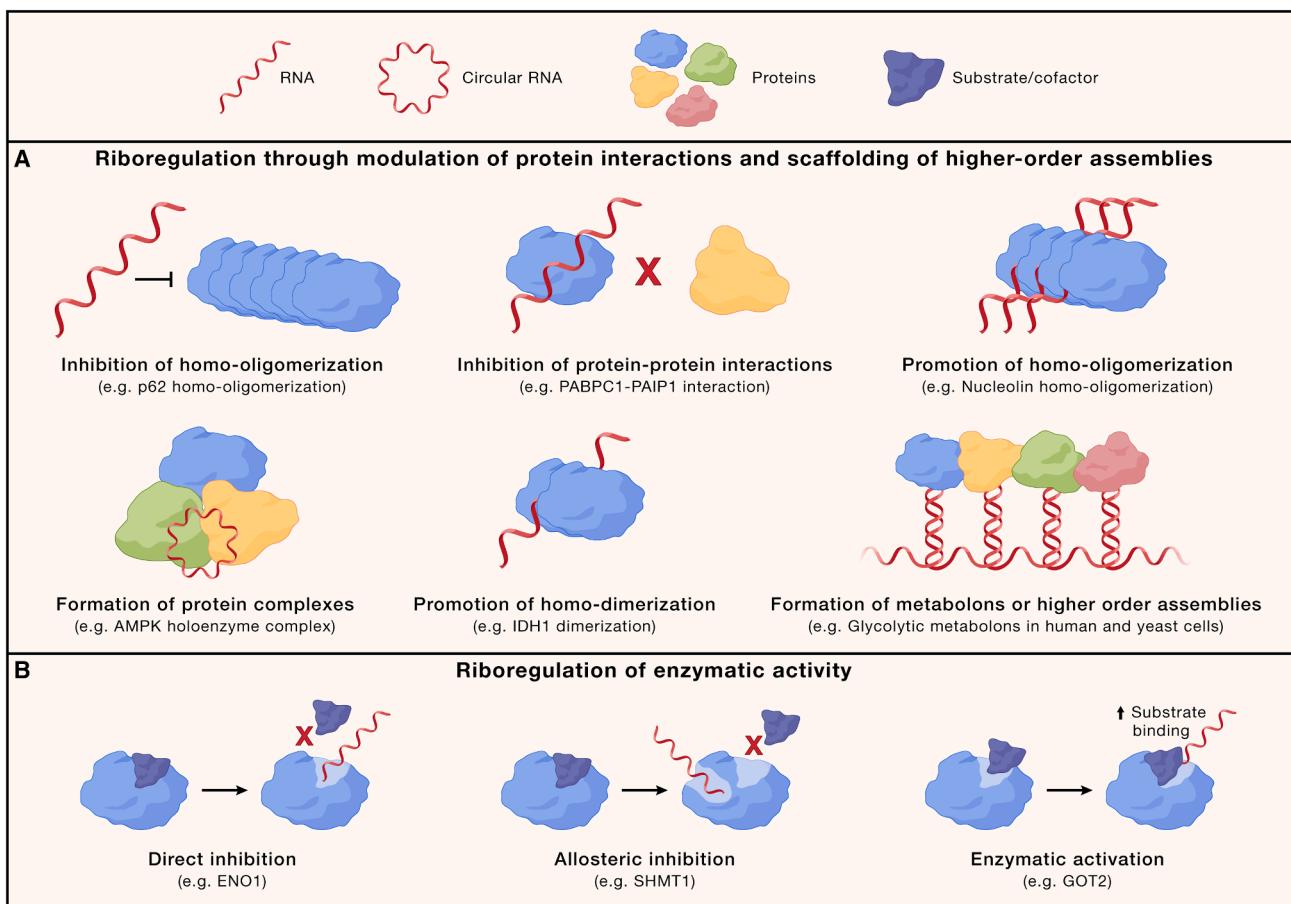


Figure 3. Examples of riboregulation

(A) RNA can influence protein function by promoting or inhibiting homo- or hetero-dimerization/oligomerization of proteins. This interaction can yield distinct functional outcomes, including direct regulation of enzymatic activity, protein stability, or modulation of downstream cellular functions. In addition, RNA can facilitate the formation of metabolon-like assemblies by positioning successive enzymes of a metabolic pathway in close proximity, as observed with glycolytic metabolons/granules in human or yeast cells.

(B) RNA can also inhibit enzymatic activity either by directly competing with substrate or cofactor binding, as seen in the IRP and ENO1 paradigms, or through allosteric modulation of substrate binding, as exemplified by the SHMT1 paradigm. Alternatively, RNA can enhance enzymatic activity by promoting substrate binding, as observed in the case of GOT2.

transcriptome, mostly localized in the 5' or 3' UTRs of mature cytosolic mRNAs. Nonetheless, binding is specific because the affinity for ligand RNAs is about two orders of magnitude higher than that for size-matched controls.¹¹⁵ This specific, yet highly redundant form of riboregulation has been described as “crowd control,” with reference to the collective regulatory impact of the overall cellular transcriptome. An elegant parallel for such crowd control can be found in microRNA (miRNA) biology, where a multitude of specific miRNAs often collectively regulate the expression of specific target transcripts, sharing the regulatory load.¹¹⁶ While such regulation may seem intuitively attractive, we still lack understanding of the structural details of the ENO1—riboregulatory RNA ligand interactions that explain the specificity underlying the observed effects, and such insight will be critical to illuminate the mechanism underlying this biologically interesting example.

Serine hydroxymethyltransferase 1 (SHMT1), a key enzyme of one-carbon metabolism, catalyzes the folate-dependent inter-

conversion of serine and glycine. It was recently found to be riboregulated by the 5' UTR of the mRNA encoding its mitochondrial counterpart, SHMT2.^{117,118} Structural studies revealed that the RNA binds to SHMT1 tetramers near the folate binding site to selectively riboregulate the serine-to-glycine cleavage reaction but not the reverse reaction, serine synthesis. RNA contacts the holoenzyme, a dimer of dimers, via a surface of the tetramer that is formed by subunits from opposite dimers, thus requiring the tetrameric assembly for binding. The RNA binds to a flexible flap that partially overlaps with the folate binding site and competes with folate for binding. RNA binding also induces an allosteric shift that interferes with the productive binding of serine to the active site, offering a molecular basis for the selective inhibition of serine cleavage.¹¹⁷ This pioneering work elaborates a detailed mechanism of how RNA can exert riboregulation of an enzyme that completely lacks the features of a classical RBP.

Some RNA-enzyme interactions could be important only in special cellular contexts, such as infection or stress, and thus

not be detectable under control culture conditions. The host lncRNA-ACOD1, whose expression is induced by infection with a variety of RNA and DNA viruses, directly binds to the metabolic enzyme glutamic-oxaloacetic transaminase (GOT2),¹¹⁹ a mitochondrial enzyme connected to amino acid metabolism, long-chain fatty acid uptake, and the TCA cycle. The lncRNA-ACOD1 binds close to GOT2's substrate binding site and enhances GOT2 activity *in vitro*, as well as promotes viral replication *in cellulo* and mice.¹¹⁹ Notably, several host metabolic enzymes have also been identified as viral RBPs (vRBPs) in system-wide screens, including cells infected with SARS-CoV-2 and Chikungunya viruses.^{120,121} Whether viral RNA directly riboregulates host metabolic enzymes to alter host cell metabolism remains an intriguing question for future research. Finally, very recent work has uncovered that TRIM25, a non-canonical RBP and E3 ubiquitin ligase with anti-viral activity, mediates degradation of exogenous (m)RNAs delivered by endosomal release into cells unless these carry N1-methylpseudouridine modifications.¹²² Apparently, TRIM25 RNA binding is stimulated by the drop in pH near ruptured endosomes, allowing discrimination from cellular RNAs, and recruits endoribonucleases that trigger RNA degradation. Bound RNA stimulates the ubiquitin ligase activity of TRIM25, which is required for RNA degradation.¹²² providing an exciting additional example of riboregulation of an enzyme.

Riboregulation of higher-order assemblies

Extending the role of riboregulation in controlling protein-protein interactions, RNA has also been reported to promote the formation of higher-order polypeptide assemblies (Figure 3A). The c-Myc-induced lncRNA glycoLINC stimulates glycolytic flux under conditions of serine deprivation by scaffolding together multiple enzymes of the glycolytic "payoff phase"—phosphoglycerate kinase 1 (PGK1), phosphoglycerate mutase 1 (PGAM1), ENO1, and pyruvate kinase M2 (PKM2)—along with lactate dehydrogenase A (LDHA), forming a so-called metabolon.¹²³ Metabolon formation under serine deficiency is critical for ATP synthesis and cell survival, because it counteracts the shunting of glycolytic precursors toward *de novo* serine synthesis, protecting the glycolytic payoff phase and mitochondrial ATP synthesis.¹²⁴ A similar paradigm was recently reported in yeast,¹²⁵ where glycolytic enzymes were identified as non-classical RBPs.¹⁶ RNA was found to promote phase separation of glycolytic enzymes into so-called glycolytic (G) bodies under hypoxic stress, accompanied by increased glucose consumption and cell survival.¹²⁵ Dynamic organization of metabolons by RNA may therefore represent a broader mechanism by which cells rewire metabolism, ensuring cell survival and metabolic homeostasis.

Another example of RNA-mediated formation of higher-order assemblies is the recently reported Fragile X mental retardation syndrome-related protein 1 (FXR1) network.¹²⁶ FXR1 is a classical RBP with three KH domains. In addition, it has an arginine-rich IDR as well as several domains involved in protein-protein interactions. The FXR1 network was described to require unusually long mRNAs for its assembly that permeates the whole cytoplasm and is required for RhoA/ROCK-mediated signaling to reorganize the cellular actomyosin cytoskeleton.¹²⁶ Future work may resolve whether the role of RNA is regulatory *sensu stricto* or whether RNA serves as a constitutive scaffold for FXR1 network formation. This example also nicely highlights how classical RBPs in combination with IDRs (as well as non-canonical RBPs) and RNAs can serve to organize cellular assemblies that fulfill critical biological functions. As such, the example also reflects the importance of RBPs, RNA, and riboregulation for the formation and dynamics of phase-separated structures that organize subcellular biology.

Regulation of transmembrane transport

Many dozens of the non-canonical RBPs represent proteins known to function in organelles, extracellularly, or as transmembrane proteins.^{3,62} ATP synthase is a multi-subunit enzyme that catalyzes a key reaction in energy metabolism, the mitochondrial generation of ATP.¹²⁷ ATP5A1 is a subunit of the so-called F1 stalk in the mitochondrial matrix that also binds RNA. Curiously, it was found that the RNA ligands of ATP5A1 are predominantly (86%) cytosolic mRNAs, suggesting that the nuclear-encoded precursor form of ATP5A1 may be bound. Indeed, functional studies *in vitro* and cell-based assays show that these RNA ligands facilitate the mitochondrial import of the ATP5A1 precursor.¹²⁸ Although ATP synthase has been studied in great detail in the past, this example reveals that RNA can regulate transmembrane protein transport, hence adding a further example of an unexpected RNA function. With several transmembrane, extracellular, and organelle-resident proteins identified as potential RBPs by omics screens, it remains to be explored whether these findings point to a much broader role of riboregulation in the transmembrane transport of proteins.

Taken together, the above examples show that some non-canonical RBPs are subject to riboregulation that has eluded decades of research. The expanding footprint of RNA as a regulatory molecule forces us to rethink some of the conventional notions about RBPs, especially where they become the subjects of riboregulation. Once viewed as a mere carrier of genetic information or fulfilling structural roles as part of large ribonucleoprotein complexes, RNA increasingly emerges as a potent regulatory entity that actively contributes to real-time cellular decision-making in yet another way.

QUESTIONS AND CONCERNS

As discussed above, comprehensive omics approaches for the experimental identification of RBPs have uncovered a plethora of non-canonical RBPs that lack discernible RBDs. Many of them are abundant proteins known for other functions in the cell, raising critical questions that deserve in-depth discussion.

First, if some of the non-canonical RBPs are so abundant, why were their RNA-binding activities not discovered earlier? One possibility is that only a minor fraction of the RBP in question may bind RNA, and this binding may be highly dynamic or dependent on cellular conditions. IRP1 only binds RNA in iron-deficient cells, and the oxidoreductase PDIA6 is mostly located in the endoplasmic reticulum (ER) lumen, which is thought to be an RNA-poor environment.^{129,130} Another possibility is that interactions are restricted to very specific RNAs. For instance, the autophagy regulator p62 preferentially binds vaultRNA1-1, and while it also interacts with a few dozen other RNAs, this is modest compared with the several thousand RNAs that many

classical RBPs bind to.¹⁰⁵ The RNA binding of non-canonical RBPs may thus require enrichment procedures such as RIC for their detection.

Riboregulation may be modulatory, rather than “all-or-none,” and hence escape detection by functional studies more readily. Where riboregulation is exerted by redundant regulatory RNAs (e.g., ENO1), loss-of-function analyses are prone to yield falsely negative results. Together with their main roles unrelated to RNA biology and their lack of predictable RBDs, RNA binding was certainly out of focus for non-canonical RBPs until their *en masse* detection by RIC.^{10,11}

Second, is RNA binding by non-canonical RBPs specific? Recent RNAcompete experiments, where a recombinant protein is mixed with a designed, non-randomized RNA pool followed by detection of bound RNAs, led to the conclusion that most non-canonical RBPs bind RNA in a non-sequence-specific fashion.¹³¹ In addition, 3 out of 6 putative RBPs failed *in vitro* validation by NMR spectroscopy, whereas a fourth acted as a rather opportunistic, non-specific binder.¹³² While these studies suggest that the intrinsic RNA-binding properties of non-canonical RBPs differ from those of their classical counterparts, *in vitro* studies may not fully capture the *in vivo* conditions necessary for RNA binding. Specific target recognition might require complex formation with other RBPs, be influenced by the RNA sequence or structural context, or depend on the cellular microenvironment or metabolic state. These scenarios are, in fact, also observed with classical RBPs. One example is the Sex-lethal:Upstream of N-Ras (SXL:UNR) complex, where UNR binds with high affinity to a specific sequence in *msl-2* mRNA only when SXL is present.¹³³ Furthermore, RNA context dependency for binding is also a common phenomenon among classical RBPs.¹³⁴

Regardless of these considerations, RNA-binding analyses of a small group of functionally validated non-canonical RBPs have revealed that expectations raised by classical RBPs may be false and that a wider perspective may be warranted. Although it is a non-canonical RBP, IRP binds IREs with affinities in the picomolar range, and even single nucleotide deletions or changes can have profound effects on binding affinity.¹³⁴ On the other end of the spectrum, ENO1 binds RNA with much lower affinity (in the high nanomolar to micromolar range) and can recognize hundreds of different RNAs that lack a common binding motif, yet RNA binding is specific, with two orders of magnitude difference between target and non-target sequences.¹¹⁵ Somewhat intermediate, p62 binds to a central loop of vaultRNA1-1 in the nanomolar range, and this central loop seems to be a distinguishing feature compared with other vault RNAs.¹⁰⁶

Another important consideration concerns functionality versus specificity. The critical question to be answered for each non-canonical RBP is whether RNA binding fulfills a biologically important, functional role. Since binding to RNA in a sequence-non-specific manner is important for the function of many, including classical RBPs (e.g., helicases, exon-junction complex proteins, translation factors, and many others), assessment of binding specificity cannot serve as a shortcut to functional relevance.

Third, how do non-canonical RBPs bind RNA, and why do they lack RBDs? Proteome-wide identification of RBDs in human cells using RBDmap has revealed major roles for IDRs and the

Rossmann fold in RNA binding.¹⁵ In fact, nearly half of the RNA-binding regions revealed by RBDmap are located in IDRs. These regions are rich in charged and aromatic residues that may non-specifically interact with nearby RNA in a crowded environment. However, the example of FMRP described above shows that RG-rich regions can engage in specific interactions.^{41,42} Furthermore, although IDRs are flexible in solution, they may undergo induced-fit adaptation upon binding to their RNA counterparts.⁶ A prominent example is that of ribosomal proteins, many of which are largely disordered in isolation but become ordered upon interaction with rRNA, forming domains that are exposed on the ribosome surface.¹³⁵

Fourth, may non-canonical RBPs be artifacts from high-throughput procedures? Surely, any large-scale procedure has the potential to generate false positive hits. While the abundance and biophysical properties of non-canonical RBPs might introduce discovery artifacts, these can be minimized by thorough experimental design and meaningful specificity controls as well as multi-method validation. Such validation includes employing alternative assays to confirm direct RNA binding (e.g., T4 polynucleotide kinase [PNK], proximity ligation assay [PLA], OOPS, and NMR), identifying RNA targets, and mapping the protein residues involved in RNA interaction for the generation of mutants (Figure 4). To uncover the functionally relevant non-canonical RBPs from proteins that have no biological meaning of their interaction with RNA, we have no high-throughput method to propose. Unfortunately, *in vitro* RNA-binding assays or present-day computational predictions fail to deliver reliable indicators for *in vivo* functions, and the lack of large datasets on this new class of RBPs does not lend them to AI-based approaches anytime soon. For now, rigorous systematic functional studies inspired by the known functions of a given non-canonical RBP appear to be the best, though a work-intensive way to make this distinction. An open-minded, critical approach will serve to avoid both falsely positive assumptions of biological relevance as well as falsely negative dismissal of an unexpected property waiting to be discovered. Hopefully, a rich harvest of unpredicted and exciting new functions of RNA combined with an understanding of how RNA binding intersects with the protein’s known roles will be the reward. Our experience so far suggests that this may well be the case.

PERSPECTIVES

RNA is everywhere in the cell. Apart from its role as the carrier of genetic information, it is a structural component of essential molecular machines and an integral part of biomolecular condensates, which play roles in intracellular compartmentalization and, more recently, in the composition and integrity of membrane-bound organelles.^{136–138} The types of condensates detected in cells are steadily growing, including the recent incorporation of TPA-inducible sequence 11B protein (TIS) and Fragile X-related protein 1 (FXR1) granules playing roles in translation and signaling, respectively.^{126,139} The formation of condensates is supported by weak multivalent protein-protein and RNA-protein interactions largely provided by IDRs that are overrepresented in RBPs, including many non-canonical ones.^{6,15,140} Given the structural plasticity and abundance

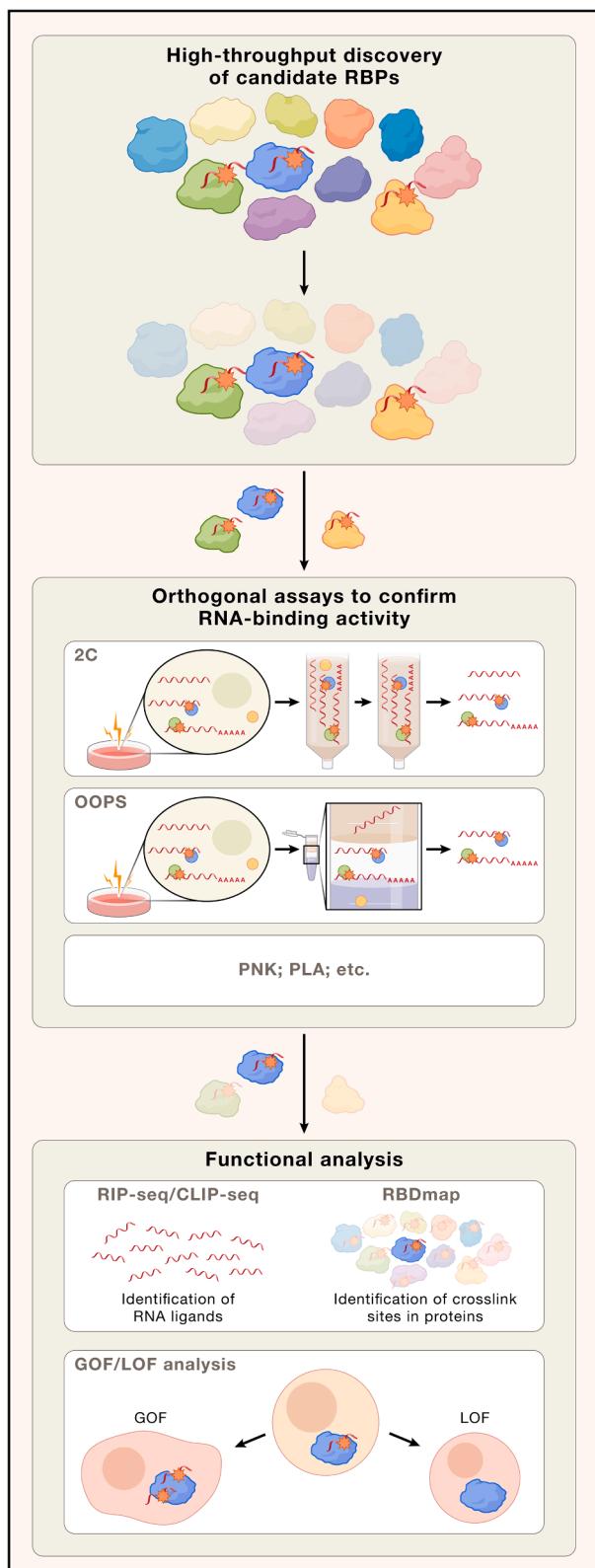


Figure 4. Possible workflow for the validation and functional evaluation of non-canonical RNA-binding proteins identified by omics approaches

Following high-throughput discovery, orthogonal RNA-binding assays, such as 2C, OOPS, PNK assay, or PLA, are recommended to validate RNA binding. Subsequently, functional characterization can proceed via identification of RNA targets (e.g., crosslinking and immunoprecipitation followed by sequencing [CLIP-seq] and RNA immunoprecipitation followed by sequencing [RIP-seq]) and the RBP crosslinking region(s) (e.g., RBDmap). Generation of gain- and loss-of-function (GOF/LOF) mutants enables dissection of the biological relevance of the RNA-binding activity and its phenotypic consequences.

of RNA, one might expect that interactions with proteins are frequent, even if they do not involve classical specificity determinants such as short RNA sequence or structure motifs. Supporting this notion, differential fractionation studies performed in the presence and absence of RNase treatment uncovered hundreds of proteins not previously linked to RNA and indicated that about 20% of all known human protein complexes contain RNA.^{141,142}

Established examples of non-canonical RBPs already evidence how RNA can regulate enzyme activity, control protein oligomerization, or simply act as a scaffold within metabolic pathways.^{105,115,123} RNA binding of the enzymes of intermediary metabolism is conserved between yeast and mammals, very common in organs, and pervasive for those enzymes that bind nucleotides as cofactors.^{16,62,143} Extrapolating from the universality of these enzymes as non-canonical RBPs, along with the broad distribution and diversity of RNA types and their versatility as regulators, we propose that non-canonical, functionally relevant RBPs may be far more common than previously recognized. Riboregulation might be the norm rather than an exception, and non-canonical RBPs may represent fundamental elements that have existed since the origins of life, with much still to be uncovered. We hope that this review could illustrate that the world of physiologically relevant RBPs includes far more than the classical RBPs.

Non-canonical RBPs and riboregulation are still a young field that will hopefully progress rapidly beyond cell biological descriptions of new RNA functions. As the SHMT1 example illustrates, we will greatly benefit from insights gained from high-resolution structural studies. We anticipate that in addition to crucial mechanistic insights, these will illuminate the secrets behind the RNA-binding specificity of non-canonical RBPs. We also predict that such insights will highlight a currently underappreciated aspect of RNA: its structural plasticity and its ability to evolve snug fits with organic surfaces that have not themselves evolved to bind RNA.

From an evolutionary point of view, riboregulation might be widespread. The “RNA world” theory posits that RNA was the primordial self-replicating molecule and that DNA and proteins appeared later.¹⁴⁴ It is attractive to suggest that the capacity of RNA to regulate proteins might be ancient. Thus, non-canonical RBPs might represent an evolutionary tip of the iceberg of an evolving RNA world. Another possibility when considering RNA binding by non-canonical RBPs is that they could utilize multiple low-affinity surfaces distributed across the protein structure, much like classical RBPs use multiple RBDs to enhance affinity and specificity.¹ Whether RNA recognition by non-canonical

RBPs follows similar rules as those used by classical RBPs is to be determined, but in principle, there is no need to have a well-folded RBD to bind RNA. This has been proven by the aptamer field, where small RNAs can be evolved and selected to bind with relevant affinity to all kinds of biological and synthetic surfaces, very different from RBDs.¹⁴⁵ Future structural studies of non-canonical RBPs bound to RNA will help us understand the underlying RNA-binding principles. It will also be interesting to determine whether riboregulation of “ancient” non-canonical RBPs, such as glycolytic enzymes, is conserved. What will the RNA-binding proteomes of prokaryotes and archaea, followed by functional studies in these organisms, reveal? May riboregulation be a primordial form of biological control? All of these exciting questions await experimental answers that should be within reach.

The (non-exhaustive) list of examples discussed above largely comes from cell-based studies. An important future goal must be to assess non-canonical RBPs and riboregulation in their organismal context. Is riboregulation affected in the context of human disease? The frequency of mutations in non-canonical RBPs documented in OMIM is higher than that for classical RBPs and similar to that of transcription factors.^{146,147} While it is presently unknown how many of these mutations affect an RNA-related function, their high number per se suggests a solid likelihood of disease-relevant discoveries. A similar prediction can be made on the basis of recent studies that link non-canonical host cell RBPs to the replication of many viruses.^{120,121} These reports imply that novel antiviral strategies could be developed by targeting non-canonical RBPs. Furthermore, the riboregulation of the ubiquitin ligase activity of TRIM25 discussed above has clear implications for RNA therapeutics. Thus, non-canonical RBPs are predicted to become a future entity in human medicine. Knowledge of the mechanism(s) by which non-canonical RBPs are riboregulated and of the surfaces and pockets that they involve undoubtedly will uncover new targets and pharmaceutical modalities for future exploitation.

Finally, riboregulation illustrates another interesting aspect of RNA-protein interactions. For the most part, classical RBPs exert regulatory dominance over their RNA targets. Riboregulation balances this picture and shows that the biology of RNA-protein interactions is not one of dominance but more like an elegant *pas de deux*.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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