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REVIEW

Advances and challenges towards the study of RNA-RNA interactions in a transcriptome-wide scale

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***Background*: RNA molecules play crucial roles in various biological processes. Their regulation and function are mediated by interacting with other molecules. Among them RNA-RNA interactions (RRIs) are important in many basic cellular activities including transcription, RNA processing, localization, and translation. However, we just start to unveil the complexity of the knowledge and underlying mechanisms of RRIs.**

***Results*: In this review, we will summarize approaches for RRI identifications, including both conventional, focused biophysical and biochemical methods and recently developed large scale sequencing-based techniques. We will also discuss discoveries per RRI type revealed by using these technologies, as well as challenges towards a systematic and functional understanding of RRIs.**

***Conclusions*: The development of sequencing-based techniques has revolutionized the study of RRIs. Applying these techniques in multiple organisms has identified thousands of RRIs, many of which could potentially regulate multiple aspects of gene expression. However, despite the great breakthrough, the RNA-RNA interactome of any species remains far from complete due to intrinsic complex nature of RRI and limitations in current techniques. More efficient experimental methods and computational framework are needed to obtain the full image of RRI networks, and their possible regulatory roles in biology and medicine.**

Keywords: RNA-RNA interactions; PARIS; SPLASH; LIGR-seq; next generation sequencing

Author summary: RNA molecules play crucial roles in various biological processes by interacting with molecules including other RNAs. The recent breakthrough in resolving the transcriptome-wide RNA-RNA interaction (RRI) networks has identified a huge amount of data of RRIs and greatly advanced our understanding to the regulation of various RNAs.

INTRODUCTION

In addition to transferring genetic information, RNA molecules play important roles in various biological events, including transcriptional regulation, alternative splicing, and protein synthesis [1–6]. During the past decades, it has been shown that the genome is largely transcribed and most transcripts do not encode proteins [7,8]. Throughout their life cycle, RNAs function by interacting with different molecules (*e.g.*, proteins, DNA, RNA). For instance, hundreds of RNA binding proteins (RBPs) have been discovered in prokaryotes and eukaryotes. RBPs interact with RNAs and form ribonucleoprotein (RNP) complexes to perform specific functions in RNA processing and post-transcriptional regulation [9,10]. In nucleus, some lncRNAs can regulate the expression of specific genes by interacting with the chromatin and modulating the epigenetic states of

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neighboring genomic loci [11,12]. Another important type of RNA interactions is between two RNA molecules, *i.e.*, RNA-RNA interactions (RRIs) [13]. For instance, in splicing, small nuclear RNAs (snRNAs) can recognize intronic regions of precursor messenger RNAs (pre-mRNAs), a critical step in determining RNA splicing products [14,15]. In translation, amino-acylated transfer RNAs (tRNAs) interact with mRNAs by reading the three-letter code and define protein amino acid sequences [16,17]. In microRNA (miRNA) targeting, the base pairing between an miRNA and 3′ UTR of an mRNA can lead to the degradation or translation inhibition of the mRNA [18,19]. And in RNA modification, small nucleolar RNAs (snoRNAs) guide the modification of ribosomal RNAs (rRNAs) by base pairing [20,21]. All these examples highlight the significance of RRIs for RNA function and regulation.

Much effort has been devoted to decoding the RRIs (Table 1). Methods have been long designed to detect RRIs based on biophysical and biochemical features that distinguish RNA interacting duplexes from non-interacting ones. For instance, RNA duplexes usually migrate slower in electrophoresis gel, allowing them to be separated from non-interacting RNA fragments [22,23]. Recently, with the advent of next generation sequencing, new techniques based on deep sequencing have been developed to reveal the full network of RRIs, *i.e.*, the interactome, for all transcripts in a cell [41,43,45,46]. They can also be used to study all interactions concerning a certain RNA, usually combined with RNA pull-down with a set of target-specific antisense probes [38,39].

To clarify, RRIs can be used to represent interactions between two RNAs (inter-molecular) or between different regions of one RNA molecule (intra-molecular). Intra-molecular RRIs is the basis of forming RNA secondary structures. Varieties of methods, like enzymatic probing (*e.g.*, PARS, Frag-seq) [47–49] or chemical probing methods (*e.g.*, DMS-seq, Structure-seq, icSHAPE) [50–52] have been designed to identify and study intra-molecule RRIs, or RNA secondary structures. The methods and biological insights from RNA secondary structure probing have been extensively reviewed and discussed [53–59]. Here we only focus on the methods to identify inter-molecular RRIs, with their underlying principles, advantages and limitations.

OVERVIEW OF EXPERIMENTAL METHODS FOR RRIs IDENTIFICATION

Low-throughtput biophysical, biochemical and cellular methods

A particularly useful scenario of RRI detection or validation is to confirm a specific interaction for further research. Computational prediction methods can be used to predict whether and how two RNA molecules could form inter-molecular base-pairing [60,61]. RRIs then can be directly tested by many biophysical and biochemical methods. For example, in electrophoretic mobility shift assay (EMSA), RNA fragments are extracted from cells or synthesized according to the potentially interacting regions. A *bona fide* RRI has a larger molecular mass and thus migrates slower in electrophoresis gel than non-paired RNAs [23–25] (Figure 1A). In surface plasmon resonance (SPR) experiments, one RNA fragment is immobilized on a sensor chip by streptavidin-biotin coupling and the interaction with potential targets is monitored in real-time [26,27] (Figure 1B). And in single molecule Forster resonance energy transfer (FRET) studies, RNA molecules are fixed on surface of quartz or encapsulated in lipid vesicle. Then real-time monitoring can be achieved by two fluorescent dyes labeled respectively on specific position of the two RNAs. If two fluorescent dyes interact with each other in a close space, then proper fluorescent signals will be produced and recorded [28,29] (Figure 1C). In co-sedimentation assays, RNA extracts are fractioned on sucrose/glycerol gradients and different fractions are examined by Northern blot. RNAs existing in the same gradient fractions are reckoned as having interactions [30,31] (Figure 1D).

Cellular methods to investigate certain RRIs also have been designed. For instance, an RNA-hybrid system in *Saccharomyces cerevisiae* has been developed to detect a certain RRI by using a reporter gene whose activation depends on the interaction of two RNAs [32,33] (Figure 1E). Another way to validate RRIs is to construct mutants that disrupt the putative base-pairing, and more importantly, rescue mutants that restore the disrupted base-pairings by complementary mutations. This mutation-and-rescue strategy is particularly useful in validating functional RRIs.

In summary, for the study of a specific RRI, an array of biophysical, biochemical and cellular methods have been developed with varying strengths. Usually these methods can only detect interactions at a molecular level and cannot reveal the precise regions that mediate the interaction.

High-throughput targeted methods

Next generation sequencing technologies have been developed to study RNA molecules at transcriptome level [62]. One of the first sequencing-based techniques to decode the RNA interactome of a specific RNA is CLASH (crosslinking, ligation, and sequencing of hybrid) [34–36] (Figure 2A). As many RNA interactions are mediated by proteins (*e.g.*, AGO in miRNA-mRNA interaction), UV can be used to crosslink a RRI duplex

Table 1 Summary of approaches for RNA-RNA interaction identification

Type	Method	Main procedure	*In vivo*/*in vitro*	Species	Features and limitations	Refs.
Biophysical	EMSA	Prepare RNA fragments; Electrophoresis gel analysis	*In vitro* and *in vivo*	–	F: Simple and rapid; L: No interacting region information	[22–25]
Biophysical	SPR	RNA immobilization; Surface plasmon resonance analysis	*In vitro*	–	F: Label-free; assess affinity dynamically; L: Susceptible to hydrolysis; no interacting region information	[26,27]
Biophysical	Single molecule FRET	Fluorescent dye labeling; Fluorescence signal monitoring	*In vitro*	–	F: Capture RRIs at single molecule level in real time; L: High requirement in sample purification and labeling; no interacting region information	[28,29]
Biophysical	Co-sendimentation	Sucrose gradient fractioning; Northern blot	*In vitro* and *in vivo*	–	F: Simple and rapid; L: May disassociate low binding affinity duplex; no interacting region information	[30,31]
Cellular	Yeast RNA hybrid system	RNA fusion; Reporter signal detection	*In vitro*	–	F: Measure interaction stability quantitatively; L: Low level of reporter activation; no interacting region information	[32,33]
Targeted high-throughput	CLASH	UV crosslinking; Proximity ligation; RT and sequencing	*In vivo*	Human; Yeast; *E. coli*	F: First high-throughput method; L: Only resolve protein mediated RRIs; limited ligation efficiency; limited sensitivity	[34–37]
Targeted high-throughput	RIA-seq	UV crosslinking; Target RNA capture; Elution and sequencing	*In vivo*	Human	F: Systematically investigate all interactions of a target RNA; L: Only resolve RRIs of a specific RNA	[38]
Targeted high-throughput	RAP	UV crosslinking; Target RNA capture; Elution and sequencing	*In vivo*	Mouse	F: Systematically investigate all interactions of a target RNA; use long capture probes to reduce non-specific interactions; L: Only resolve RRIs of a specific RNA;	[39,40]

(*Continued*)

Type	Method	Main procedure	*In vivo*/*in vitro*	Species	Features and limitations	Refs.
Transcriptome-wide	PARIS	AMT crosslinking; Rnase and protease digestion; 2D gel purification; Proximity ligation; RT and sequencing	*In vivo*	Human; Mouse	F: Resolve RRI duplexes directly; duplex enrichment by 2D gel; L: Limited crosslinking and ligation efficiency; only intercalate uridine	[41,42]
Transcriptome-wide	SPLASH	Biotinylated psoralen crosslinking; Fragmentation; Beads purification; Proximity ligation; RT and sequencing	*In vivo*	Human; Yeast; *E. coli*	F: Resolve RRI duplexes directly; duplex enrichment by biotin isolation; L: Limited crosslinking and ligation efficiency; only intercalate uridine	[43,44]
Transcriptome-wide	LIGR-seq	AMT crosslinking; Digestion by nuclease circularization and enrichment by RNase R; RT and sequencing	*In vivo*	Human	F: Resolve RRI duplexes directly; duplex enrichment by RNase R; L: Limited crosslinking and ligation efficiency; only intercalate uridine	[45]
Transcriptome-wide	MARIO	RNA-protein crosslinking; Fragmentation; Beads purification; Proximity ligation; RT and sequencing	*In vivo*	Mouse	F: Resolve protein mediated RRIs; enrichment by biotin isolation; L: Limited crosslinking and ligation efficiency	[46]

For biophysical or cellular methods, the experiments have been conducted in many species.

Abbreviation: EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; FRET, Forster resonance energy transfer; CLASH, crosslinking, ligation, and sequencing of hybrids; UV, ultraviolet; RIA-seq, RNA interactome analysis followed by deep sequencing; RAP, RNA antisense purification; PARIS, psoralen analysis of RNA interactions and structures; SPLASH, sequencing of psoralen crosslinked, ligated, and selected hybrids; LIGR-seq, ligation of interacting RNA followed by high-throughput sequencing; MARIO, mapping RNA interactome *in vivo*; AMT, psoralen-derivative 4′-aminomethyltrioxsalen; RT, reverse transcription; RRI, RNA-RNA interaction.

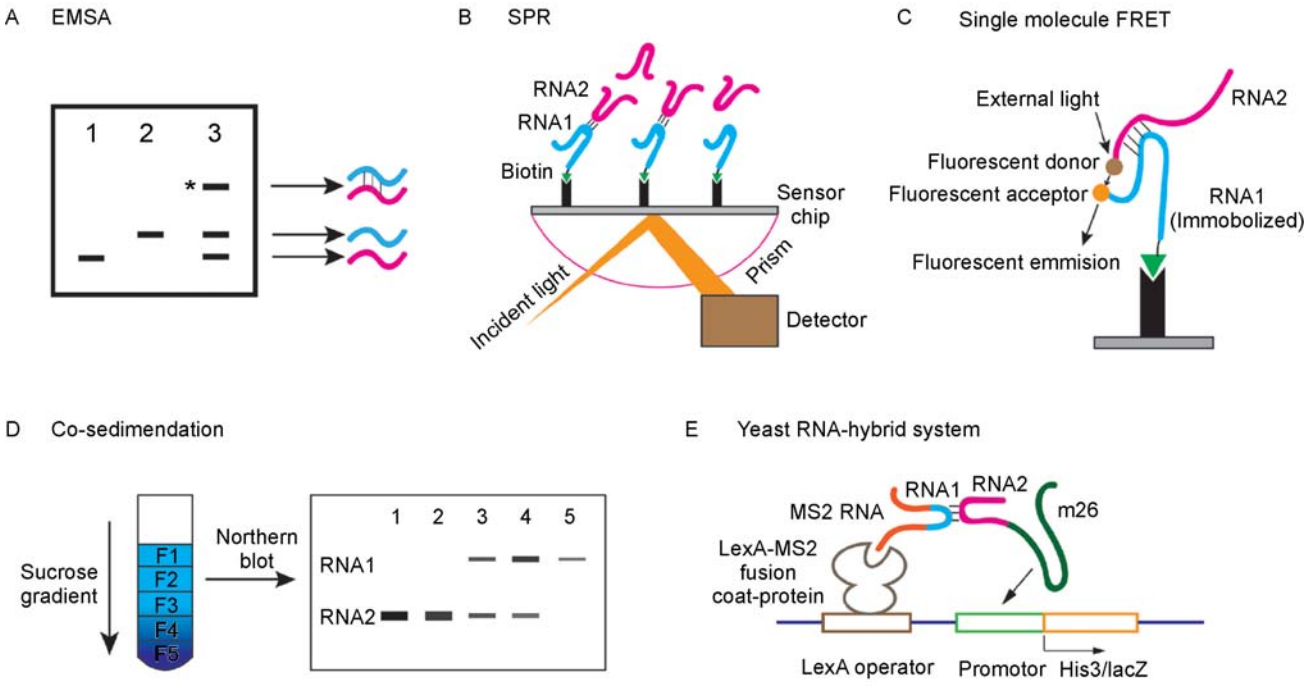

Figure 1. Traditional biophysical and cellular methods for specific RNA-RNA interaction identification. (A) Electrophoretic mobility shift assay (EMSA). An interacting RNA duplex is revealed as a retarded band with star labelled. (B) Surface plasmon pesonance (SPR). One of two RNAs is immobilized on a sensor chip while the other is in the solution. (C) Single molecule fluorescence resonance energy transfer (FRET). The donor and acceptor fluorescent dyes are attached directly to the RNA molecules and the distance between dyes can be revealed by the efficiency of energy transfer between them. (D) Co-sedimentation. Extracts containing different RNAs are fractioned by sucrose gradient and interacting RNAs that stay in the same fraction is revealed by Northern blotting. (E) Yeast RNA-hybrid system. The bait RNA is fused with MS2 RNA that bind LexA-MS2 hybrid protein and subsequently the LexA operator. And the prey RNA is linked to m26 that can bind to the promoter of a reporter gene. The interaction between the bait and prey RNAs will bring m26 to the proximity of the promoter and activate the expression of the reporter gene.

with proteins that bind to the duplex. Then the RNA-protein complexes are affinity-purified, RNA-RNA duplex are ligated for subsequent library construction and finally sequencing. After bioinformatics analysis of chimeric reads that correspond to RNA-RNA duplexes, the RNA interactions can be identified. CLASH has been applied to characterize different types of RRIs among diverse species, including small RNA centered interactions in bacterial [36], snoRNA-rRNA interactions in yeast [34], and miRNA-mRNA interactions in human [35]. Recently, CLASH was slightly modified to detect RRIs in *Escherichia coli* [37], in which AMT molecule was also used to crosslink RRIs that are not mediated by proteins. Another method called RNA interaction by ligation and sequencing (RIL-seq), using strategy like CLASH, has been applied to reveal Hfq-associated small RNA (sRNA) interactions in *E. coli* [63]. It is worthy to mention that, similar to CLASH, HiCLIP (RNA hybrid and individual-nucleotide resolution ultraviolet cross-linking and immunoprecipitation) can also identify duplexes of two ligated RNAs, but it achieves higher specificity by adding an adaptor in ligation. HiCLIP has been used to decode RNA secondary structure bound by a double strand RNA binding protein Staufen1, but in principle it can also identify inter-molecular RRIs [64,65].

Another similar approach for RRI discovery is RNA walk. This method utilizes AMT-induced UV cross-linking and affinity purification just as CLASH does. But instead of ligation, RNA walk directly performs RT-PCR after crosslinking, with primers designed to target different parts of the RNA. Cross-linked adduct within the RNA will hinder the amplify efficiency, thus indicate the base pairing regions [66,67].

RNA interactome analysis followed by deep sequencing (RIA-seq) is approach to decipher interactome for a target RNA [38] (Figure 2B). First, antisense DNA probes are designed to target the full-length of the investigated RNA. To improve specificity, the probes are separated into two non-overlapping pools and assayed independently. Biotinylated probes are added to cell lysate and thus beads–biotin-probes–RNA adducts are formed. Then the enriched adduct RNA is isolated and converted into

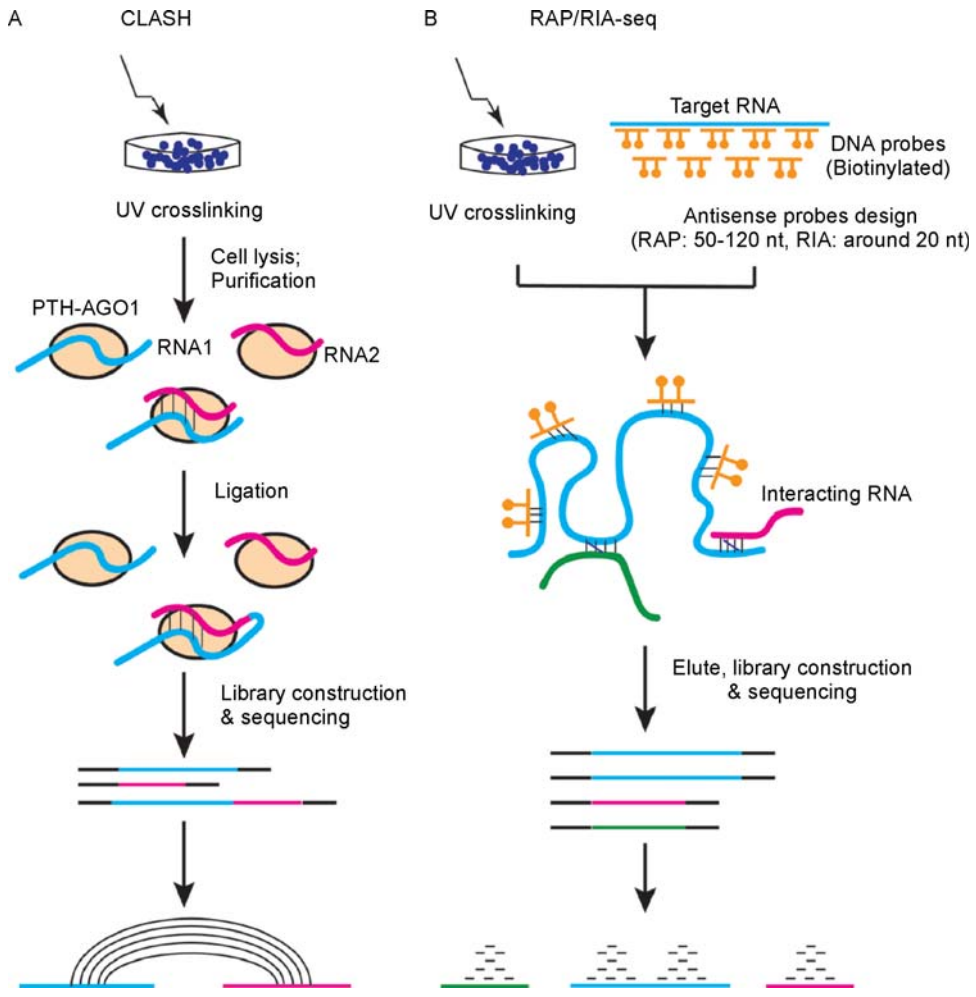

Figure 2. Sequencing-based methods to identify RNA-RNA interactions for mediated by a protein or a RNA target. (A) CLASH (crosslinking, ligation, and sequencing of hybrids): RNAs are crosslinked to proteins under UV irradiation followed by purification, fragmentation, proximity ligation, sequencing and bioinformatics analysis. An interaction between two RNAs is revealed as chimera reads mapped to two different transcripts. (B) RAP (RNA antisense purification) and RIA-seq (RNA interactome analysis followed by deep sequencing): Antisense DNA probes are designed to target and used to purify the full-length lncRNA. RNAs interacting with the target RNA are co-purified and can be identified from sequencing.

cDNA for the following library construction and sequencing. After computational analysis the interacting partners and precise duplex regions can be recovered. By using a similar strategy as RIA-seq, RNA antisense purification (RAP) is capable of identifying interacting partners for a specific RNA [39,40] (Figure 2B). The main steps of RAP consist of probe design, cross-linking, cell lysis, hybridization and elution, followed by high-throughput sequencing and bioinformatics analysis. However, the distinctive feature of RAP is that the capture probes are up to 120-nucleotides long, enabling the use of more stringent hybridization and wash conditions, thus can reduce non-specific interactions to a great extent [39].

In summary, many methods that utilize next generation sequencing have been developed to map RRIs in a large scale. Some of them can decode the RNA interactome bound or mediated by a specific protein, while others can reveal all interacting partners for a certain RNA.

Transcriptome-wide methods

The methods aforementioned can only detect RRIs between specific types of RNAs or for one target RNA. More recently some new methods have been developed to discover the transcriptome-wide RNA interactome that has the potential to cover all RNAs in a cell. These include PARIS (psoralen analysis of RNA interactions and structures) [41,42] (Figure 3A), SPLASH (sequencing of psoralen crosslinked, ligated, and selected hybrids) [43,44] (Figure 3B), LIGR-seq (ligation of interacting

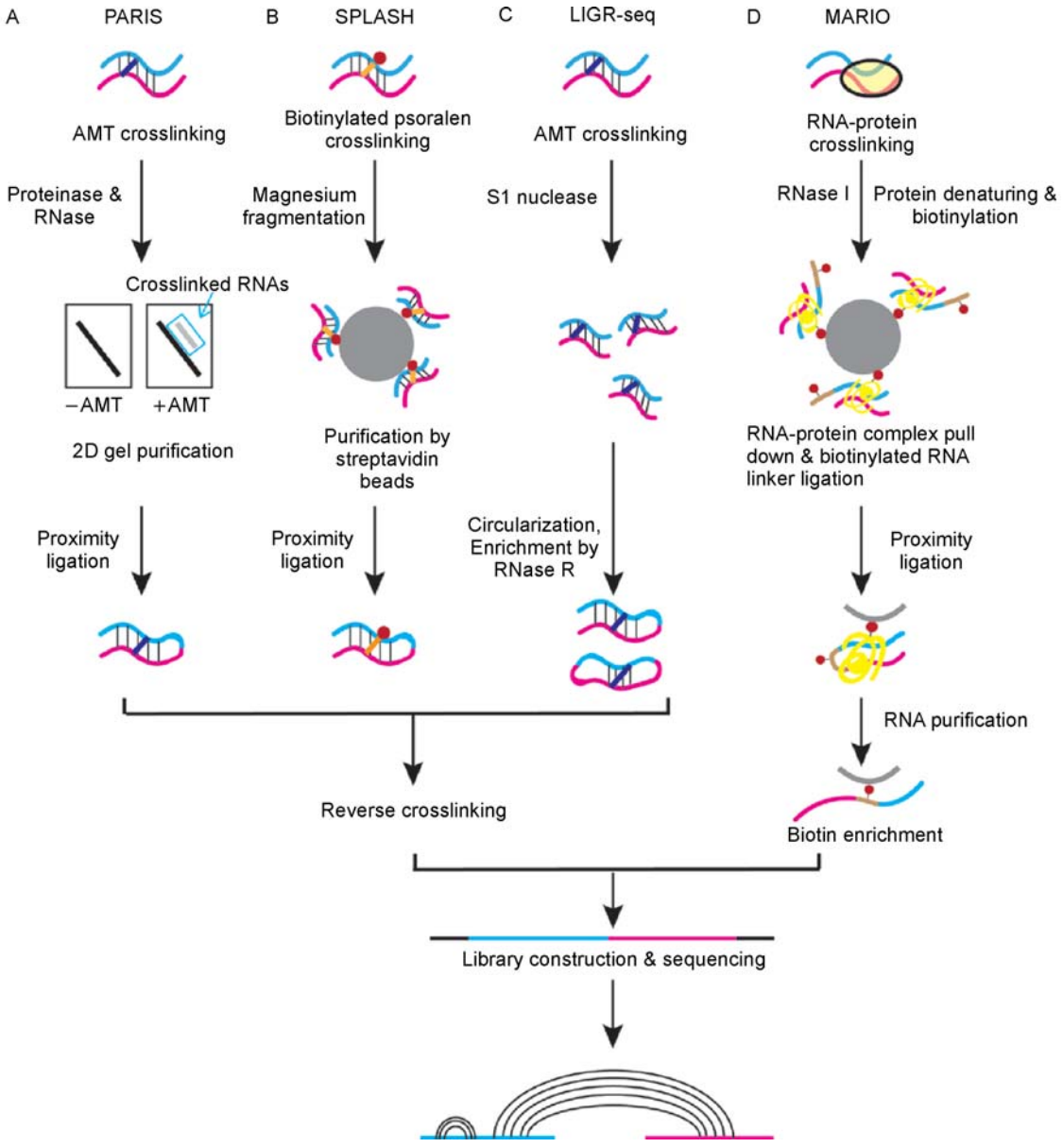

Figure 3. Sequencing-based methods for transcriptome-wide RNA-RNA interaction identification. (A) PARIS (psoralen analysis of RNA interactions and structures): Interacting RNAs are crosslinked with AMT and purified through two-dimension electrophoresis separation, followed by proximity ligation, crosslinking reverse, reverse transcription and library construction and sequencing. RRIs are identified from chimeric reads (a chimeric read is a read mapped to two different transcripts). (B) SPLASH (sequencing of psoralen crosslinked, ligated, and selected hybrids): Similar to PARIS, except that biotinylated psoralen is used in crosslinking and to purify cross-linked duplex. (C) LIGR-seq (ligation of interacting RNA followed by high-throughput sequencing): Similar to PARIS, except that cross-linked duplex is circularized and enriched with RNase R digestion. (D) MARIO (mapping RNA interactome *in vivo*): RNA interactions mediated by proteins are crosslinked to the proteins and then fragmented, and co-purified following protein denaturation and biotinylation. RNAs are then proximity ligated and subjected to sequencing and bioinformatics analysis.

RNA followed by high-throughput sequencing) [45] (Figure 3C), and MARIO (mapping RNA interactome *in vivo*) (Figure 3D) [46]. PARIS employs a psoralen-derivative 4′-aminomethyltrioxsalen (AMT) as the nucleic acid cross-linker to cross-link RNA base pairs in living cells [41]. RNA fragments are then purified with partial RNase and complete proteinase digestion, and cross-linked duplxes are enriched through two-dimension electrophoresis. The next few steps include proximity ligation of enriched RNA duplexes, reversal of cross-linking, high-throughput sequencing and bioinformatics analysis [41]. The other three methods are structurally

similar, with the main process consisting of cross-linking, purification of base-paired RNA, proximity ligation of enriched duplexes, high-throughput sequencing and bioinformatics analysis. Differences between these methods mainly lie in the step of purification and enrichment of RNA-RNA duplexes. SPLASH and MARIO employ biotinylated RNA linkers and the linker-containing chimeric RNAs are isolated using streptavidin-coated magnetic beads. In LIGR-seq, the RNA is digested with S1 nuclease thus can be ligated by circRNA ligase and enriched by RNase R. It is worthy to mention that in addition to identifying RRIs by base-pairing, MARIO is capable of discovering RRIs mediated by proteins. In all these methods, proximity ligation serves as an important step with its efficiency greatly affects the number of interactions that could be detected. RNA proximity ligation (RPL) is a relevant technique that also highly relys on proximity ligation [68]. But the main focus of RPL is to determine RNA secondary structures. Similarly, after treating with an endogenous RNase, RNA fragments are ligated and subjected to sequencing library construction. Analysis of the sequencing result then reveals the structural information of spatially neighboring nucleotides that are ligated to form a chimeric sequencing read. The method has been applied to map RNAs structures of the ribosomal and other abundant RNAs, but essentially analysizing the sequencing data will also reveal informations of RRIs [68].

All these methods can massively identify all types of RRIs at transcriptome level. They have uncovered many previously known or unexplored RRIs, and greatly improved our knowledge on the RNA interactome. One advantage of sequencing-based techniques is that they can map the interaction region with relative high resolution. They can be used to obtain the binding sites of previously known interactions [41], or refine the base pairing details by combination with prediction tools [38].

INSIGHTS FROM RRI DETECTION METHODS

The successful applications of these methods have revealed many insights into the essential roles of RRI regulation. Here we first summarize some of the findings classified into different types of RNAs, and then network features obtained from global RNA interactome analysis.

miRNA related interactions

miRNAs are endogenous small non-coding RNAs that plays an essential role in post-transcriptional regulation by targeting mRNA for cleavage or translational repression [18,69]. Whether mRNA is degraded or prevented from translation depends on the complementary degree between mRNA and miRNA. Studies demonstrated that sufficient complementarity favors cleavage of mRNA, otherwise translational repression is more likely to occur [70,71]. The targeting principle and regulation mechanism of miRNA has been extensively discussed and well-reviewed in [18,69]. Recently CLASH has been used to identify the miRNA-centered interactome in human [35]. Surprisingly, the study shows that about 60% duplexes among seed regions contain bulges or mismatches, suggesting non-canonical miRNA targeting is much more widespread than previously expected [35]. Besides, only 18% of the miRNA-mRNA interactions identified by CLASH involves the 3′ portion of miRNA, which is consistent with previous findings [35,72]. By analyzing sequences of mRNA interacting sites, they identified enriched motifs for miRNA targeting. While most of those motifs are complementary to the 5′ region of miRNAs, several miRNAs favor interactions at its 3′ region (*e.g.*, *let-7a*, miR-16) [35].

snoRNA related interactions

Small nucleolar RNA (snoRNA) is a kind of small RNA that can direct chemical modifications of other RNAs, mainly including rRNAs, tRNAs and snRNAs [21,73,74]. Sequencing-based techniques has characterized more and more novel snoRNA-involved RRIs [34,41,43,45]. In Ref. [34] the authors used CLASH to resolve RRIs mediated by snoRNP proteins (*i.e.*, Nop1, Nop56 and Nop58) in yeast. They found that snoRNAs are more likely to interact with other RNAs rather than to fold into self secondary structure. Among all interacting partners, snoRNAs show a preference for rRNAs. Some snoRNAs interact with rRNAs in multiple regions, emphasizing their essential roles in rRNA processing and modification. For instance, U3 snoRNA can not only bind to the 5′ region of the 18S rRNA for pre-rRNA processing, but also pair with the central regions of 18S rRNA to facilitate the formation of a key pseudoknot [34]. There are many helicases to disassociate snoRNA-rRNA interactions in rRNA biogenesis [75,76]. Some snoRNAs are identified to interact with the helicase Prp43 [77,78]. The authors found many novel snoRNA-rRNA interactions by the Prp43 mutant, suggesting that Prp43 may be another helicase for pre-rRNA releasing from snoRNA [43,77,78]. In Ref. [45], the authors recover many known snoRNA-snRNA and snoRNA-rRNA interactions. By aligning the interacting regions of snoRNA, they revealed that the duplex sequences are located upstream of D or D' boxes, agreeing well with their modification roles. Some orphan snoRNAs are also found to interact with snRNAs [43], like the experimentally verified SNORD83, SNORA51 and SNORD89 [43]. Another orphan snoRNA

SNORD44 can interact with mRNA, such as the telomerase RNA component (TERC) which is highly mutated in cancer cells [45].

lncRNA related interactions

lncRNAs refers to those non-coding RNAs of length more than 200 nt. LncRNAs play a role in various biological processes including epigenetic, transcriptional and post-translational regulation [11,12,79,80]. Interacting with other RNAs is important for the regulatory role of lncRNAs. For instance, in Ref. [38] the authors found that the terminal differentiation-induced ncRNA (TINCR) can facilitate differentiation by binding to target mRNAs to regulate their expression level. The interaction region of mRNA partners contains a 25-nucleotide motif, called "TINCR box", that directly interacts with TINCR. At the same time, TINCR interacts directly with a double strand RNA binding protein STAU1 and this ncRNA-protein complex helps to stabilize duplex structure [38]. Sirt1 antisense (AS) lncRNA can form duplex with Sirt1 3' UTR and prevent binding with miR-34a which may act as a transcriptional suppressor [81]. In Ref. [82], the authors characterize a number of lncRNAs, whose Alu element can base pair with that of 3′ UTR of mRNA. Then STAU1 is recruited and triggers the RNA decay process. By comparing PARIS data with RAP-RNA data, the authors revealed the precise interacting regions between MALAT1 and U1. They also found that the duplex is conserved in both human and mouse [41]. In mouse Matlat1 can interact with the pre-mRNAs of some active genes, especially the genes encoding RBPs, with the help of proteins. This RRI mechanism can give an explanation for the regulatory role of Malat1 in RNA processing [39].

mRNA related interactions

The major role of mRNAs is to encode proteins, and in this fundamental biological process mRNAs interact with tRNAs to guide protein translation [17,83]. However, mRNAs can also interact with other types of RNA molecules in multiple biological processes. For example, they interact with snRNAs in alternative splicing [15], and with miRNA in translation regulation [18,19]. Many new types of mRNA-involved RRIs are identified from sequencing-based techniques. For instance, both PARIS and SPLASH revealed a big number of mRNA-lncRNA and mRNA-mRNA interactions. Besides, many sRNA-mRNA interactions have been revealed by CLASH [36] and a similar technique called RIL-seq [63]. Interaction between sRNA and other kinds of RNAs like ncRNA and tRNA have also been identified but the most enriched ones are sRNA-mRNA interactions [36,63]. Some disease-related sRNA-mRNA interactions have been characterized in these studies. For example, the mRNA targets of an enterohemorrhage *E. coli* (EHEC)-specific sRNA, Esr41, were identified. It is shown that Esr41 can interact with the transcripts of iron transport and storage proteins CirA, ChuA and Bfr, thus influence their expressions [36]. In Ref. [43] the authors found that mRNA-mRNA is generally more stable than intra-molecules interaction (*i.e.*, RNA secondary structure) due to a lower folding energy. They verified 12 pairs of them by qPCR experiments. Interaction between different mRNAs have distinguishing features from intra-molecular interactions. Instead of base pairing within the same genomic context (*e.g.*, 5′ UTR), interaction regions of two mRNAs are more likely from different domains. There was no surprise that analyses using network clustering and functional annotation suggest that RRIs within the same cellular compartment tend to have a higher interacting probability [43].

Regulation of ceRNA network

RRIs aggregate into RRI networks, in which a node represent an RNA and an edge represents an interaction. A competing endogenous RNA (ceRNA) network refers to the complex regulation interplays among different RNAs (such as messenger RNAs, transcribed pseudogenes, circular RNAs and long noncoding RNAs) by competition for miRNA binding [84,85]. Abundant functional RNAs such as tRNAs and rRNAs can also act as ceRNAs by forming duplexes with miRNAs. The amount of these RNAs often much exceeds that of their miRNA cognates thus can prevent inappropriate miRNA binding and premature degradation of the other potential miRNA targets [35]. The key factors of ceRNA activity mainly include the abundance and subcellular location of ceRNAs, binding affinity of miRNAs to their miRNA response elements (MREs), RNA editing and secondary structures, etc [85]. The repression degree on a certain mRNA depends on the ratio of a subset of miRNAs to the corresponding ceRNA's MREs [86]. High-throughput validation has been conducted in breast and prostate adenocarcinomas and indicates that a majority of cancer genes are regulated by ceRNA networks in the tumor cells [87].

Characteristics of global RRI network

The development and successful applications of high throughput sequencing-based RRI identification technologies have accumulated a huge amount of interaction data [88]. Studies have been carried out to collect the data and analyze them from a perspective of systems biology. It is found that, very similar to protein-protein interaction (PPI) networks [89], RRI networks are also scale-free and

modular in human [88], mouse [46], and yeast [90]. One characteristic of a scale-free network is the existence of a set of nodes with relatively high interaction degrees [89,91]. These nodes are called hubs, which are often associated with important regulatory roles in a cell. The loss of hubs usually causes the breakdown of the whole interaction network into disconnected subnetworks [92]. These subnetworks usually correspond to local modules with higher density of interactions relative to the whole network. In many cases these modules are enriched with specific functions, *e.g.*, RNA binding, translation or RNA metabolism [43]. RRI networks may also associate with cell states. Compared with retinoic acid (RA) treated human embryonic stem cells (hESC), the RRI network density in hESC tends to be much more interconnected, suggesting that RRI networks may be highly dynamic and indicate different states for a cell [43]. However, we are just started to unveil the complexities of these networks. Cautious should be taken when interpreting data that might be incomplete, biased and contain much noise [88].

CHALLENGES IN RNA-RNA INTERACTION STUDIES

Despite the recent big progresses on RRI discovery, especially those sequencing-based methods that can approach the RNA full interactome in one experiment, the image on functional RNA interactions inside a cell remains far from clear and complete.

First, the coverage of current technologies are very limited in identifying all RRIs. The recent breakthrough in transcriptome-wide methods for RRI discovery depends on the crosslinking of interacting duplexes by small molecules including psoralen and its derivates [41,43,45,46]. However, psoralen and AMT can only crosslink pyrimidines, and with very limited efficiency [93,94]. For instance, the cross-linking efficiency is less than 6% in most related techniques [41,43,45,46]. In addition, the efficiency of proximity ligation is also low and influenced by the secondary and tertiary structures of target RNAs, which may result in deteriorated performance on small ncRNAs [41,45]. As a consequence, a high sequencing depth is usually required to achieve a broad coverage on RNA interactomes in these methods. There is a pressing need to develop new molecules of higher crosslinking efficiency and no nucleotide bias.

Second, the dynamic nature of RRIs adds additional difficiulties in revealing RRIs and their biological roles. RRIs are frequently modulated by RNA interacting with other molecules (*e.g.*, DNA, RNA, protein), thus specific RRI relationship may be gained or lost in different biological process. This concept has been confirmed by large-scale studies which showed that alternative secondary structures or inter-molecular interactions is common for many RNAs [41]. One convincing example is the lncRNA Xist. The study by PARIS demonstrated that alternative structures are supported by a large fraction of helices detected as conflicting duplexes, which is consistent with co-variation analysis [41]. Although this is an example of intra-molecular interactions, the biophysical basis is the same for inter-molecular RRIs. Studies have now started to understand the regulation and functional relevance of alternative RRIs. For example, different cell states may have distinct global distributing patterns of RRIs. It is shown that many RRIs are lost in differentiated human ES cell, thus the interaction network is less interconnected [43]. However, more in-depth investigations should be taken to validate and interpret the observations.

Third, in particular for high-throughput sequencing-based studies, the algorithms and parameters to call interactions from sequencing data vary from technology to technology [41,43,45,46]. In many cases, these contribute to the ambiguity in comparing data sets across different studies. To date, every published high-throughput technology came with an analysis pipeline to identify RRIs. However, there is no systematic testing on the strategy of reads mapping, the algorithm of duplex calling, and the scoring functions of interaction evaluation. It is in great desire to develop a computational framework that could uniformly process the raw data from different technologies and identify RRIs. We envision that the framework could consist of (i) data preprocessing to account for different sequencing setups, (ii) a statistically solid algorithm to call interactions, (iii) and a confidence measurement to assign a score to each individual RRI with careful benchmarking.

Last and most importantly, it is difficult to distinguish specific and non-specific RRIs and even more challenging to elaborate the precise functions of a certain RRI. RRIs can be formed by random collision and pairing of RNA molecules. The energy differences between specific and non-specific are often not significant and the function of a specific interaction sometimes only depends on certain proteins and other trans-factors that read out the interaction. The dilemma is, non-specific RRIs are also captured in most of current technologies. For example, random ligations in many sequencing-based methods will result in non-specific interactions that are difficult to be distinguished from specific ones. Even if a interaction has been validated to be specific, usally it needs a lot of research to confirm its functional role in gene regulation. For example, thousands of mRNA-involved interactions have been detected in different transcriptome-wide studies [41,43,45,46]. In SPLSH [43] the authors validated 12 out of 13 mRNA-mRNA interactions using qPCR. However whether and how these mRNA-mRNA affect their localization, translation, or degradation

remains to be answered. Hythothesis has been proposed for their collaborative regulations. There is a chance that we are at the door of revealing a hidden layer of complexity in genetic regulations.

CONCLUSIONS AND OUTLOOK

Interactions between different RNA molecules play crucial roles in RNA regulations and functions. With the advent of different techniques, there have accumulated a great number of RRIs identified *in vitro* and *in vivo*. This rich resource has led to many novel findings of RRI's role in gene expression regulation. For example, in Ref. [43] the authors found that a module of RRI network is enriched in correlated translation efficiency, thus RRI may be another factor in regulating protein abundance. Nevertheless, more regulatory roles and functional effects of RRIs remain to be explored. Previously RNA modifications were found to be able to switch the conformations of local RNA secondary structures [52]. As the duplex formation is essentially the same for local RNA secondary structures and also inter-molecular RRIs, RNA modifications may also regulate RRI duplexes, or vice versa. There are many protein mediated RRIs *in vivo* [46,95]. The crosstalk between RRIs and proteins is largely unknown. Focused association analysis is in great desire to reveal the underlying mechanism of their interplay.

Efforts have been devoted to the curation of RRIs. NPInter [96], RAID [97] and RAIN [98] collected both experimental and predicted data of RNA-associated interactions. However, RRIs aggregated in these resources are largely miRNA related; and computational predicted interactions contribute most of the data. Recently, a new comprehensive database of RNA interactions, RISE [88], was constructed to collect RRI data with an emphasis on those from the new sequencing-based studies. It provides a centralized repository to explore, compare, and analyze RNA interactions in a convenient way. As the development and application of sequencing-based methods, more and more RNA interaction data will be generated. A unified and comprehensive platform like RISE will facilitate the functional exploration of RRIs. We envision that we can start to decipher the functional roles and the underlying mechanisms of RRIs as a new paradigm of gene expression regulation.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Jing Gong, Yanyan Ju, Di Shao and Qiangfeng Cliff Zhang declare that they have no conflict of interests.

This article is a review article and does not contain any studies with human or animal subjects performed by any of the authors.

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