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Review Article

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Advanced Bioinformatics and Biotechnology Approaches in Identifying and Targeting RNA Regulatory Networks

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ABSTRACT

Background and Objective: RNA regulatory networks are of fundamental importance in gene expression, cellular homeostasis, development, and disease. Recent advances have improved the identification, characterization, and potential therapeutic targeting of these complex regulatory systems. This narrative review summarizes the computational and experimental approaches available for the study of RNA-mediated regulation with a particular focus on non-coding RNAs (ncRNAs), including miRNAs, lncRNAs, circRNAs, siRNAs, and piRNAs.

Materials and Methods: The review discusses computational tools and databases available for the prediction of RNA-RNA, RNA-protein, and transcription factor-RNA interactions, as well as the integration of multi-omics data to build regulatory networks. It goes on to consider the experimental technologies available for the profiling and validation of RNA interactions (bulk and single-cell RNA-seq, long-read sequencing, reporter assays, RIP, CLIP, and RNA-centric capture methods). Functional validation is also a major theme, with discussion of loss-of-function and gain-of-function experiments, CRISPR-based perturbation, rescue experiments, and the validation of feed-forward regulatory motifs. The review also considers the emerging biotechnologies for modulation of RNA regulatory networks (RNA-targeting technologies, CRISPR-based RNA modulation, delivery systems, and chemical modification of RNA therapeutics).

Results and Conclusion: By integrating computational prediction, multi-omics network construction, experimental validation, and emerging RNA-targeting biotechnologies, this review provides a comprehensive framework for translating RNA regulatory network discoveries into mechanistically supported and therapeutically relevant applications. Particular emphasis is placed on distinguishing predicted molecular associations from experimentally validated interactions and causal regulatory mechanisms.

What is "already known":	• ncRNAs regulate gene expression at multiple molecular levels • Computational tools predict RNA interactions but can generate false positives. • RNA therapeutics face delivery, specificity, and off-target challenges
What this article adds:	• Integrates computational, multi-omics, and experimental approaches • Compares methods to distinguish RNA binding from functional regulation. • Connects regulatory network discovery with emerging RNA-targeting therapies

1. INTRODUCTION

The classical view of molecular biology is based on the assumption that the genome provides templates for the synthesis of subsequent proteins. This view of genome function has evolved substantially through genome-wide sequencing and transcriptomic studies. With the advent of this information, it has become clear that only a small fraction of the human genome encodes proteins, while extensive regions generate non-coding transcripts, many of which remain incompletely characterized [1]. Although some non-coding transcripts were initially regarded as transcriptional noise, many characterized ncRNAs have important regulatory functions [2]. Rather than acting as passive bystanders, ncRNAs function as dynamic and interconnected components of complex regulatory networks, exerting control over cellular processes through intricate molecular interactions. NcRNAs act as central regulators of gene expression and regulate biological processes at three different levels: transcriptional, post-transcriptional, and epigenetic levels through various mechanisms including mRNA splicing, translational inhibition, and chromatin remodeling. The noncoding RNA family includes several key subgroups such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs), each of which is characterized by distinct biogenesis pathways and functional modes [2]. Maintaining cellular homeostasis is the result of complex interactions between these diverse classes, ranging from small RNA-mediated silencing to large-scale chromatin remodeling by lncRNAs. Any disruption in the regulation of these regulatory modules can be a hallmark of various pathologies [3]. Any disruption and disorder in the regulation of ncRNA networks is intricately linked to the pathogenesis and development of various human diseases, including cancer, neurological disorders, cardiovascular diseases, and metabolic syndromes [4]. In addition to their role in regulating gene expression, their tissue-specific expression patterns and remarkable stability in biological fluids have made ncRNAs powerful non-invasive biomarkers for disease diagnosis, prognosis, and treatment monitoring [5]. Despite significant therapeutic promise, the challenge of translating RNA-based discoveries into clinical applications remains, requiring advanced biotechnologies to overcome the barriers of delivery, stability, and specificity of these molecules. In addition, computational intelligence, particularly artificial intelligence (AI) and machine learning (ML), has revolutionized the way we interpret these complex networks, predict RNA structures, and develop highly specific RNA therapies [6]. In this context, the integration of cutting-edge bioinformatics methods with experimental biotechnology provides a very attractive avenue for the systematic identification, characterization, validation, and targeting of ncRNA-mediated regulatory networks; thus, linking computational predictions to real mechanistic understanding and application potential.

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2. METHODS

2.1. Review design

This study was conducted as a narrative review with the aim of providing an overview of bioinformatics advances and biotechnological approaches to identify, characterize, validate, and target RNA regulatory networks.

2.2. Literature search strategy

Literature searches were conducted using PubMed, Scopus, and Google Scholar without restrictions on publication date. The search focused on articles related to non-coding RNAs (ncRNAs), RNA regulatory networks, computational prediction of RNA interactions, multi-omics integration, experimental validation, and emerging RNA targeting technologies. A wide range of keywords and their combinations were used to identify publications relevant to the objectives of this review.

2.3. Eligibility criteria

Original research articles and review articles that addressed the biological functions, computational analysis, experimental characterization, validation, and therapeutic targeting of RNA regulatory networks were considered relevant. Articles related to miRNAs, lncRNAs, circRNAs, siRNAs, piRNAs, and their molecular interactions were all within the scope of the review.

2.4. Literature selection and data integration

Articles were evaluated for their relevance to the objectives and scope of this review. Relevant findings were grouped into general themes: computational approaches, experimental validation technologies, and biotechnological approaches to targeting RNA regulatory networks. A narrative review of the evidence was then conducted to identify key issues and future directions.

3. RESULTS AND DISCUSSION

3.1. Functional classification and biogenesis of ncRNAs

To understand the complexity of RNA-based regulation, it is essential to adopt a comparative and functional perspective on ncRNAs. The classification of ncRNAs cannot be said to be based solely on size, but is deeply rooted in their biogenesis, the ways in which they interact with proteins and nucleic acids, and their specific roles in the cellular regulatory hierarchy. The regulatory capacity of ncRNAs is also defined by their ability to act as scaffolds, decoys, guides, or signals. By finely regulating the transcriptome and proteome, ncRNAs coordinate these two layers and serve as central actors that connect different biological signaling pathways [7]. The functional diversity of ncRNAs is a direct result of their structural complexity and ability to interact with various molecular partners, including proteins, DNA, and other RNA species, and so, understanding the distinction and demarcation between these categories requires the definition of each class, comparative analysis of their biogenesis pathways, and regulatory mechanisms, which are presented separately below.

3.1.1. Major classes of non-coding RNAs

Due to their significant structural diversity and various biological functions, non-coding RNAs can be grouped into several broad functional categories (Figure 1). Classification generally reflects transcript length, structure, biogenesis, and molecular function. The major classes are summarized below.

3.1.1.1. MicroRNAs (miRNAs)

MicroRNAs (miRNAs), a category of non-coding RNAs are single-stranded RNAs of 18 to 24 nucleotides in length that regulate gene expression at the post-transcriptional level, primarily by directing argonaute-containing silencing comp-

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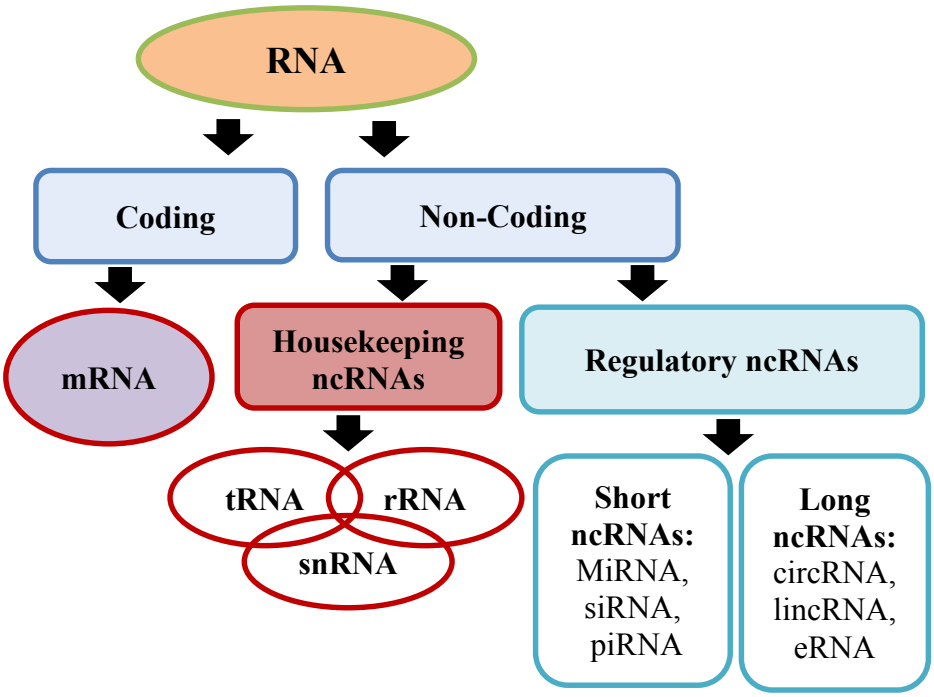


Figure 1. Classification of non-coding RNAs

lexes to relatively complementary sites on target messenger RNAs.

This class of non-coding RNAs was first discovered and characterized in 1993 through studies of lin-4 in Caenorhabditis elegans, which led to the opening of a new layer of gene expression regulation through post-transcriptional control [8]. Most miRNAs are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs) containing one or more stem-loop hairpins. In the nucleus, a complex containing from the RNase III enzyme Drosha and its cofactor DGCR8 cleaves these hairpins into approximately 55-70-nucleotide that calls as precursor miRNAs (pre-miRNAs). The pre-miRNA is exported to the cytoplasm by Exportin-5 in a Ran-GTP-dependent state. Then Dicer cleaves it into a short miRNA duplex. The guide strand is loaded into an Argonaute (AGO) protein to create the miRNA-induced silencing complex (miRISC) and the passenger strand is discarded [9]. Mature miRNAs usually recognize targets by their seed sequence through base pairing, most often with the 3′ untranslated region (3′ UTR) of mRNAs. This interaction facilitates translational repression, mRNA deadenylation, decapping, and exonucleolytic decay; as a result, miRNAs diminish protein production from numerous target transcripts. Specifically, the mechanism of repression varies based on the degree of complementarity. For example, near-perfect complementarity often triggers direct mRNA cleavage by AGO2, whereas partial complementarity typically results in translational inhibition and accelerated decay through deadenylation [10]. Individual miRNAs can regulate multiple mRNA targets, and multiple miRNAs can converge on a single pathway, enabling combinatorial and context-dependent control of cellular processes such as development, differentiation, proliferation, apoptosis, and immune responses [11]. Recent studies show that miRNA production and its stability are controlled by RNA modifications, precursor structure, tailing reactions, and target-directed miRNA decay. In addition, miRNA dysregulation is increasingly implicated in cancer and other

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human diseases [8]. From a therapeutic standpoint, while the ability of miRNAs to target entire gene networks is a significant advantage, it also presents a major challenge regarding specificity, as unintended off-target interactions can lead to unintended biological consequences in clinical applications [12].

3.1.1.2. Long non-coding RNAs (lncRNAs)

Long non-coding RNAs (lncRNAs) are RNA molecules longer than 200 nucleotides that have little or no protein-coding capacity but function as versatile regulators of gene expression at epigenetic, transcriptional, and post-transcriptional levels [2]. Most lncRNAs are transcribed by RNA polymerase II and can undergo canonical processing, including 5′ capping, splicing, and 3′ polyadenylation [13]. In the nucleus, lncRNAs serve various roles such as guides, scaffolds, decoys, or signals to attract or sequester transcription factors and chromatin-modifying complexes. These interactions can influence processes including histone modifications, DNA methylation, chromatin loops, topologically associating domains, and the organization of nuclear compartments [2]. For instance, lncRNAs can act as scaffolds to assemble multiple proteins including chromatin-modifying enzymes and guide these complexes to specific genomic loci, or as decoys to sequester transcription factors away from their target DNA, thereby functioning as molecular switches in gene regulation [7]. Additionally, in the cytoplasm, lncRNAs regulate mRNA stability, splicing, translation, and protein activity [14]. Their role as competing endogenous RNAs (ceRNAs) is a prominent cytoplasmic mechanism, in which they act as molecular sponges to sequester miRNAs, leading to their non-binding to their target mRNAs and indirectly enhancing those targets. Through these diverse interactions with DNA, RNA, and proteins, this class contributes to development, cell identity, immune responses, stress adaptation, and homeostasis. Their dysregulation is linked to diseases such as cancer, developmental disorders, and other human-related diseases. Therefore, lncRNAs constitute an essential regulatory machine that connects chromatin organization, transcriptional oversight, and RNA-mediated gene regulation in a highly context-specific state [2].

3.1.1.3. Circular RNAs (circRNAs)

Circular RNAs (circRNAs) are a class of covalently linked, single-stranded, non-coding RNAs that are primarily produced by back-splicing of precursor mRNAs, in which a downstream splice donor is joined to an upstream splice acceptor, creating a stable loop without free 5' and 3' ends [15, 16]. Their closed structure makes them relatively resistant to exonucleases, allowing many circRNAs to persist in the cytoplasm and act as durable regulators of gene expression [15]. Compared to linear RNAs, this unique circular topology provides enhanced stability, making them exceptionally robust biomarkers in clinical diagnostics. Some circRNAs can act as competing endogenous RNAs for microRNAs, potentially reducing miRNA-mediated repression of target mRNAs when expression and binding-site abundance are sufficient. They can also interact with RNA-binding proteins as decoys, scaffolds, or adaptors, and regulate transcription and splicing processes by interacting with the transcriptional machinery [15, 16]. Additionally, certain circRNAs contain internal ribosome entry sites or RNA modifications such as N6-methyladenosine that enable cap-independent translation, producing peptides or proteins with regulatory functions [17]. Through these pathways, circular RNAs participate in fundamental cellular processes such as proliferation, differentiation, immune signaling, and stress adaptation while ensuring homeostasis. Conversely, their aberrant expression is strongly linked to oncogenesis, neurological disorders, and immune-mediated conditions [16, 18].

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3.1.1.4. Small interfering RNAs (siRNAs)

Small interfering RNAs (siRNAs) are 21-24 nucleotide double-stranded RNAs that mediate sequence-specific post-transcriptional silencing of complementary target mRNAs through the RNA interference pathway. After loading into RISC, the guide strand directs AGO2-mediated cleavage of the target transcript, thereby reducing protein expression. This mechanism is particularly valuable for targeting transcripts encoding proteins that are difficult to inhibit pharmacologically [19, 20]. In the RNAi pathway, long double-stranded RNA precursors are cleaved into siRNA double-stranded RNAs by the RNase III enzyme Dicer and then loaded into the RNA-induced silencing complex (RISC) containing Argonaute proteins [19]. The resulting siRNA duplex is stabilized within RISC, where the degree of thermodynamic asymmetry between its two ends contributes to accurate guide-strand selection. During RISC loading, the passenger strand is removed while the guide strand directs the complex to complementary target mRNAs. Through extensive base pairing with the target transcript, the guide strand positions the catalytic Argonaute protein, particularly Argonaute2, for sequence-specific endonucleolytic cleavage. Argonaute-mediated cleavage promotes target mRNA degradation [19]. This process decreases the cellular abundance of the corresponding mRNA and consequently suppresses protein coding, thereby linking RNA recognition and molecular cleavage to the downstream biological effect of gene silencing. According to studies, siRNAs function as key components of antiviral defense and transposon silencing in many organisms, and help limit the activity of viral RNAs and genetic elements [3, 19]. In mammalian systems, synthetic siRNAs have emerged as widely used therapeutic agents, although significant challenges remain, including efficient delivery, off-target effects, and immune system activation [3]. Furthermore, their clinical application may be limited by the following reasons: limited extracellular and intracellular stability, inefficient tissue-specific distribution, and transient activity. While partial sequence complementarity can cause unintended transcriptional repression. Accordingly, the therapeutic performance of siRNAs depends not only on the selection of the target sequence, but also on chemical modification, design of the delivery system, and careful evaluation of on-target efficacy, off-target effects, and immunogenicity [21].

3.1.1.5. PIWI-Interacting RNAs (piRNAs)

PIWI-interacting RNAs (piRNAs) represent a unique category of small non-coding RNAs, generally ranging from 24 to 32 nucleotides in length. They associate with PIWI-family Argonaute proteins and function as sequence-specific guides for both transcriptional and post-transcriptional gene silencing. Compared to conventional RNA-targeting platforms like siRNAs, which primarily operate post-transcriptionally, piRNAs possess the unique capability to regulate gene expression at both the epigenetic and post-transcriptional levels, offering a broader, albeit more complex, regulatory landscape. They are primarily recognized as guardians of the germline genome: by guiding PIWI-piRNA silencing complexes to transposable-element transcripts and genomic locations, they inhibit transposon mobilization through target-RNA cleavage, the formation of heterochromatin, and, in mammals, DNA methylation, thus safeguarding genome integrity and fertility [22]. This dual-action capacity to intervene directly at the chromatin level represents a fundamental mechanistic distinction that differentiates piRNAs from purely post-transcriptional silencers. In addition to transposon repression, piRNAs have the ability to regulate protein-coding gene expression, mRNA turnover, translation, chromatin status, and epigenetics, and in recent years have shown emerging roles in

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somatic cells and disease contexts. This functional versatility has sparked significant interest in utilizing piRNA dysregulation in oncology as a potential avenue for diagnostic profiling or future therapeutic intervention. Their biogenesis mechanism differs from the conventional pathway of other non-coding RNAs such as miRNA: piRNAs are generally derived from long single-stranded precursors and are processed via primary rather than Dicer-dependent processing [23]. While their potential as precision medicine tools is substantial, particularly for targeted cancer therapy, they currently remain in the early experimental and preclinical stages compared to established siRNA-based therapeutics. Consequently, the clinical translation of piRNA-based approaches faces substantial hurdles, including the need to elucidate their complex biogenesis and to develop tissue-specific delivery systems capable of modulating these pathways safely in somatic tissues.

3.1.2. Comparative overview of ncRNA classes

Non-coding RNAs constitute a heterogeneous but functionally interconnected group of regulatory molecules that can be broadly divided into small ncRNAs of less than 200 nucleotides, and long ncRNAs of more than 200 nucleotides, mainly lncRNAs, and circRNAs represent a distinct structural class defined by their complex secondary and tertiary folds that enable interaction with diverse cellular machinery. While small ncRNAs serve as precision tools for sequence-directed silencing, long ncRNAs function as multidimensional scaffolds, transcriptional modulators, and competitive endogenous RNA (ceRNA) sponges, thereby orchestrating gene networks rather than acting on single targets. This functional distinction is crucial: small ncRNAs offer high specificity for knockdown applications, whereas long ncRNAs represent a largely untapped therapeutic frontier requiring strategies to modulate protein-RNA or RNA-DNA interactions [24]. Small ncRNAs mainly mediate sequence-directed silencing, miRNAs and siRNAs use argonaute-containing complexes to repress or degrade complementary mRNAs, while piRNAs associate with PIWI proteins to silence transposable elements and regulate chromatin-related processes, particularly in the germline. In contrast, lncRNAs and circRNAs can modulate gene expression by interacting with proteins, DNA, chromatin, and various RNAs, offering a broader regulatory scope but posing significant challenges regarding target specificity, structural prediction, and delivery [2, 23, 25]. The characteristics of each of the noncoding RNA classes are compared in Table 1.

3.1.3. From individual ncRNAs to integrated regulatory networks

Increasing evidence suggests that all classes of ncRNAs function as interconnected components of dynamically interacting gene regulatory networks. From a systems biology perspective, characterizing these networks is essential for predicting the systemic response to therapeutic RNA manipulation. Within these networks, an individual ncRNA can concurrently affect transcription, RNA stability, translation, chromatin organization, and signaling pathways, while its function is influenced by the quantity, location, and interacting partners of other RNAs. A central example is the competing endogenous RNA (ceRNA) network, in which lncRNAs, circRNAs, pseudogenes, and mRNAs containing shared miRNA response elements compete for a limited pool of miRNAs. By sequestering miRNAs, these transcripts can indirectly relieve repression of common target mRNAs and coordinate gene expression [26].

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Table 1. Comparative analysis of non-coding RNA classes: mechanisms, biogenesis, and therapeutic status

Non-coding RNA	Size & Structure	Biogenesis	Regulatory State	Biomarker Potential
miRNAs	18-24 nt/ Single-stranded	Pri-miRNA → Drosha/DGCR8 → pre-miRNA → Exportin-5 → Dicer	Translational repression and mRNA decay	Detectable in biological fluids including plasma, serum, and extracellular vesicles
siRNAs	21-24 nt/ Double-stranded duplex	Dicer cleavage of long dsRNA or exogenous/synthetic duplexes	Sequence-specific mRNA cleavage/degradation	Therapeutic biomarkers
piRNAs	24-32 nt/ Single-stranded	Long single-stranded precursors; Dicer-independent processing and ping-pong amplification	Transposon silencing; transcriptional and post-transcriptional repression	cancer-related contexts
lncRNAs	>200 nt/ Linear (capped & polyadenylated)	RNA polymerase II transcription with variable RNA processing	Chromatin remodeling, transcription, splicing, mRNA stability, miRNA decoying	tissue- and disease-specific circulating and exosomal forms
circRNAs	several hundred nt to >1 kb	Back-splicing of pre-mRNA	miRNA sponging, protein sequestration, transcription/splicing regulation, translation	stable in biofluids and enriched in extracellular vesicles

This molecular competition implies that the therapeutic modulation of a single miRNA may have cascading effects across the entire ceRNA network, necessitating sophisticated computational models to predict resultant therapeutic outcomes. This type of crosstalk establishes regulatory pathways such as lncRNA-miRNA-mRNA, circRNA-miRNA-mRNA, and more intricate lncRNA/circRNA-miRNA-mRNA modules. In these pathways, lncRNAs and circRNAs function as miRNA sponges, protein scaffolds, or decoys, whereas miRNAs act as post-transcriptional hubs that connect coding and non-coding transcripts. The biological impact of a non-coding RNA (ncRNA) cannot solely be deduced from its specific interactions with individual targets; instead, it arises from its role within a larger RNA interactome. Therefore, evaluating the therapeutic potential of an ncRNA requires moving beyond singular target validation toward integrating multi-omics data and structural interactions. This network-level perspective is particularly relevant in disease settings, when disruption and dysregulation in a single ncRNA can be transmitted through ceRNA modules and protein interactions, altering entire molecular pathways involved in the development and progression of diseases including cancer, neurological dysfunction, immune signaling, and metabolic imbalances [27]. Consequently, the future of RNA therapeutics lies in integrating computational prediction, experimental validation, and network-level analysis to design interventions that minimize systemic disruptions while maximizing therapeutic specificity [28].

3.2. Computational tools for predicting RNA interactions

Most computational approaches assume that RNA molecules are able to interact when they have corresponding complementary base-pair regions. In miRNA-mediated regulation, the

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important region of interest is the seed sequence, which is generally nucleotides 2-8 at the 5′ end of a mature miRNA [29]. When the seed is complementary to some sequence in the 3'UTR of mRNA, the miRNA may recruit RISC (RNA-induced silencing complex) that inhibits tran-slation and degrades mRNA [30]. However, seed complementation alone is not enough to ensure biological relevance and often leads to high false-positive rates in predictive models. A useful tool should also consider such factors as evolutionary conservation of the binding region, thermos-dynamics (whether local RNA structure allows miRNA to bind), whether the site is available within the 3' UTR of the message, and whether the local environment contains a high content of adenine and uracil, which promotes miRNA binding [31]. These factors help to differentiate real interaction from mere complementation, thereby increasing the specificity of the pre-diction. Concerning lncRNAs and circRNAs, pre-diction becomes difficult due to various effects on the regulation of gene activity that may be exerted by these ncRNAs [32]. The effect is mediated either by acting as ceRNAs (competing RNAs) and capturing miRNAs; in this case, miRNA-targeted mRNAs will be more functional; or by interacting with mRNAs, proteins, or DNAs, altering pack-aging and gene expression [33]. Therefore, anal-yzing lncRNAs and circRNAs with computers needs to mix sequence predictions with infor-mation about how much RNA is around, its sub-cellular localization, and results from experiments that show where it binds, as structural complexity and stoichiometric abundance are as critical as sequence complementarity in these classes.

The most advanced area of bioinformatics in ncRNA is the prediction of miRNA targets. The major algorithms evaluate the following broad characteristics: Seed complementarity, conserva-tion, thermodynamic stability, and accessibility of the target site [30]. Due to the various assum-ptions each algorithm applies, it is better to utilize several tools together to achieve a consensus-based approach, which mitigates the inherent biases and limitations of individual compu-tational models.

3.2.1. TargetScan, miRanda, miRmap, and miRDB

TargetScan is one of the widely used methods for miRNA target prediction. This method looks for conserved 8-mer and 7-mer sites in the 3'UTR that can pair with the seed of the given miRNA. In simple terms, TargetScan identifies conserved sites in the mRNA that can pair with the seed of this miRNA [34]. Besides the site itself, TargetScan also takes into account the contextual factors that affect the efficiency of repression. For example, the presence of A/U rich residues nearby, the position of the site in the 3'UTR, and the presence of 3' compensatory pairing outside of the seed [35]. The major advantage of TargetScan is that it focuses on conserved sites, therefore improving the biological plausibility. However, as a limitation, many functional miRNA/mRNA interactions are either not well conserved or are not in the 3'UTR canonical site [36], thus, potentially missing species-specific or non-canonical regulatory networks. By comparison, miRanda estimates miRNA-mRNA interactions based on sequence complementarity, binding free energy, and evolutionary conservation. The main difference from TargetScan is that miRanda can recognize non-perfect seed pair interactions. Hence it is good for finding non-canonical miRNA-mRNA interaction, but more false positive prediction is expected [37]. miRMap is a more comprehensive scoring system. The score integrates 11 features, including seed match quality, target accessibility, local A/U enrichment, 3′ compensatory match, and the distance from the 3′ end of the 3′ UTR, calculated based on thermo-dynamic, conservation-based, probabilistic, and sequence-based methods. In practice, it's not just

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whether the miRNA can bind the target, but more like how probable the binding will bring detectable repression [38]. Moreover, miRDB functions based on MirTarget, a machine learning algorithm trained on experimental sequencing data by identifying miRNA targets and predicting their functional relevance scores [39]. In contrast to methods that rely only on seed matches, MirTarget identifies patterns of experimental mi RNA/mRNA regulation, leveraging predictive modelling to bridge the gap between static sequence matching and dynamic cellular activity. miRDB is especially suitable for researchers who need to prioritize their targets based on their predicted functional significance [40]. Like other prediction methods, many candidate targets are identified, with many potential false positives still a major problem. Accordingly, the use of miRDB requires the filtering of the predictions using either expression data, experimental databases, or functional assays, reinforcing the necessity of an integrative bioinformatics pipeline.

3.2.2. miRNet and DIANA Tools: (microT-CDS and TarBase) as integrated platforms for miRNA regulatory network analysis

Integrated platforms are defined as combining databases, expression datasets, functional annotations, and network visualization to identify regulatory modules [41]. miRNet is a tool for building and visualizing miRNA-centric regulatory networks that include miRNAs, mRNAs, TFs, lncRNAs, circRNAs, proteins, and diseases. It is particularly valuable for identifying hub molecules and disease-related regulatory modules, offering a systemic view that transcends single-molecule interactions. Note that not all network hubs necessarily mean causal regulators and can be affected by database bias or unvalidated predictions [42], which underscores the need for cross-platform validation. DIANA-microT-CDS can predict miRNA binding sites in 3'UTRs as well as coding sequences, taking into account conservation and accessibility information [43]. By extending the search space to coding sequences, microT-CDS addresses a critical gap in traditional 3'UTR-centric models. TarBase provides experimentally validated miRNA-mRNA interactions from luciferase reporter assays, western blot, qRT-PCR, RNA affinity purification, and high-throughput sequencing methods [44]. Using both resources enables separate predicted and experimentally supported interactions, thereby allowing researchers to distinguish between high-confidence validated targets and computationally predicted candidates.

3.2.3. DIANA-LncBase, starBase, and RNAInter: analysis of lncRNA- and circRNA-associated interactions

LncRNAs and circRNAs participate in the ceRNA network by sharing the same miRNA response elements (MREs). By binding miRNAs, ncRNAs can indirectly regulate target gene expression by reducing miRNA availability for mRNA targets [45]. This competitive landscape necessitates tools that can accurately map these intricate interaction networks. In this regard, DIANA-LncBase is aimed at miRNA-lncRNA interactions (both experimentally and computationally predicted). The integration of Argonaute CLIP-seq information is very useful since Argonaute binding supports miRNA regulation more strongly [46], providing a biochemical basis for interaction that pure sequence complementarity lacks. starBase is integrated into ENCORI, and it is an extensive source for CLIP-seq-supported miRNA-mRNA, miRNA-lncRNA, miRNA-circRNA, and ceRNA interactions. The interaction data are combined with RNA expression data and pan-cancer datasets. The CLIP-seq evidence shows the physical binding of Argonaute or other RBPs to the RNA region. It supports but does not prove the functional relevance of the predicted interaction [47], as high-throughput physical

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binding does not always equate to a measurable repression of the target transcript. On the other hand, RNAInter is an extensive RNA interactome database that contains both experimentally validated and computationally inferred RNA-RNA, RNA-protein, RNA-DNA, and RNA-compound interactions. The database includes annotations of RNA editing, localization, modification, secondary structure, tissue specificity, and involvement in diseases [48]. The confidence scoring system based on experimental evidence and tissue specificity makes it appropriate for ncRNA analysis, bridging the gap between static interaction lists and biologically context-dependent regulatory insights.

3.2.4. Integration of transcription factor-RNA regulatory interactions

The miRNAs themselves are involved in transcriptional regulatory networks. Transcription factors (TFs) regulate the expression of miRNAs, whereas the miRNAs themselves can regulate mRNAs coding for TFs and/or regulatory proteins. This reciprocal interaction often manifests as complex feed-forward or feedback loops that maintain cellular homeostasis or drive pathological states. TransmiR is a curated database of associations between TFs and miRNAs with experimental support [49]. This database assists in identifying upstream transcriptional regulators of disease-associated miRNAs and placing them in larger regulatory networks, moving the analysis beyond isolated molecular pairs to a systems-biology perspective.

3.2.5. RNA-RNA interaction prediction

Not all lncRNA-mediated, circRNA-mediated, antisense RNA-mediated, and viral RNA-mediated interactions obey canonical miRNA seed principles. The deviation from sequence-based seed matching necessitates a shift toward structural-based paradigms, where RNA structure and accessibility play an important role. RNAfold estimates RNA secondary structure by finding minimum free energy structures and base-pairing probabilities. This method is useful in identifying exposed sites that can interact with other RNAs or proteins [50, 51], though its predictive accuracy is often constrained by the inherent flexibility and dynamic folding of RNA molecules in vivo. IntaRNA predicts direct RNA-RNA interactions by combining information on sequence complementarity, hybridization energies, and target accessibility. As accessibility-based tools perform better than sequence-based tools, IntaRNA is a good choice for predicting lncRNA-mRNA, circRNA-miRNA, and antisense RNA-mRNA interactions [52]. By integrating thermodynamic stability with local accessibility, IntaRNA mitigates the high false-positive rates typically encountered in purely complementarity-based algorithms.

3.2.6. Prediction of RNA-protein interactions

The RNA-binding proteins (RBPs) have important functions such as the regulation of RNA splicing, stabilization, localization, translation, and degradation of RNA, whereas the noncoding RNAs can bind the RBPs or sequester them at specific sites [53]. This bidirectional regulatory loop underscores the necessity of defining precise binding modalities to understand cellular homeostasis. RNA-protein interaction (RPI) predictions computationally depend on RNA sequence features, RNA secondary structure, protein-binding sites, conservation, expression correlation, and CLIP-Seq data. However, achieving high predictive accuracy remains challenging due to the dynamic and context-dependent nature of RBP-RNA complexes, which are often sensitive to physiological and environmental conditions. The direct [25] or indirect detection of RPI is done through experiments like HITS-CLIP, PAR-CLIP, iCLIP, eCLIP, and RIP-seq [54]. While these high-throughput technologies have revolutionized the interactome landscape, they primarily capture

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transient associations, necessitating integrative computational validation to establish functional relevance. RNAInter and NPInter are currently two major integrative databases for RPI [55], serving as essential scaffolds for systems-biology approaches despite the potential reporting biases inherent in aggregated experimental datasets.

3.2.7. Integration of multi-omics data for RNA regulatory network construction

According to a growing body of evidence, building an ncRNA regulatory network necessitates combining interaction predictions with multiple omics data [25] . In general, it involves the following steps: (i) to detect differentially expressed miRNAs, lncRNAs, circRNAs, and mRNAs, (ii) predict miRNA-mRNA, miRNA-lncRNA, and miRNA-circRNA interactions, (iii) filter based on experimentally supported data (e.g., TarBase, miRTarBase, ENCORI, RNAInter, etc.), (iv) filter based on expression data (e.g., inverse correlation between miRNA and mRNA), (v) build a ceRNA network and detect hub molecules, (vi) functional annotation of target genes [56]. While this pipeline provides a robust framework, the integration of multi-omics data must account for tissue-specific expression profiles to avoid false-positive network topologies. To build a ceRNA network, it is likely that for the lncRNA/circRNA-miRNA-mRNA axis we could observe inverse miRNA-lncRNA/circRNA and miRNA-mRNA correlations and positive lncRNA/circRNA-mRNA correlations with the same miRNA. In circRNA-mediated sponging, a potential regulatory relationship could be inferred from an inverse circRNA-miRNA correlation when a predicted miRNA response element (MRE) exists [57]. However, statistical correlation alone does not establish causality, since co-expression patterns may result from shared upstream transcriptional regulation rather than direct RNA-RNA interactions. But correlation doesn't mean causality. The potential interaction should be validated by CLIP-seq/Argonaute binding or other suitable subcellular localization and functional validation to distinguish between direct sponging effects and indirect regulatory associations.

3.2.8. Limitations of computational prediction

The key drawback of miRNA target prediction is a high false positive rate, as some approaches have been reported to reach false positive rates of up to 50%, and even the most reliable predictors can still yield significant false positives in experiments [37]. This inherent uncertainty stems from the fact that sequence complementarity alone is insufficient to guarantee functional repression in a complex cellular environment. Consequently, computational predictions should be seen merely as hypothesis generation rather than definitive regulatory evidence. An axis for lncRNAs-miRNAs-mRNAs with confidence requires experimentally supported miRNA binding sites, coexpression or inverse expression correlation, tissue-specific expression, CLIP-seq or Argonaute binding support, and functional validation. Integrating these multifaceted layers of evidence is essential to transition from purely predictive models to biologically validated regulatory networks.

3.2.9. Artificial intelligence and machine learning in RNA therapeutics

Artificial intelligence and machine learning are increasingly being used in RNA research and therapeutic development. By combining nucleotide sequences, predicted RNA structures, chemical modifications, expression profiles, and experimental interaction data, these methods can help predict RNA structures, identify RNA-protein and RNA-ligand interactions, and prioritize potential binding sites for therapeutic targeting [58-60]. For oligonucleotide therapeutics, computational models can help select and

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optimize antisense oligonucleotide (ASO) and siRNA sequences by predicting target accessibility, silencing potency, stability, and potential off-target effects [60, 61]. Such predictions may reduce the need for experimental screening; however, their reliability depends on the quality of the training data and their biological relevance. Hence, computational scores should be considered as a tool for prioritization, not as evidence of therapeutic efficacy or specificity. Experimental confirmation, including on-target interaction, assessment of off-target effects, and functional rescue, remains essential [60, 61]. Furthermore, integrating multi-omics data with perturbation datasets allows linking RNA expression and molecular interactions to cellular phenotypes and therapeutic responses. Machine learning methods can be used to identify regulatory networks, prioritize therapeutic targets, and investigate cellular responses to genetic and drug perturbations [62, 63]. However, challenges remain, including model interpretability, bias in the dataset, reproducibility, and the need for independent validation. In general, AI-based methods are useful for formulating hypotheses and prioritizing potential RNA targets, but they alone cannot reveal direct molecular interactions or causal biological roles. Therefore, it is essential to conduct experimental validations to confirm the predicted relationships and assess their functional significance.

3.3. Experimental technologies for identifying and validating RNA regulatory interactions

3.3.1. Introduction to experimental validation of RNA regulatory networks

Computational analyses can identify and predict various interactions, including miRNA-mRNA, lncRNA-miRNA, circRNA-miRNA, RNA-protein, and RNA-chromatin, but their experimental validation remains indispensable to discern their direct and functional regulatory relationships. While computational models provide the breadth of potential interactions, experimental assays provide the necessary depth and biological truth. A robust experimental validation strategy is usually a combination that can include transcript-level discovery, physical interaction mapping, disruption of the candidate RNA or protein, and assessment of downstream molecular and phenotypic consequences. Such a multi-layered approach is required to overcome the limitations of individual assays, such as the high background noise in pull-down methods or the transient nature of some binding events. These explanations highlight that techniques like RNA-seq, CLIP-based methods, RNA pull-down, ChIRP/CHART/RAP, and CRISPR-based disruption offer strategies for building dependable RNA regulatory networks [64].

3.3.2. High-throughput RNA profiling technologies

High-throughput RNA profiling technologies facilitate an unbiased, genome-wide assessment of RNA levels, transcript structures, and expression patterns specific to various cell types. Although these techniques provide a comprehensive “snapshot” of the cellular transcriptome, they capture steady-state RNA levels, which may mask rapid regulatory dynamics or transient transcriptional events. These methodologies are frequently employed as the basic layer in RNA regulatory research for the identification of differentially expressed non-coding RNAs (DEncRNAs), transcript isoforms, and co-expression modules that can later be subjected to experimental and *in vitro* validation [65]. Researchers must be cautious, as transcriptomic data alone does not distinguish between primary transcriptional changes and secondary effects resulting from feedback loops, necessitating downstream functional verification to establish

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the directionality of the observed regulatory interactions [66].

3.3.2.1. Bulk RNA sequencing (RNA-seq)

In general, bulk RNA sequencing examines and measures the average transcriptome of a specific cell population and is widely used to identify mRNAs, lncRNAs, and circRNAs with differential expression between laboratory conditions [67]. When combined with other computations, such as pathway enrichment and co-expression analyses, RNA-seq methodology can also reveal candidate regulatory relationships and functionally coordinated gene modules [68]. These computational associations are hypothesis-generating and do not, by themselves, establish direct regulatory interactions or causal relationships. Nevertheless, due to its method of averaging signals from numerous cells, bulk RNA-seq can potentially mask effects that are specific to certain cell types or rare populations, especially in diverse tissues like tumors or samples rich in immune cells [69]. Accordingly, findings from bulk profiling may require complementary approaches, such as cell-type-resolved analyses or single-cell sequencing, followed by targeted functional validation to determine whether the inferred signals reflect genuine regulation within specific cellular populations.

3.3.2.2. Small RNA sequencing (small RNA-seq)

Small RNA-seq is optimized for the detection and quantification of short RNAs, typically 18-35 nucleotides in length, including miRNAs, siRNAs, piRNAs, and other small regulatory RNAs. This method is particularly useful for identifying circulating or tissue-specific small RNAs associated with a disease and for constructing regulatory networks that focus on miRNAs. Nevertheless, detection alone does not establish that a disease-associated small RNA is functionally involved in the relevant regulatory network; predicted targets and expression changes therefore require independent experimental validation. However, bias in library preparation and the need for careful normalization remain significant challenges in this method [70, 71]. In particular, sequence- and ligation-dependent differences in RNA recovery can affect the relative abundance estimates, while normalization choices may influence which RNAs are classified as differentially expressed. These technical factors should therefore be considered when comparing studies or interpreting candidate biomarkers [72].

3.3.2.3. Single-cell RNA sequencing (scRNA-seq)

Single-cell RNA-seq is designed to measure transcript abundance in individual cells. This sequencing method allows researchers to identify cellular heterogeneity and rare cell populations and reconstruct the regulatory states specific to each cell type. However, these regulatory states are reconstructed from transcriptional similarity rather than from direct measurements of physical interaction; co-expression of an ncRNA and its putative targets within the same cell indicates compatibility, not proven regulation. In ncRNA research, scRNA-seq can reveal whether a candidate lncRNA or miRNA-related program is restricted to a specific cell type or disease subpopulation. Yet cell-type restriction is a descriptive observation and does not by itself demonstrate that the ncRNA is required for the program, so perturbation experiments remain necessary to establish functional dependency. In this method, the challenges of detecting low-abundance, non-polyadenylated RNAs remain [73, 74]. These limitations are compounded by the shallow sequencing depth per cell, which increases dropout events and can systematically underrepresent short-lived or low-copy transcripts, and by the fact that most standard protocols rely on poly(A) selection and 3′-end capture. Consequently, negative findings in scRNA-seq should be interpreted cautiously, and

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comparisons across samples require explicit correction for batch effects before cell-type-specific regulatory conclusions are drawn [75].

3.3.2.4. Long-read RNA sequencing

Long-read RNA sequencing generates reads spanning entire or nearly complete transcripts, significantly enhancing the annotation of isoforms, alternative splicing events, fusion transcripts, and complex lncRNA architectures. This technology is particularly advantageous for characterizing lncRNAs and circRNAs, whose structural diversity and isoform complexity often elude short-read sequencing. However, long-read platforms are frequently constrained by higher error rates in homopolymer regions and greater costs per read. Additionally, while providing superior structural resolution, long-read sequencing lacks the quantitative precision of high-depth short-read platforms for absolute transcript abundance. Consequently, integrating long-read sequencing for structural discovery with short-read sequencing for accurate quantification represents the most robust strategy for a comprehensive characterization of the non-coding RNA (ncRNA) landscape [76].

3.3.3. Experimental validation of RNA-RNA interactions

To validate RNA-RNA interactions, reporter systems, biochemical enrichment, sequencing-based crosslinking techniques, and spatial visualization approaches can be employed. Combined, these approaches assist in identifying whether predicted interactions are direct, occur within cells, and display measurable regulatory effects [77, 78]. A major obstacle is that interactions must be validated at two levels: physical association and biological effect. For example, although reporter assays confirm interaction potential in vitro, they often overlook how RBP localization and availability modulate the efficiency of RNA-RNA duplexes in a living cellular environment.

3.3.3.1. Dual-luciferase reporter assays

Dual-luciferase reporter assays are widely employed to determine if a miRNA directly targets a predicted binding site within a 3'UTR, lncRNA, or circRNA. While a reduction in reporter activity with the wild-type sequence suggests direct miRNA-mediated repression, this assay does not reflect regulation within the endogenous genomic context. Moreover, the artificial nature of plasmid-based expression often results in the overexpression of both the miRNA and the reporter, potentially inducing false-positive interactions that are absent at physiological concentrations. These discrepancies stem from the absence of native regulatory architecture, such as chromatin states and endogenous RNA competition, which modulate interactions in vivo. Consequently, while indispensable for confirming sequence complementarity, reporter assays should be regarded as a preliminary screening tool rather than definitive evidence of physiological regulatory dynamics [79].

3.3.3.2. RNA pull-down assays

RNA pull-down assays employ biotinylated antisense probes or labeled RNA constructs to enrich for specific RNAs and their associated molecular partners. In investigating RNA-RNA interactions, these assays, followed by RT-qPCR or sequencing, can validate the physical association of a candidate lncRNA or circRNA with a miRNA or other RNA species. However, pull-down assays are inherently susceptible to non-specific interactions, particularly those mediated by ubiquitous RNA-binding proteins (RBPs) that may act as unintended bridges. Furthermore, assay efficiency is heavily contingent upon target RNA accessibility and the stringency of washing steps. Consequently, to differentiate direct RNA-RNA duplexes from indirect, protein-mediated associations, pull-down data must be rigorously interpreted in

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conjunction with orthogonal validation methods [80].

3.3.3.3. RNA-RNA interaction sequencing (e.g., CLASH and CLEAR-CLIP)

The CLASH and CLEAR-CLIP methods identify miRNA target RNA chimeras on a large transcriptional scale. These methods use a combination of cross-linking, safe precipitation of argonaute-containing complexes, and high-throughput sequencing. These approaches provide direct evidence of miRNA binding to mRNAs, lncRNAs, or circRNAs in cells and can be powerful tools for validation and subsequent confirmation of computationally predicted miRNA-target relationships [81, 82]. Although high-throughput techniques using chimeric reads offer direct proof of interaction, these techniques pose quite a bit of technological complexity. The computational processes used should accurately differentiate true chimeras from those that are created artificially while preparing the library. Furthermore, the fact that these methods favor highly abundant RNAs and efficient cross-linking may impede their ability to identify low-abundance RNAs [81].

3.3.3.4. RNA fluorescence in situ hybridization (RNA-FISH)

RNA-FISH uses fluorescently labeled probes to assess the abundance and subcellular localization of specific RNA molecules in healthy cells or tissues, which is particularly useful for confirming whether a lncRNA or circRNA is nuclear or cytoplasmic. It is also useful for examining whether a lncRNA or circRNA co-localizes with a suspected interacting partner [83]. It is crucial to distinguish co-localization from actual physical interaction, as proximity in the same cellular region does not guarantee duplex formation. Additionally, the diffraction limit in standard RNA-FISH often makes it impossible to differentiate between molecules that are simply near each other and those that are directly interacting. Therefore, RNA-FISH spatial data should be integrated with complementary methods to validate direct interactions [84].

3.3.4. Identification of RNA-protein interactions

Identifying RNA-protein interactions requires choosing between protein-centric or RNA-centric approaches, depending on whether the goal is tomap RBP targets or RNA partners. Protein-centric methods provide global breadth but may overlook transient interactions, whereas RNA-centric methods offer high specificity for individual transcripts but have a narrower scope and higher non-specific binding risks. For a comprehensive view of the interactome, both approaches should be integrated to account for both stable and transient complexes [85].

3.3.4.1. RNA immunoprecipitation (RIP)

An antibody against an RNA-binding protein is used to separate native ribonucleoprotein complexes in RNA immunoprecipitation, followed by RT-qPCR, microarray, or even sequencing to identify associated RNAs. RIP is a relatively straightforward method that detects both direct and indirect interactions. In contrast, direct binding of proteins in the same complex is not detected in this method [86]. A significant limitation of this method is that it is usually performed under natural conditions and without crosslinking, which may cause redistribution of RNA-protein complexes after lysis, a phenomenon that can often lead to the detection of “false positive” interactions. Furthermore, since it is unable to distinguish between direct protein-RNA binding and the presence of RNA in a large macromolecular assembly. With these reasons, although RIP is a useful and inexpensive tool, but the absence of crosslinking requires cautious interpretation and the findings should ideally be validated with methods that record the physical interaction in real time [86].

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3.3.4.2. Cross-linking and immunoprecipitation (CLIP-seq)

CLIP-seq employs UV crosslinking to covalently secure direct RNA-protein interactions prior to immunoprecipitation, RNA fragmentation, adapter ligation, and deep sequencing. In contrast to RIP, CLIP offers greater confidence in direct interactions and facilitates the mapping of protein-binding locations on RNAs throughout the transcriptome. CLIP-seq substantially improves the resolution of direct binding-site identification, it also presents technical challenges. UV cross-linking may vary in efficiency, potentially causing certain accessible or highly structured regions of RNA molecules to be over-represented in the resulting data. Furthermore, CLIP-seq involves a complex workflow that requires the fragmentation and ligation of cross-linked molecules. These steps can introduce library-related biases, making specialized bioinformatic methods necessary to define binding-site boundaries precisely [87].

3.3.4.3. Enhanced CLIP (eCLIP)

The enhanced cross-linking and immunoprecipitation (eCLIP) protocol represents a significant technological advancement over conventional CLIP methods by integrating several critical methodological refinements. By incorporating size-matched input controls and improved library preparation, eCLIP helps distinguish specific enrichment from technical background. Furthermore, the streamlined library preparation process enhances the efficiency and scalability of the workflow. These refinements improve the robustness of transcriptome-wide binding-site identification. eCLIP provides a high-resolution map of RNA-binding-protein occupancy, serving as a robust and widely adopted tool for the precise identification and characterization of direct binding sites for various regulatory proteins across the entire transcriptome [88]. Even with the improvements made to the signal-to-noise ratio, eCLIP technology is still hampered by the dynamics of RNA-protein interactions. Due to the fact that the crosslinking will only provide a static 'snapshot' of the cell's condition, the interaction may not capture rapidly changing RNA-protein interactions. In addition, even though the use of size matched controls makes sure that there is less noise in the analysis process, the complexity of the data requires special bioinformatics processing techniques for the data [88].

3.3.4.4. RNA pull-down and mass spectrometry

RNA pull-down assays, when combined with mass spectrometry-based proteomics, constitute a powerful RNA-based approach to systematically identify proteins that are physically associated with a target RNA molecule. This method is powerful for elucidating the complex protein-protein interactions of non-coding RNAs that often act as molecular scaffolds [89]. Even though this technique proves to be very efficient for interactome mapping, non-specific binding in the pull-down procedure is likely to cause significant noise in the mass spectrometry data. Therefore, it becomes necessary to conduct strict statistical analysis as well as negative controls, including scrambled RNA sequences, to separate real binding partners from those proteins which can bind non-specifically. In addition, mass spectrometry is capable of recognizing even such protein molecules that can associate temporarily and have no role in regulation at all. Therefore, although RNA pull-down coupled with mass spectrometry is necessary for scaffold-based modeling, it is still necessary to conduct some other techniques to verify its results [89].

3.3.5. Experimental characterization of lncRNA and circRNA

Because lncRNAs and circRNAs are structurally diverse and often low-abundance, their characterization requires methods that confirm transcript identity, abundance, localization, stru-

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cture, and molecular interactions. Combining expression validation with interaction mapping and functional perturbation provides the strongest evidence for their regulatory roles. The intrinsic complexity of non-coding RNAs creates substantial experimental challenges. In circRNA studies, specific validation is essential to distinguish circular isoforms from their linear counterparts. Likewise, because most lncRNAs are present at low abundance, highly sensitive detection methods are needed to reduce the likelihood of false-negative results. Moreover, transcript co-localization or correlated expression alone cannot establish functional interaction. A robust characterization framework must go beyond correlation and demonstrate causality through rigorous loss-of-function and gain-of-function studies [90].

3.3.5.1. Northern blotting and RT-qPCR

Northern Blotting and RT-qPCR techniques offer orthogonal yet complementary approaches to RNA characterization. Northern blotting enables the direct visualization of transcript size, isoform structure, and RNA integrity, thereby confirming the presence of the expected molecular species. In contrast, RT-qPCR provides a highly sensitive and quantitative measure of transcript abundance. Together, they constitute a robust validation framework, widely utilized to confirm RNA-seq-derived expression changes and to authenticate the differential expression of candidate lncRNAs and circRNAs under varying experimental conditions. [91]. Method selection involves a trade-off between structural resolution and sensitivity. Northern blotting provides essential information on transcript length and isoforms but requires high RNA concentrations and has low sensitivity. Conversely, RT-qPCR is highly sensitive but blind to transcript architecture, potentially misidentifying degraded fragments as full-length. For robust validation of novel RNAs, Northern blotting should be used for structural profiling, while RT-qPCR provides quantification [92].

3.3.5.2. Validation of circRNA back-splice junctions

Given that circular RNAs (circRNAs) are fundamentally generated through non-canonical back-splicing events, their robust identification critically depends on the empirical validation of their signature back-splice junctions (BSJs). To achieve this, researchers typically employ a comprehensive methodological pipeline: outward-facing (divergent) primers for targeted RT-qPCR amplification, Sanger sequencing to confirm the back-splice junction, and RNase R digestion to selectively enrich for exonuclease-resistant circular transcripts. Collectively, these complementary approaches help distinguish authentic circRNAs from linear RNA isoforms, trans-splicing artifacts, or bioinformatic read-mapping ambiguities [93]. Pipeline reliability depends on stringent control procedures. Although RNase R is standard for circRNA identification, it is not foolproof; complex secondary structures or protein binding can protect certain linear RNAs from digestion, leading to false-positive circRNA calls. Similarly, divergent primers for RT-qPCR must be rigorously validated to prevent unintended amplification of linear RNAs caused by genomic DNA contamination or alternative splicing. Consequently, authentic circRNA identification requires combining these methods with nucleotide-level junction sequencing [94].

3.3.5.3. Subcellular localization of regulatory RNAs

Elucidating the subcellular compartmentalization of an RNA molecule, whether it is nuclear, cytoplasmic, membrane-associated, or packaged into extracellular vesicles, is paramount for deciphering its functional mechanism of action. For instance, nuclear enrichment is consistent with potential roles in transcriptional control or

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chromatin regulation. Conversely, cytoplasmic localization is consistent with potential post-transcriptional regulatory functions, such as miRNA sponging, mRNA stability modulation, or translational regulation [95]. Although bulk fractionation is widely used, it may produce incomplete or misleading results because leakage or contamination between nuclear and cytoplasmic fractions can obscure precise transcript localization. The dynamic organization of subcellular compartments and the movement of RNA between them further complicate localization. To address these limitations, high-throughput methods such as RNA-FISH and subcellular RNA-seq (e.g., APEX-seq) should be used to map transcripts accurately within their native cellular architecture [96].

3.3.5.4. RNA structure probing

Through the application of chemical modification or enzymatic cleavage, RNA structure probing methods allow for the high-resolution mapping of base-pairing patterns and single-stranded regions. These experimental insights are crucial for validating in silico structural models. Because the conformational state of an RNA molecule directly modulates its functional repertoire, including its capacity for protein interaction, miRNA-mediated regulation, and its propensity for circularization, structural probing is valuable for linking primary sequence information to actualized biochemical functions [97]. The structural approaches currently being used often yield population-averaged results that obscure the conformational variability within an RNA. In addition, these in vitro approaches ignore the effects of factors such as molecular crowding and binding by RBPs on RNA folding, which are encountered in vivo. Hence, the experimental information should only be considered constraints for the computation of the structure [98].

3.3.6. Validation of transcription factor-RNA regulatory interactions

Transcription factors can control the expression of non-coding RNAs (ncRNAs) by binding to regulatory regions located at ncRNA loci [99]. On the other hand, lncRNAs can bind to and assemble chromatin-regulatory complexes, thereby influencing transcriptional regulation [100]. Complementary experimental methods are thus needed to determine transcription-factor binding at non-coding RNA loci and to delineate the genomic areas linked to chromatin-bound ncRNAs. To move from association to causation in feedback mechanisms, ChIP-seq (protein-DNA) must be integrated with RNA-centric proximity assays (ChIRP-seq, Capture Hi-C). This overcomes ChIP-seq's inability to distinguish active regulation from inactive complex membership and confirms direct RNA-protein-DNA interactions [101].

3.3.6.1. Chromatin immunoprecipitation sequencing (ChIP-seq)

ChIP-seq maps and reconstructs the genomic occupancy of transcription factors and other chromatin-associated proteins, as well as the distribution of histone modifications [102] that when applied to the regulatory regions of lncRNA or miRNA genes, can identify candidate transcriptional regulators of ncRNA expression [99]. Integration of occupancy profiles obtained with this method with ncRNA expression measurements can support the inference of transcription factor-ncRNA regulatory networks [103]. ChIP-seq interpretation for ncRNA regulation should be done with a few caveats in mind. Firstly, there is a problem with protein occupancy being an association by nature; presence at the promoter site does not mean there is any functional regulation going on. Secondly, DNA fragmentation may lower the resolution of binding sites. To switch from correlation to causation, functional perturbation experiments need to be carried out in addition to

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ChIP-seq analysis, like CRISPR-based depletion experiments [104].

3.3.6.2. Chromatin isolation by RNA purification (ChIRP)

ChIRP employs pools of biotinylated antisense DNA oligonucleotides to selectively enrich a target RNA along with its associated chromatin from crosslinked cellular material [105]. Associated DNA can subsequently be analyzed to pinpoint potential genomic occupancy sites, while suitable downstream workflows can also examine co-purified RNA and protein components [106]. ChIRP-seq delineates the genomic distribution of chromatin-associated lncRNAs, offering localization evidence pertinent to their suggested transcriptional roles [105].

3.3.6.3. Capture hybridization analysis of RNA targets (CHART)

The CHART technique employs affinity-labeled antisense oligonucleotides to enhance the enrichment of target RNA, along with chromatin and its related proteins [107]. Probe-accessible regions of the RNA are typically identified using RNase H sensitivity assays. Sequencing of enriched DNA-CHART-seq maps genomic regions associated with the target RNA and has been used to characterize roX2 and Xist localization. CHART's specificity is limited by RNase H digestion sensitivity, where improper digestion levels bias enrichment toward accessible RNA regions. It is also prone to off-target probe binding and genomic noise. Overcoming these drawbacks requires stringent washing, bioinformatic analysis, and validation through high-resolution techniques such as CLIP-seq or ChIRP [107].

3.3.6.4. RNA antisense purification (RAP)

RAP employs extended biotinylated antisense probes to selectively enrich an endogenous target RNA under rigorous hybridization conditions. In RAP-DNA, the sequencing of copurified DNA reveals genomic regions linked to the RNA. The initial setup employed antisense RNA probes, about 120 nucleotides in length, to identify Xist's distribution across the X chromosome [108]. Related RAP workflows can investigate RNA or protein partners, depending on the crosslinking conditions and downstream analytical approach [101, 106]. While RAP offers superior specificity and signal-to-noise ratios compared to affinity-based methods like CHART, it remains constrained by formaldehyde cross-linking. This can artificially expand footprints by stabilizing transient or indirect RNA-DNA-protein complexes. Furthermore, using long biotinylated probes requires careful optimization to prevent steric hindrance and enrichment bias toward exposed RNA regions. Consequently, RAP identifies proximity markers rather than direct interactions, functional perturbations or structural analyses are necessary to confirm regulatory roles [109].

3.3.7. Functional validation of RNA regulatory networks

Physical association alone cannot imply regulatory or causative function, and perturbation experiments, along with appropriate rescue and mechanistic analyses, are required to determine whether an RNA or a proposed RNA-mediated interaction is causally involved in gene expression or cellular phenotype [110]. For lncRNAs, these tests must differentiate the RNA molecule's roles from the impacts of its DNA origin, transcription process, or RNA modifications [108].

3.3.7.1. Loss-of-function and gain-of-function experiments

Loss-of-function approaches, including siRNA-mediated inhibition, antisense oligonucleotides, and CRISPR-based disruption, examine whether reducing ncRNA expression or disrupting its locus alters target gene expression or cellular behavior [110]. Genomic deletion may also interfere with regulatory DNA or transcription-related functions; therefore, its impacts should not be solely

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ascribed to the loss of the RNA molecule [108]. Conversely, ectopic overexpression or CRISPR-mediated activation can be used to assess whether increased ncRNA expression is sufficient to induce a predicted regulatory effect under in vitro conditions [110, 111]. Each approach to inducing loss of function has distinct limitations for establishing causality. RNA interference (RNAi) effects are transient and may involve off-target silencing, saturation of the RNAi machinery, or activation of an interferon response. Antisense oligonucleotides (ASOs), meanwhile, can act through mechanisms that are complex and difficult to distinguish, including RNase H-mediated cleavage and steric blocking. Although CRISPR produces permanent loss of function, it can generate heterogeneous indels and trigger clonal adaptation, in which observed phenotypes result from compensatory changes in the cell. Proving causality requires caution: rescue experiments must use mutation-resistant constructs to confirm on-target effects, while gain-of-function methods (overexpression or CRISPRa) risk creating artifacts through non-physiological levels or unintended genomic effects. Thus, sufficiency evidence must be physiologically titrated and treated as complementary to loss-of-function results.

3.3.7.2. CRISPR-based RNA perturbation

CRISPR interference and activation influence transcription at native loci, while Cas13 systems facilitate programmable RNA targeting and degradation [111, 112]. Catalytically inactive Cas13 can also recruit effector domains to target RNAs, enabling targeted RNA modification [113]. These complementary approaches facilitate functional interpretation [108]. The specificity and effectiveness of both techniques depend on distinct biochemical constraints that must be considered in functional experiments. CRISPRi represses transcription by targeting a promoter or a transcription start site near the promoter. Its effects depend on the transcription machinery and may be confounded by changes in the regulation of neighboring genes within the same topologically associating domain. In contrast, Cas13 directly cleaves RNA without affecting DNA. However, in some cell types, Cas13 may cause collateral cleavage, and complete transcript depletion may be difficult when the target RNA is tightly bound in RNP complexes or RNA granules. Recruiting different effectors with dCas13, a non-cleaving Cas13, enables targeted RNA editing or modification. This approach, however, depends heavily on RNA-scaffold accessibility, and the large Cas13 protein may hinder effector recruitment. To establish a high-confidence and functional role, CRISPRi-mediated transcriptional inhibition should be compared with depletion of Cas13-mediated RNA [114].

3.3.7.3. Rescue experiments and mechanistic validation

Rescue experiments examine whether or not a phenotype is reversed by restoring RNA expression or function that has been disrupted by RNA depletion or disruption [110]. Analyzing wild-type RNA alongside interaction-defective mutants can assist in identifying whether a specific sequence or interaction domain is essential for activity, particularly when biochemical interaction assays are conducted in conjunction with the functional analysis [100]. However, a function that depends on transcription or RNA production at the endogenous locus is not ruled out if ectopic RNA expression fails to rescue a condition [108, 110].

3.3.7.4. Validation of miRNA-mediated feed-forward loops

The validation of miRNA-mediated feed-forward loops can be achieved by assessing the expression levels of the transcription factor, miRNA, and common target genes in response to perturbation conditions. A coherent feed-forward loop is provided when the transcription factor controls both the miRNA and the target gene,

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while the miRNA inhibits the same target, resulting in coordinated and often strong regulation of pathway output. Steady-state expression profiling is insufficient for confirming Feed-Forward Loops (FFLs) due to the complex dynamics of gene regulatory networks. Purely descriptive methods fail to capture the temporal delay between miRNA activation and target repression, which is essential to distinguish FFLs from simple co-expression. Furthermore, steady-state data overlook stoichiometric requirements and the necessity of sufficient miRNA concentrations for effective regulation. To move beyond correlation, functional validation requires time-course assays (e.g., synchronized TF activation) and sequestration or sponge experiments to demonstrate the FFL's buffering capacity and its impact on transcriptional noise. [115].

3.3.8. Spatial and single-cell technologies for context-specific RNA analysis

Spatial transcriptomics and single-cell technologies facilitate the investigation of RNA regulatory interactions in their natural tissue and cellular contexts. These techniques can identify the specific cell types that generate a candidate ncRNA, ascertain whether its expression is confined to particular anatomical regions, and elucidate the evolution of regulatory networks throughout disease progression [116].

3.3.9. Integration of computational predictions with experimental evidence

Computational analyses can prioritize potential RNA regulatory interactions from extensive sequencing data; however, predicted interactions necessitate experimental validation instead of being assumed as confirmed regulatory relationships [117, 118]. Importantly, different experimental approaches provide distinct levels of evidence. RNA-seq identifies transcript abundance and expression changes, whereas CLIP-seq, RIP, and RNA pull-down assays provide evidence of RNA-protein or RNA-RNA associations, depending on the assay. However, neither expression correlation nor physical association alone establishes a direct functional or causal regulatory relationship. Demonstrating such relationships generally requires targeted perturbation experiments, appropriate controls, and, where feasible, rescue experiments to confirm the proposed molecular mechanism. The confidence in inferred RNA regulatory networks is enhanced by the integration of expression measurements, predictions based on sequences or binding preferences, evidence of physical interactions, and molecular responses that depend on perturbations. Additionally, functional and phenotypic assays offer further support for the biological relevance of specific regulatory connections. [110, 118].

3.3.10. Technical limitations, reproducibility, and best practices in experimental validation

Experimental methods for mapping RNA regulatory interactions have specific assay limitations, such as antibody-dependent specificity, cross-link selectivity, probe-dependent artifacts, and biases introduced during library preparation and amplification [101, 118, 119]. In addition, physical association and perturbation-induced expression changes do not necessarily establish direct functional regulation [118]. It can be concluded that reliable validation requires biological replicates, appropriate assay controls, reproducibility assessment, and complementary experimental approaches. For example, for eCLIP, size-paired input controls help to distinguish specific enrichment from background signal, while the use of independent perturbation strategies enhances the interpretation of the functional effects [110, 118].

3.4. Biotechnological strategies for targeting RNA regulatory networks

3.4.1. RNA-targeting technologies

RNA-targeting technologies offer a way of functionally altering regulatory networks that

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have been identified by computational analyses (Figure 2). Unlike approaches focused primarily on proteins or genomic DNA, these technologies can directly affect RNA stability, translation, splicing, and regulatory activity. Main strategies for manipulating RNA-mediated regulation include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and microRNA (miRNA) mimics or inhibitors [19, 24, 120, 121]. ASOs are short nucleic acid sequences chemically modified to hybridize with complementary RNA molecules. Depending on their chemical structure and the RNA region they bind, ASOs can degrade RNA via RNase H1, alter pre-mRNA splicing, or disrupt RNA-protein interactions. Because of these properties, ASOs can target both protein-coding transcripts and regulatory non-coding RNAs, including long non-coding RNAs (lncRNAs) [24, 120].

In contrast, siRNAs function via the RNA-induced silencing complex (RISC), which binds to complementary transcripts and causes their degradation. This mechanism allows specific suppression of certain genes and thus offers a valuable method for experimentally testing regulatory interactions identified through network analysis [19, 121].

MiRNA-based interventions provide greater regulatory control. Synthetic miRNA mimics can be used to restore the activity of miRNAs that have been reduced in disease conditions, while "anti-miRNA" molecules (anti-miRs) can inhibit the activity of miRNAs whose overexpression leads to abnormal gene regulation. Because a single miRNA may regulate multiple transcripts, these methods can simultaneously affect multiple parts of a regulatory network. However, this same feature makes it difficult to predict treatment outcomes and increases the likelihood of unwanted effects. Therefore, when choosing a strategy for targeting RNA, one must consider factors such as the biological role of the target RNA, its expression pattern, the desired direction of change (increase or decrease in activity), and the degree of specificity required for the therapeutic intervention [24, 122].

3.4.2. CRISPR-based RNA modulation

CRISPR systems that target RNA, especially those based on Cas13, have broadened the range of methods available for manipulating RNA molecules. Unlike CRISPR-Cas9, which mainly acts on DNA, Cas13 uses a guide RNA to identify complementary RNA sequences and can cut the target RNA. This ability to act in an RNA-directed manner provides a possible way of reducing disease-linked transcripts without making direct changes to the original genomic DNA [123]. Nevertheless, Cas13-mediated targeting is not equivalent to a fully transcript-specific intervention, because the observed phenotype may also depend on guide-dependent off-target activity, collateral RNA cleavage in certain Cas13 subtypes or cellular contexts, and incomplete depletion of structurally protected or protein-bound transcripts. Therefore, the absence of genomic DNA modification should not be interpreted as evidence that the approach is free from broader transcriptomic effects. Cas13-based methods can be used to perform functional assays on candidate mRNAs, lncRNAs, and other regulatory transcripts identified through transcript profiling or network analysis. If candidate lncRNAs are depleted by Cas13 and their predicted downstream targets are then examined, this could help clarify their potential regulatory functions. Likewise, disrupting disease-associated transcripts may provide further evidence for their role in proposed regulatory pathways. Therefore, these types of approaches can help bridge the gap between computational predictions and experimental functional validation [123-125].

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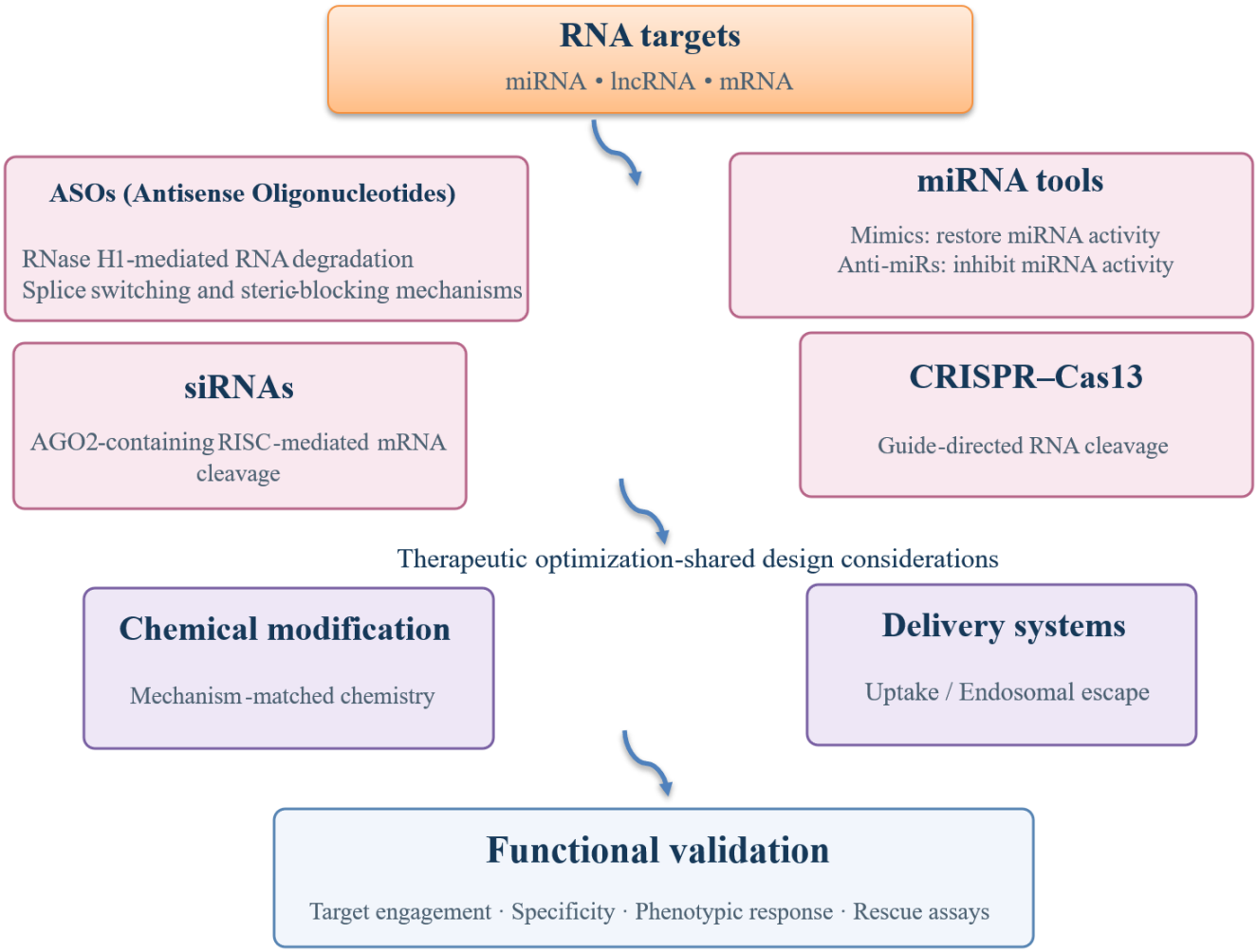


Figure 2. Biotechnological strategies for targeting RNA regulatory networks. From computationally prioritized RNA targets to experimental validation.

Deletion of a candidate transcript is only an indication that its expression is essential under the experimental conditions tested; it does not in itself indicate whether the RNA function is through nucleotide sequence, secondary structure, protein binding, subcellular localization, or transcription of an endogenous gene. This is particularly true for lncRNAs, where reduced transcription and gene silencing can lead to different phenotypes. Functional interpretation should therefore take into account isoform-specific measurements, subcellular localization studies, and orthogonal reduction approaches, where possible [2]. However, challenges remain in Cas13-based RNA editing, including the need for precise design of guide sequences, efficiency of transduction into the cell, target accessibility, potential for off-target effects, and consequences that depend on cellular context. Furthermore, RNA degradation does not necessarily establish the direction or molecular mechanism of a regulatory interaction; therefore, when interpreting results, it is important to use appropriate controls, employ independent guide sequences, perform rescue experiments, and simultaneously measure the target molecule and downstream transcripts [123]. Validating Cas13 specificity requires monitoring off-target effects, RNA integrity, and cellular stress. Stronger causal proof is provided when Cas13-mediated effects

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are corroborated by ASOs or CRISPRi, or reversed by guide-resistant RNA. Due to potential transcriptional adaptation, time-course and single-cell analyses are preferred over endpoint measurements to ensure that Cas13 provides meaningful biological insights within a broader validation context [126].

3.4.3. RNA delivery systems

The therapeutic efficacy of RNA-targeting technologies depends not only on sequence specificity, but also on the successful delivery of nucleic acids to target cells and their entry into appropriate intracellular compartments. Since unprotected RNA molecules can be rapidly degraded, have only limited ability to enter cells, or fail to get to the cytoplasm, where many RNA-targeting mechanisms function, delivery systems are necessary if the stability of RNA, its uptake by cells, and its presence inside cells are to be improved [19, 127].

Lipid nanoparticles (LNPs) are one of the most well-established methods for nucleic acid delivery, as they are able to carry RNA molecules, protect them from degradation, and facilitate cellular uptake as well as exit from endosomes. As alternative approaches, polymer nanoparticles and hybrid nanocarriers offer additional possibilities, as the carrier composition can be modified to influence RNA loading, release rate, stability, and tissue distribution. Extracellular vesicles have also attracted attention as potential biocarriers, although challenges remain in their reproducible production, cargo loading, purification, and industrial-scale production [124, 128]. The LNPs have seen significant success in hepatic delivery, their application in extrahepatic tissues remains a significant obstacle, primarily due to the sequestration of these particles by the mononuclear phagocyte system and the lack of innate tropism for non-liver organs. Likewise, although extracellular vesicles offer excellent biocompatibility and low immunogenicity, variation in their cellular origins and cargo-loading efficiencies leads to considerable population heterogeneity, making standardization and regulatory approval more challenging [129]. When designing a delivery strategy for use in a regulatory network, the choice should be based on the target tissue, the type of cell, the RNA payload, and the desired mechanism of action. Oligonucleotides that are conjugated to a ligand can enhance uptake in cells that have the corresponding receptors, and in cases where the target is located within a particular tissue, locally administered formulations may be of use. Yet, successful delivery to one organ does not mean that efficient delivery will occur in other tissues or cell groups. It is therefore necessary to take into account biodistribution, endosomal escape, immune responses, toxicity, and manufacturing consistency when moving RNA-targeting strategies into therapeutic applications [124, 128, 130, 131]. Given the complexity of regulatory RNA networks, delivery must also take into account the cellular localization of the target. In cases where the therapeutic target requires the regulation of a lncRNA that remains in the nucleus, the transporter must not only be able to escape endosomes but also transport the cargo into the nucleus, which is much more difficult than delivering it to the cytoplasm.

3.4.4. Chemical modification of RNA therapeutics

Chemical modification is a key method for enhancing the stability, target affinity, pharmacokinetic properties, and tolerability of therapeutic oligonucleotides; Modifications to the sugar backbone, the bonds between nucleotides, or nucleobases can increase resistance to nuclease-mediated degradation and affect how they interact with proteins and the intracellular RNA processing machinery. Typical chemical modifications are phosphorothioate (PS) backbone linkages, 2′-O-methyl modifications, 2′-O-

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methoxyethyl (2′-MOE) modifications, and locked nucleic acids (LNAs) [24, 120, 132, 133]. Although such changes help to avoid nucleolytic degradation, they cause significant changes in pharmacodynamics. In this regard, the use of phosphorothioates causes a marked increase in protein plasma binding that extends systemic exposure but leads to altered accumulation and potential liver toxicity and immunogenicity [134].

The effects of these modifications vary according to the oligonucleotide design. PS linkages are capable of enhancing nuclease resistance and affecting protein binding, while 2′-O-methyl and 2′-MOE modifications can improve stability and alter the way they interact with cellular components. LNA residues can increase the affinity for hybridization and are therefore useful in certain antisense and anti-miRNA designs. However, the best chemical structure will depend on the mechanism intended. For instance, an ASO intended to recruit RNase H1 must keep the structural features needed for enzyme-mediated cleavage, whereas a steric-blocking ASO is mainly designed to interfere with RNA interactions without necessarily causing degradation [120, 132]. For RNase H1-binding gapmers, the central DNA core must remain accessible to the enzyme, while the high-affinity modified flanking regions must not unduly impede formation of the enzyme-substrate complex. Conversely, for steric-blocking ASOs, the challenge lies in creating a highly stable, high-affinity interaction capable of displacing RNA-binding proteins or masking splice sites without triggering an innate immune response or interfering with the cell's native splicing machinery. Hyper-affinity in these molecules can lead to persistent binding to unintended targets, creating a molecular glue effect that may induce toxicity entirely unrelated to the intended regulatory pathway [135]. Therefore, chemical optimization should be an integral part of treatment design, not a one-size-fits-all corrective strategy. Since excessive affinity, an inappropriate sequence composition, or adverse protein interactions might affect specificity, distribution, or safety, the oligonucleotides under consideration should be assessed for target engagement, off-target activity, cellular toxicity, and functional efficacy in exp-erimentally relevant biological systems before progressing with their development [24, 120]. Achieving this requires a systems-pharmaceutical approach, in which the chemical structure is optimized not only for target interaction but also for minimal disruption of cellular interactions. Given that chemical modifications can alter the intracellular distribution and protein binding profile of oligonucleotides, simple in vitro binding assays are not sufficient for this purpose, and it is crucial to evaluate these compounds using high-throughput profiling for off-target effects across the transcriptome and to validate their pharmacokinetic behavior in complex physiological models relevant to disease [136].

3.4.5. Challenges and future perspectives

Although RNA-targeting technologies have advanced significantly, obstacles still limit their wider use as therapeutics. These obstacles include: inefficient delivery to specific tissues and cell groups, inadequate degradation or release of therapeutic molecules within cells, immune system activation, off-target effects, and differences in target expression levels among different individuals and at different stages of the disease. Moreover, the regulatory function of an RNA molecule may vary depending on its cellular environment, the molecules with which it interacts, and the expression level of other network components. As a result, a change in an RNA can have different consequences in different biological systems [24, 127]. This context-dependency highlights a main limitation in the current strategies: a single RNA-targeting intervention

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may lead to highly divergent effects across different cell types, potentially resulting in unexpected side effects or therapeutic failure in specific subpopulations. A major challenge is to translate computationally predicted regulatory interactions into experimentally validated targets that can be used in therapy. Using network-based analyses, miRNAs, transcription factors, and target genes can be ranked based on their connectivity and the predicted regulatory functions they play. However, no gain from network centrality alone proves causal significance, druggability, or therapeutic safety. Although targeting hub nodes may have a substantial impact, it also carries a high risk of toxicity, since disrupting highly connected components could cause the catastrophic failure of essential cellular networks. Therefore, the transition from network topography to therapeutic target must move beyond identifying highly connected nodes toward identifying controllable nodes, those whose modulation can steer the network toward a healthy state without inducing global dysregulation [137].

It is only by combining expression data with experimentally supported RNA-target interactions, disruption experiments, and functional rescue studies that stronger evidence for selecting potential interventions can be obtained. Future research could make use of combining network-based target selection with single-cell transcriptomics, spatial profiling, CRISPR-based disruption, and high-throughput functional screening. This type of integration may help to discern cell-specific regulatory mechanisms, identify disease-associated cell populations, and determine whether modulating a specific RNA leads to expected downstream effects. Furthermore, the integration of multi-omics data with machine learning-driven predictive models could accelerate the identification of these targets by uncovering non-linear relationships in RNA-protein-DNA interactions that traditional statistical methods often overlook [138]. Ultimately, effective RNA-based therapeutics require a coordinated approach that includes computational prioritization, mechanistic validation, chemical optimization, delivery system development, and preclinical evaluations [24, 123, 127]. In the case of complex diseases, the goal is not necessarily to suppress the largest number of network components. A more promising strategy might be to find regulatory nodes such that their modulation results in a beneficial biological effect without compromising essential physiological functions. This approach offers a way of turning RNA regulatory network analyses into more selective and experimentally supported therapeutic strategies.

4. CONCLUSION

RNA regulatory networks are complex systems in which non-coding RNAs, protein-coding transcripts, transcription factors, and RNA-binding proteins interact to regulate gene expression across multiple molecular levels. This review describes how computational prediction, multi-omics integration, and AI can be used to identify candidate regulatory interactions and prioritize those that may be of relevance. Of course, computational predictions and evidence of molecular association are not sufficient by themselves to establish direct regulation or causation. Therefore, robust characterization requires complementary experimental approaches, including RNA profiling, interaction mapping assays, targeted perturbation, and rescue experiments. Emerging RNA targeting technologies, such as antisense oligonucleotides, siRNAs, miRNA-based approaches, and CRISPR-Cas13 systems, offer opportunities to modulate disease-associated regulatory networks. However, their therapeutic efficacy depends on achieving a

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high level of target specificity, delivery, intracellular activity, and safety. Overall, the integration of bioinformatics with rigorous experimental validation and context-aware therapeutic design provides a framework for translating RNA regulatory network discoveries into biologically supported and potentially actionable applications.

5. DECLARATIONS

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5.2. Conflicts of Interest

The authors declare no conflicts of interest.

5.3. Author Contributions

All authors participated in project administration and writing of the first draft of the manuscript, providing critical revision and editing. All authors approved the final version of the manuscript.

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5.5. Data Availability Statement

Data will be made available on request.

5.6. Ethics Declaration

Not applicable. This review did not involve human participants or animal experiments.

5.7. Use of Artificial Intelligence

During the preparation of this manuscript, ChatGPT (OpenAI) was used as an auxiliary tool for language refinement and improving manuscript readability. Consensus (https://consensus.app/) was used as an AI-assisted literature search and research discovery tool to support the identification and review of relevant scientific literature.

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