Trends in Genetics

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REVIEWS

Review

miRNA Targeting: Growing beyond the Seed

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miRNAs are small RNAs that guide Argonaute proteins to specific target mRNAs to repress their translation and stability. Canonically, miRNA targeting is reliant on base pairing of the seed region, nucleotides 2–7, of the miRNA to sites in mRNA 3′ untranslated regions. Recently, the 3′ half of the miRNA has gained attention for newly appreciated roles in regulating target specificity and regulation. In addition, the extent of pairing to the miRNA 3′ end can influence the stability of the miRNA itself. These findings highlight the importance of sequences beyond the seed in controlling the function and existence of miRNAs.

Highlights

While canonical miRNA targeting involves pairing of the miRNA seed, nucleotides 2–7 of the miRNA, to target 3′ UTR sequences, recent studies have revealed roles for miRNA sequences beyond this region in specifying target recognition and regulation.

Auxiliary base pairing to sequences in the 3′ half of the miRNA can overcome seed imperfections and confer specificity for individual members of a miRNA family that share identical seed sequences.

Base pairing of 3′-end miRNA sequences enables targeting of protein-coding sequences that lack canonical seed-pairing interactions.

Extensive pairing interactions between a miRNA and its target can lead to target-directed miRNA degradation.

Target Recognition and Regulation by miRNAs

Since their discovery in the early 1990s [1,2], thousands of miRNAs have been identified across the plant and animal kingdoms [3]. There is now evidence that miRNAs impact every major biological pathway by regulating the expression of substantial fractions of protein-coding genes [4,5]. Given this omnipresent role in gene regulation, it is not surprising that misregulation of individual miRNAs can have dire consequences, contributing to a variety of diseases and afflictions in humans [6].

Within the miRNA-induced silencing complex (**miRISC**) (see Glossary), the ~22-nt miRNA recruits Argonaute (AGO) to specific target sites via base-pairing interactions [4,7]. Perfect base pairing of the miRNA with its target site, which is common in plants but rare in animals, results in endonucleolytic cleavage by AGO of the target RNA. Animal miRNAs typically form a partial duplex with their target site, which prevents cleavage and instead relies on AGO cofactors to regulate target expression through translational repression and mRNA destabilization [4,7]. Pairing of nucleotides 2–7 of the miRNA, called the **seed**, to its target site has generally been considered the minimal element needed to engage a target mRNA [4]. Indeed, structural studies have shown that only sequences within the seed of the AGO-bound miRNA are available for initial pairing to a target site [8–10]. In addition, single molecule studies have demonstrated the importance of the seed in stable target site engagement [11–13]. Interestingly, once miRISC binds target RNA, AGO can undergo a conformational change that allows for extended seed pairing and exposes part of the miRNA 3′ region (nucleotides 13–16) for additional interactions with the target [14]. A recent systematic investigation of pairing interactions between a miRNA and target corroborates a sequential recognition model where the miRNA seed binds first and then nucleotides in the 3′ half are able to bind the target site [15]. The relevance of pairing to miRNA 3′-end sequences has been demonstrated in several new studies showing that it can impact the specificity of targeting, the regulatory mechanism, and the stability of the miRNA itself.

Same Seed, Different Targets

Given its importance in many established miRNA–target interactions, seed-pairing potential is the foundation of popular target prediction programs [4]. As such, members of a **miRNA family** that share seed sequences are typically assigned to the same target sites. However, there is mounting *in vivo* evidence that pairing interactions beyond the seed can lead to non-overlapping target

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profiles for individual miRNAs in a family. On a genome-wide scale, crosslinking and immunoprecipitation with sequencing (**CLIP-seq**) methods have been used to identify endogenous AGO-bound target sites. In some of these studies, the target sequence became ligated to the presumptive targeting miRNA, producing miRNA-target **chimeric reads** [16–19]. These types of sequencing reads allow for the unambiguous identification of which specific miRNA recruited AGO to a particular target site. Many chimeras contained target sequences that could pair to the seed of the attached miRNA, reaffirming the prevalence of this pairing motif in endogenous miRNA–target interactions [16–19]. Contrary to expectation, miRNAs within a family did not always produce chimeras with the same target sites, suggesting that sequences beyond the seed can contribute to target recognition *in vivo* [18,19].

The examination of miRNA–target chimeras generated from CLIP-based studies in human cell culture, mouse brain, and whole larval-stage *Caenorhabditis elegans* revealed that individual miRNA family members, often called **sisters**, can exhibit biased target interactions [18,19]. With identical seed sequences, miRNA sisters apparently rely on their potential for unique 3′-end pairing interactions to engage some targets. For sister-specific target sites in both the mammalian and worm studies, the chimera-forming miRNA had a more favorable predicted binding affinity than that of its sisters [18,19]. Consistent with non-identical target preferences, sister miRNAs were also shown to differ in their regulatory capacity for targets with 3′-end pairing interactions that favored one of the family members. In mammalian cells, reporters with sister-specific target sites were generally repressed more potently by the chimera-forming miRNA versus other family members upon transfection into the culture system [18].

Taking advantage of a well-established let-7 miRNA target in *C. elegans,* Broughton *et al*. further demonstrated the importance of 3′-end pairing interactions for specific and functional targeting *in vivo* [19]. The *lin-41* 3′ untranslated region (UTR) contains two let-7 target sites that lack perfect seed pairing (one site involves a G-U pair and the other site forces a target nucleotide bulge) but support perfect pairing to nucleotides 11–19 of the let-7 miRNA (Figure 1A) [20,21]. Loss of *let-7* results in misregulation of *lin-41* and lethality, despite the expression of sister miRNAs that apparently cannot compensate [19–21]. The wild-type *lin-41* 3′ UTR only formed chimeras with let-7 miRNA, but this specificity was found to be transferrable when 3′-end pairing was designed to favor a sister [19]. **CRISPR/Cas9**-based genome editing was used to swap each let-7 target site for a site that had formed chimeras exclusively with its sister miRNA, miR-48. Importantly, these new *lin-41* 3′ UTR sites supported canonical seed pairing with any of the let-7 family members but were predicted to bind more extensively to the 3′ end of miR-48 (Figure 1B). Worms with the edited *lin-41* 3′ UTR were found now to depend on miR-48, but not let-7, for regulation of this gene and, ultimately, viability (Figure 1B) [19]. This study confirmed that pairing to sequences beyond the seed can confer specific and functional miRNA–target interactions *in vivo.*

While imperfect pairing to the miRNA seed can be compensated by extended pairing interactions with the 3′ portion of the miRNA, even seemingly perfect seed matches can depend on additional pairing [4]. One explanation for this phenomenon is that sequences immediately adjacent to the seed can influence targeting. In fact, there is a hierarchy of seed-pairing architecture wherein targets that pair to miRNA nucleotides 2–7 alone are generally less repressed than those that include pairing to the eighth position [4]. New work from Brancati and Grosshans shows that the ability to pair with nucleotide 8 can also influence the specificity of miRNA–target interactions [22]. Using the same *C. elegans* model described above, these authors demonstrated that perfect pairing of the *lin-41* target sites to nucleotides 2–8 of let-7 permits regulation by other family members, regardless of differences in potential 3′-end pairing

Glossary

Chimeric reads: contiguous sequences from CLIP-seq-based assays that contain two independently derived RNA sequences that became ligated during library preparation. The chimeric reads in AGO CLIP-seq data sets represent a miRNA associated with a specific target site. The name refers to different entities brought together into one being, as in the Chimera in Greek mythology composed of a lion's head, goat's body, and a serpent's tail.

CLIP-seq: crosslinking and immunoprecipitation with sequencing is a technique used to isolate and sequence RNA bound to a specific protein. This method has been used to identify miRNAs and target sites bound by AGO.

CRISPR/Cas9: a genome editing method that uses guide RNAs to target CRISPR (clustered regularly interspaced short palindromic repeats)-associated nuclease (Cas9) to specific DNA sequences for endonucleolytic cleavage. Repair of the cut DNA can be engineered to incorporate any new sequence of interest. This method is based on a natural prokaryotic defense system against bacteriophage that involves the integration of foreign DNA sequences between CRISPR segments in a bacterial genome.

GW182/TNRC6: alternative names for a miRISC factor that bridges AGO to proteins that promote deadenylation and translational repression of bound targets. GW182 refers to the molecular weight and glycine/tryptophan repeats that characterize this protein. TNRC6 stands for trinucleotide repeat containing gene 6.

miRISC: a complex consisting of a mature miRNA, AGO, and potentially other proteins that function in target regulation.

miRNA family: a group of miRNAs that share a seed but differ to varying degrees in the rest of their sequence.

miRNA recognition element (MRE): miRNA binding site, the region in a target RNA that can base pair to a miRNA.

Seed: nucleotides 2–7 counting from the 5′ end of a miRNA. Canonical

interactions (Figure 1C). However, seed pairing at nucleotides 2–7 with a G:U wobble pair at position 8 was sufficient to reinstate dependence on pairing to miRNA 3′-end sequences (Figure 1B). In some cases, bias for targeting by a specific sister was sensitive to the expression levels of other family members [22]. This work highlights the importance of considering overall miRNA–target pairing architecture as well as miRNA abundance in understanding target recognition and regulation *in vivo*.

Target Sites within Coding Regions Supersede Canonical Recognition and Regulation

Many of the miRISC binding sites identified through CLIP-based studies include the expected features: seed-pairing capacity, 3′ UTR residence, and an association with target mRNA destabilization (Figure 2) [16–19,23–26]. However, in all of these reports sizeable fractions of AGO-bound sites were also detected in protein-coding sequences (CDSs), often lacking in complementary seed motifs or clear effects on target mRNA regulation [16–19,23–26]. While specific examples of functional CDS-located miRNA target sites have emerged [27–29], in general this position in the mRNA has been regarded as suboptimal for eliciting a regulatory outcome because stable miRISC association would be thwarted by translating ribosomes [30]. Thus, the relevance of the thousands of CDS miRNA target sites, including some that are highly reproducible across biological replicates, has been an outstanding question.

In recent work, Zhang *et al*. provide compelling evidence that some CDS miRNA target sites actually comprise a new category of recognition elements [31]. Multiple independent studies have identified a miR-20a target site in the second exon of *DAPK* (a p53-activating kinase) [16,23,26,32]. Curiously, this site lacks seed pairing and, except for a G:U wobble at position 6 and a bulged C at position 12, is perfectly complementary to miR-20a nucleotides 5–23 (Figure 2). Within the CDS context, this pairing architecture sufficed for target regulation, but it lost functionality when inserted into in the 3′ UTR [31]. Additional examples of CDS sites with weak seed and extensive 3′-end pairing interactions for a different miRNA were also shown to mediate target regulation when placed in coding, but not untranslated, regions, leading the authors to define these as a novel class of **miRNA recognition elements (MREs)** [31].

In addition to the unusual pairing architecture and location of MREs in this class, the regulatory mechanism is also atypical. These MREs apparently repress translation without triggering mRNA destabilization (Figure 2) [31]. In contrast to miRNA seed-dependent interactions in 3′ UTRs, seedless sites in CDSs interfere with translation through a mechanism that does not rely on the AGO cofactor **GW182/TNRC6** (Figure 2) [31]. The GW182/TNRC6 protein is instrumental in recruiting deadenylation factors to initiate mRNA decay of canonical targets [4,7]. Its absence in complexes that regulate CDS targets may explain their lack of influence on mRNA levels. The stark contrasts between canonical 3′ UTR target sites and the newly described CDS sites that depend more on 3′-end pairing interactions highlight wide gaps in our understanding of functional miRNA targeting rules.

Seeding miRNA Decay with 3′-End Pairing

While miRNA pairing potential can greatly influence the specificity and mechanism of target regulation, it can also impact the fate of the miRNA. Following initial studies in *Drosophila* and mammalian cells [33,34], numerous examples of **target RNA-directed miRNA degradation (TDMD)** have emerged [35]. In this pathway, seed along with extensive pairing to the 3′ half of the miRNA to a target site can trigger decay of the miRNA itself. While the factors and mechanisms that sense this pairing structure and elicit

targeting involves perfect pairing of the miRNA seed to target sequences.
Sisters: members of the same miRNA family.
Tailing: the addition of non-templated nucleotides to the 3′ end of an RNA, usually by addition of uridines and/or adenosines.
Target RNA-directed miRNA degradation (TDMD): degradation of a miRNA caused by binding to a highly complementary target RNA sequence.

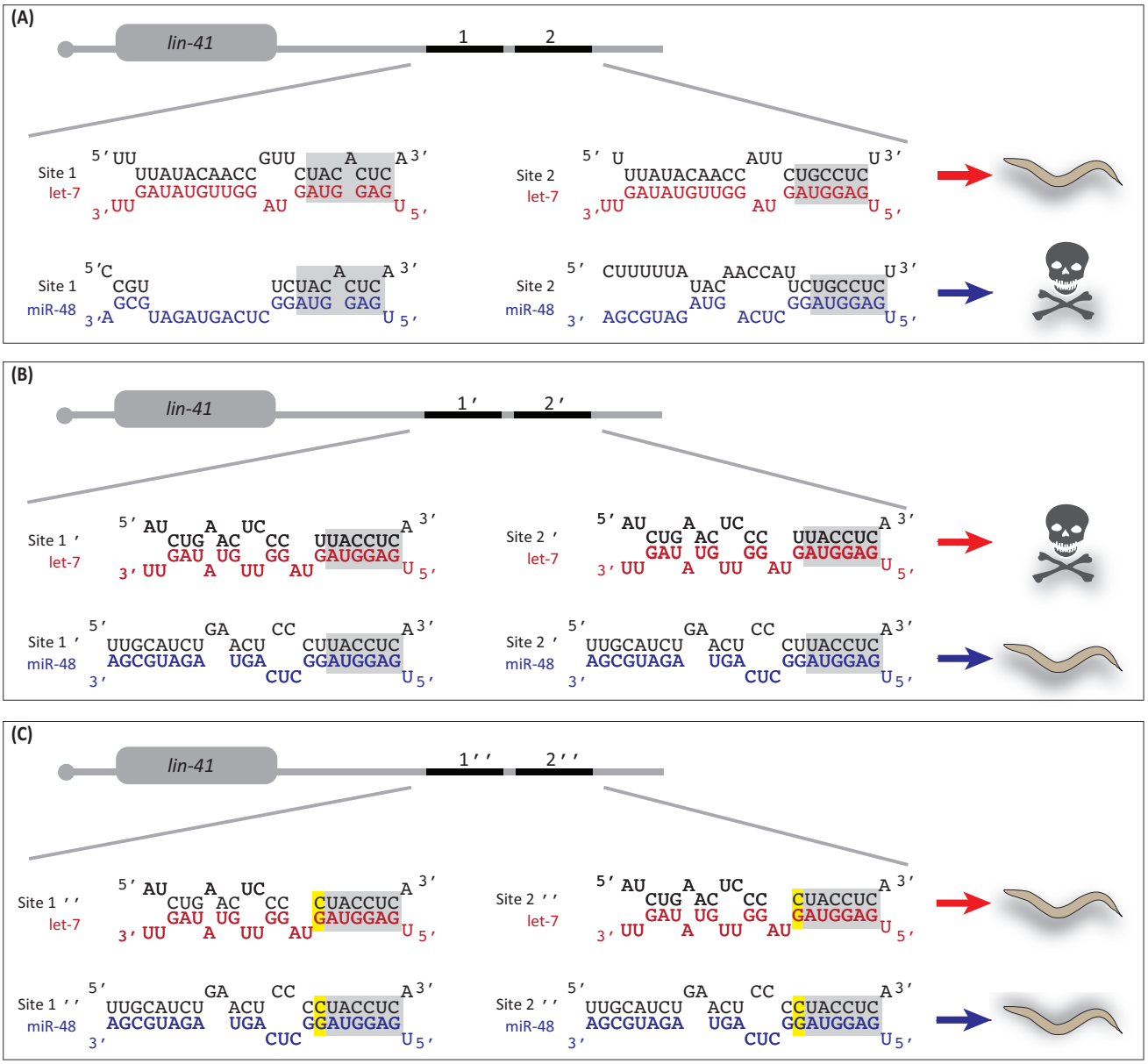

Figure 1. Auxiliary Pairing of miRNA 3′-End Sequences Can Overcome Seed Imperfections and Confer Target Specificity to miRNA Sisters. (A) In *Caenorhabditis elegans*, the *lin-41* 3′ untranslated region (UTR) contains two let-7 miRNA target sites that each feature extensive complementarity to the 3′ half of let-7 and imperfect seed-pairing potential: Site 1 forces a target nucleotide bulge and site 2 includes an unfavorable G:U base pair (pairing to the miRNA seed, nucleotides 2–7, is shaded gray). While let-7 family members, such as miR-48, can support the same seed-pairing architecture, only let-7 has sufficient 3′-end pairing capacity to regulate *lin-41*, allowing for normal worm development; loss of *lin-41* regulation by let-7 results in lethality (depicted by skull and crossbones) because the let-7 sisters cannot compensate. (B) Exchange of the let-7 sites for sequences predicted to correct the seed imperfections but pair more favorably to the 3′ end of miR-48 transfers regulation of *lin-41* from let-7 to miR-48. Sites 1′ and 2′ are duplications of a sequence in the *dot-1.1* 3′ UTR that only formed chimeras with miR-48 [19]. (C) The inclusion of pairing to nucleotide 8 (shaded yellow) in this context provides a seed architecture that allows regulation by let-7 or miR-48, regardless of 3′-pairing capacity [22]. Sites 1′′ and 2′′ are duplications of the sequence in (B) except for the substitution of U for C to enable canonical pairing to the G at the eighth position in let-7 and miR-48.

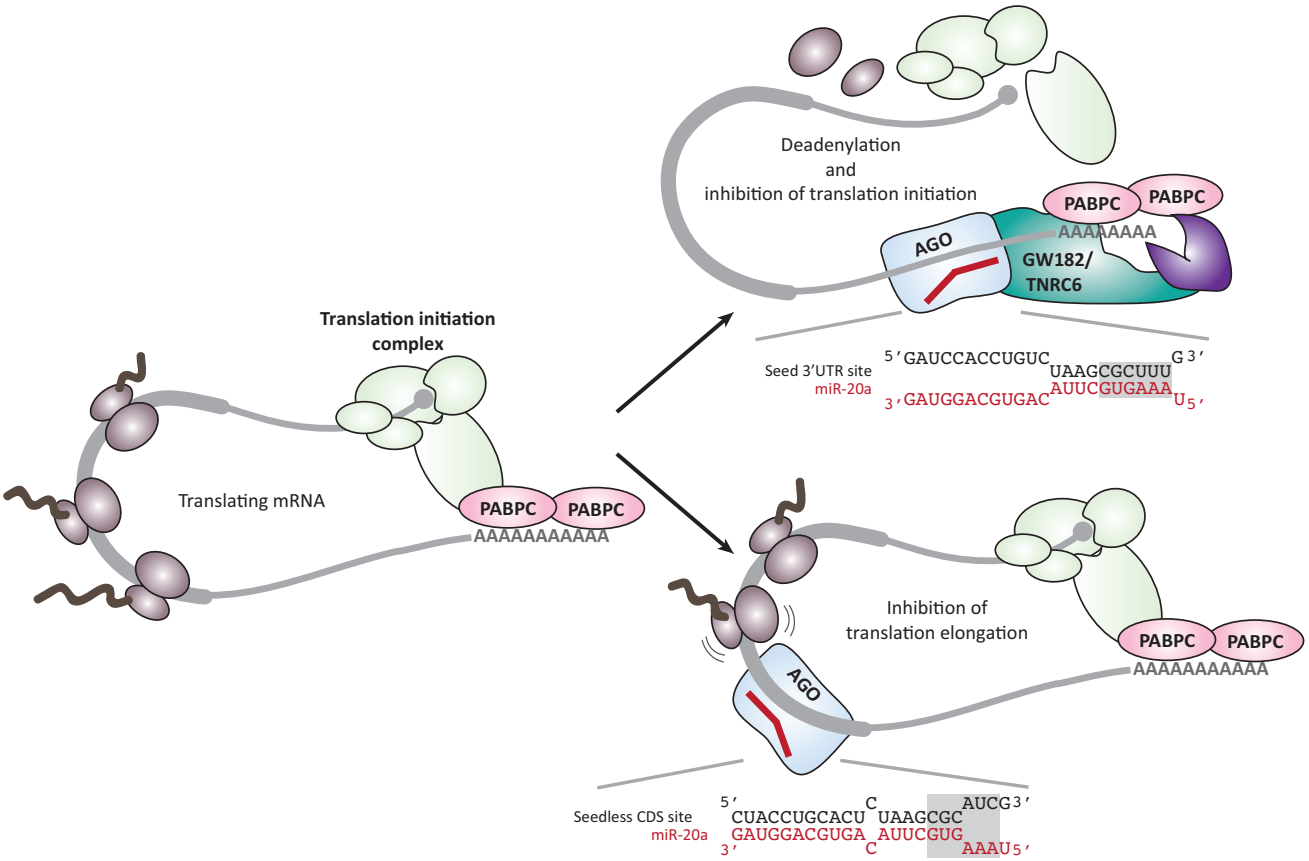

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Figure 2. The Structure and Position of miRNA–Target Interactions Impose Different Regulatory Outcomes. Canonical miRNA target sites reside in 3′ untranslated regions (UTRs), require seed pairing, and rely on the Argonaute (AGO) cofactor GW182/TNRC6 to recruit deadenylases and other factors that act to destabilize and repress translation initiation of the target mRNA (top) [4,7]. The example is a 3′ UTR site engineered to limit pairing to the miR-20a seed region [31]. A new class of target sites are located in coding sequences (CDSs), lack seed complementarity, and instead offer extensive pairing to miRNA 3′ ends; these types of sites seem to block translation elongation independently of GW182/TNRC6, resulting in reduced protein, but not mRNA, levels of the target (bottom) [31]. The example is the CDS site in exon 2 of *DAPK3* mRNA paired to miR-20a [31]. Abbreviation: PABPC, cytoplasmic poly(A)-binding protein.

destruction of the miRNA are yet to be fully revealed, TDMD often is associated with non-templated nucleotide additions, called **tailing**, and trimming at the miRNA 3′ end [35]. Since efficient TDMD seems to require an unusually high degree of pairing to nucleotides in the 3′ half of the miRNA [33,36], how often this pathway functions *in vivo* has been an open question. Nonetheless, intriguing examples of viruses expressing transcripts that trigger TDMD of host miRNAs established that this is a biologically relevant mechanism for regulating gene expression [34,37–39].

In just this past year, several examples of host-mediated TDMD of endogenous miRNAs have come to light [40–42]. In one study, a conserved block of sequence containing a highly complementary site to miR-29b was shown to regulate spatial expression of this miRNA (Figure 3) [40]. This element is present within a long non-coding RNA (lncRNA) called libra or the 3′ UTR of the *Nrep* mRNA throughout vertebrate evolution. In mice and zebrafish, high

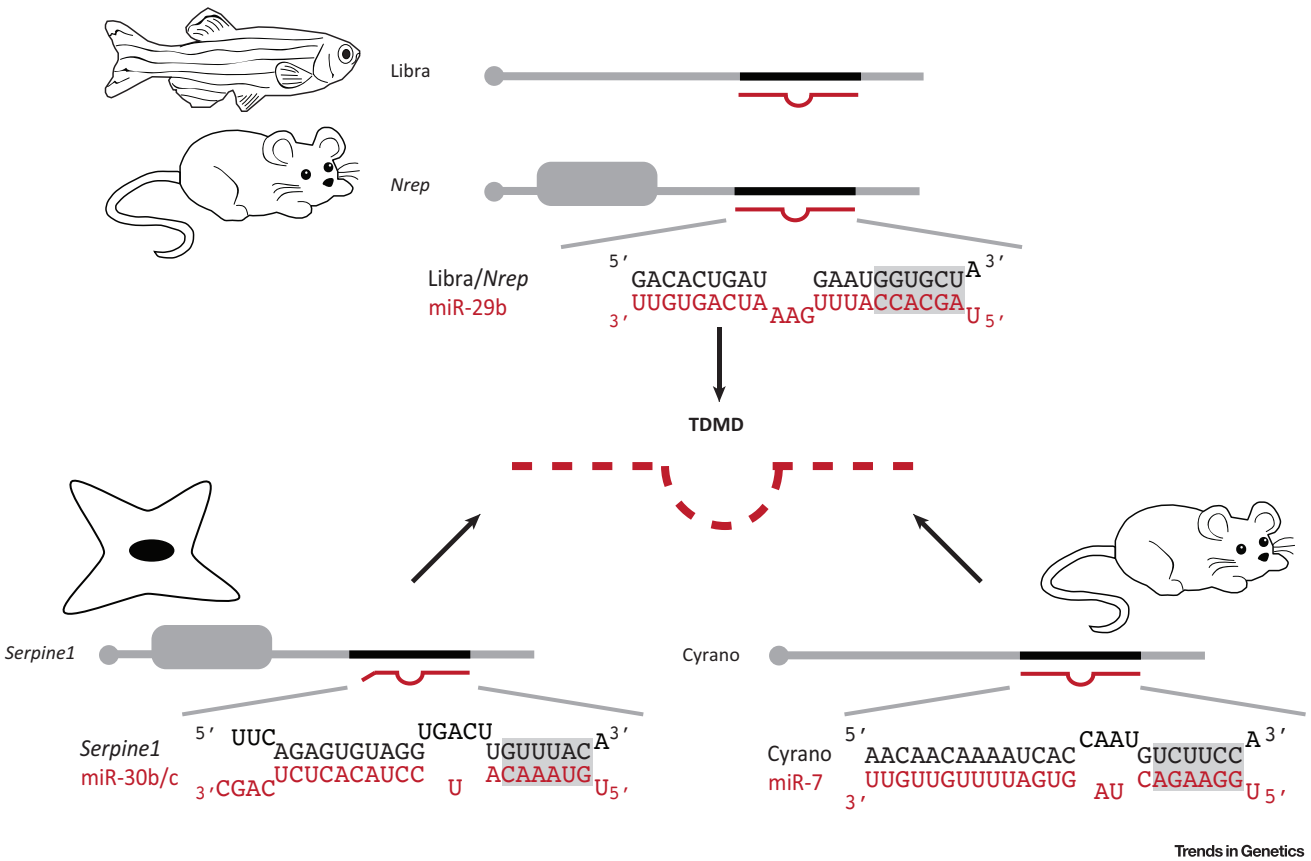

Figure 3. Extensive Pairing between a miRNA and Target Can Induce Target-Directed miRNA Degradation. Target-directed miRNA degradation (TDMD) of miR-29b can be triggered by pairing to a conserved region in the zebrafish long noncoding RNA (lncRNA), libra, or the mouse *Nrep* 3′ untranslated region (UTR) (top) [40]. A site in the 3′ UTR of *Serpine1* induces TDMD of miR-30b/c in mouse fibroblasts (bottom left) [42]. In mice, pairing of miR-7 to a site in the lncRNA Cyrano results in rapid decay of the miRNA through TDMD [41].

expression of these transcripts in the cerebellum leads to TDMD of miR-29b in this brain region [40]. Importantly, loss of this regulatory mechanism resulted in striking behavioral defects, including impaired motor functions in mice and aberrant exploratory and anxiety-like behaviors in zebrafish [40].

One advantage of using TDMD to regulate miRNA expression is that it can be selective for miRNA family members with differing degrees of 3′-end pairing interactions. Indeed, the extended complementarity of miR-29b designates this miRNA, but not its sisters, for TDMD through the pathway described above [40]. Likewise, the ability of a site in the *Serpine1* 3′ UTR to form 10 contiguous pairs with miR-30b/c triggered TDMD of those sisters, but not the less complementary miR-30a/d/e family members in mouse fibroblasts (Figure 3) [42]. When this site was removed from the *Serpine1* 3′ UTR by CRISPR/Cas9, levels of miR-30b/c increased, which impacted the degree and specificity of targeting by these miRNAs. Loss of *Serpine1*-mediated TDMD led to cell cycle and stress response defects, suggesting that this pathway plays a critical role in the regulation of miR-30b/c activity [42].

While Ghini *et al*. suggested that *Serpine1* may be just one of more than a thousand endogenous TDMD trigger mRNAs, noncoding RNAs provide another source of potential targets for

controlling miRNA stability through this pathway [42]. The Cyrano long noncoding RNA (lncRNA), which includes a highly complementary miR-7 site, is broadly conserved across vertebrates (Figure 3) [43]. While knockdown of Cyrano in zebrafish resulted in neurodevelopmental defects [43], mouse knockouts of Cyrano appear normal [41]. However, loss of Cyrano in mouse brain tissue led to increased miR-7 levels, which was associated with a general derepression of its targets [41]. While the importance of miR-7 TDMD by Cyrano in mammals awaits further studies, the conservation of this noncoding RNA and its potency in triggering miR-7 decay make it an intriguing model.

Concluding Remarks and Future Perspectives

While the seed is long recognized and well supported as a critical element in miRNA targeting [4,7], there is a growing appreciation that sequences in the 3′ half of the miRNA have roles to play as well. Members of a miRNA family can recognize unique targets depending on seed region architecture along with the potential for 3′-end pairing interactions. In parallel, specific sisters can be subjected to TDMD via differences in their 3′-end regions. Further elucidation of the pairing rules that govern selective targeting and TDMD will be needed to realize how widespread these events are (see Outstanding Questions). The demonstration of a new class of miRNA targets that depend on 3′-end interactions, but not seed pairing, suggests there is still much to be learned about how the miRNA complex engages targets *in vivo.* Furthermore, the existence of targets that lack seed complementarity, are located in CDSs, and exclusively undergo translational repression could mean that the extent of gene regulation by the miRNA pathway may be farther reaching than previously considered. With limited sequence content of only ~22 nt, it is now becoming clear that each nucleotide in a miRNA contributes to an overall pairing architecture that can influence recognition and regulation of the target, as well as stability of the miRNA.

Outstanding Questions

How common is targeting by specific miRNA family members *in vivo*? Under what conditions can family members compensate upon the loss of the target-specific miRNA?

How does miRISC recognize and stably associate with targets that lack seed complementarity? How do CDS target sites that lack seed pairing recruit AGO devoid of its GW182/TNRC6 cofactor?

What level of 3′-end complementarity is required to trigger TDMD *in vivo*? What is the extent and molecular mechanism of TDMD? How do differences in cell types and conditions affect this pathway?

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