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The biological functions of miRNAs: lessons from *in vivo* studies

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Abstract

Despite their clear importance as a class of regulatory molecules, pinpointing the relevance of individual miRNAs has been challenging. Studies querying miRNA functions by overexpressing or silencing specific miRNAs have yielded data that are often at odds with those collected from loss-of-functions models. In addition, knockout studies suggest that many conserved miRNAs are dispensable for animal development or viability. In this review we discuss these observations in the context of our current knowledge of miRNA biology and review the evidence implicating miRNA-mediated gene regulation in the mechanisms that ensure biological robustness.

Keywords

microRNAs; animal models; paralogs; network motifs; buffering

An elusive role for microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that regulate protein output post-transcriptionally [1]. Overwhelming evidence accumulated since their discovery [2,3] leaves little doubt regarding their importance. They comprise 1-2% of all genes in worms, flies, and mammals [1], and because each miRNA is predicted to regulate hundreds of targets, the majority of protein coding genes is thought to be under their control [4]. In practice, this means that virtually every biological process is subject to miRNA-dependent regulation. As additional evidence of their functional relevance, miRNAs and their targets often display striking evolutionary conservation [5-7]. Lastly, animals carrying mutations that impair miRNA processing [8-12] are not viable, indicating that complete loss of miRNA activity is incompatible with life.

Despite their clear importance as a class of regulatory molecules, determining the biological relevance of individual miRNAs has proven challenging. For the most part, the

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physiological functions of specific miRNAs have been inferred from overexpression studies in animals and cultured cells or from studies that used antisense molecules as a means of disrupting their pairing to targets. These experiments have attributed critical roles to miRNAs in processes such as cell proliferation, differentiation, and survival, and have implicated them as crucial players during normal development, homeostasis, and disease [13-17]. Surprisingly, the expectations raised by these early studies have been met by a growing number of knockout animals with very modest or no apparent phenotypes. Furthermore, so far only two miRNA genes (*miR-17∼92* and *miR-96*) have been shown to cause developmental defects in humans when mutated [18,19]. The absence of phenotypic consequences upon ablation of individual miRNAs seems to be the rule rather than the exception. In *Caenorhabditis elegans*, for example, systematic deletion of miRNAs indicates that less than 10% of them are individually required for normal animal development or viability [20], and this trend seems to be true in mice as well [21] (Table 1).

In this review we discuss these observations in the context of our current understanding of miRNA-mediated gene regulation and examine the evidence implicating miRNA activity in the processes that ensure robust animal development and homeostasis.

Endogenous miRNAs exert mild repression on many targets

miRNA processing has been extensively reviewed [22] and will be only briefly discussed here. MiRNAs are transcribed as long primary transcripts (pri-miRNAs) and cleaved in the nucleus by the Drosha/DGCR8 microprocessor complex. The resulting ∼70-nucleotide-long hairpin-shaped molecule—the pre-miRNA—is exported into the cytoplasm, where it is further processed by Dicer, bound by an Argonaute protein, and incorporated into an RNA-induced silencing complex (RISC). Metazoan miRNAs typically direct the RISC to target mRNAs through imperfect base pairing to their 3′ untranslated regions (3′UTR), leading to post-transcriptional repression mainly through mRNA destabilization, though a minor component of translation inhibition has also been detected [23] (see Box 1).

Target recognition is primarily determined by the seed-sequence, a stretch of 6 nucleotides spanning nucleotide 2-7 on the 5′ end of the miRNA [24,25]. Accordingly, targets can be confidently predicted by searching for conserved matches to this sequence in the 3′UTR of messages [26]. Prediction accuracy increases further when this search is restricted to 7-nucleotide-long motifs encompassing the seed [26] and when the sequence context within the 3′UTR is taken into consideration [27]. Non-canonical targeting through sites with mismatches to the seed has also been reported [28-32], but seems to be generally associated with lower levels of repression and its biological relevance remains unclear [4,30]. Because targeting requires the presence of such short conserved sequences, individual miRNAs have the potential to regulate hundreds of targets [4]. These computational predictions have been supported by experimental evidence showing that loss or overexpression of a miRNA in cultured cells results in the deregulation of hundreds of genes [33,34]. In both cases, the deregulated messages are enriched in conserved miRNA binding sites and their expression *in vivo* tends to be anti-correlated with that of the miRNA [33-36]. These observations suggest that both knockout and ectopic expression studies can give clues into the biological functions of miRNAs.

Nevertheless, *in vivo* studies tell a cautionary tale against taking these approaches as equivalent. In *C. elegans* for example, overexpression of *miR-61* results in vulval development defects, which seem to stem from its ability to regulate *Vav-1* and establish a feedback loop that regulates *LIN-12/Notch* expression within the vulval precursor cell population [13]. Yet, deletion of *miR-61* is compatible with normal vulval development, and even animals carrying co-deletion of *miR-61* and the closely related *miR-267* are phenotypically normal [20]. In mammals, overexpression of members of the *miR-34* family suggests a potent tumor suppressor function downstream of p53 [17,37-41]. However, mice carrying targeted deletions of all *miR-34* genes display wild type p53 responses to a variety of cellular insults, including ionizing radiation and oncogenic stress [42,43].

How can we account for these differences? Obviously, ectopic expression studies address the question of whether a miRNA can exert a specific function, while loss-of-function studies test whether it is required for that function. In addition, it is important to keep in mind that the ability of a miRNA to repress its targets crucially depends on its expression levels [44,45].

Genetic inactivation of a miRNA results in very modest de-repression of its direct targets, typically less than two-fold even for highly abundant miRNAs [33,46]. These differences are well within the range that could be attributed to fluctuations of gene expression between two genetically identical cells or between individuals [47]. For most genes such modest changes in expression can be well tolerated by the organism, which might explain why genetic inactivation of miRNAs often does not have obvious phenotypic consequences. These observations sparked the idea that rather than acting as genetic switches—where strong repression of one or few targets results in a clear phenotypic outcome [48,49]—most miRNA act as rheostats, fine-tuning the expression of hundreds of genes to reinforce cell fate decisions brought about through other mechanisms [47,50,51].

It is important to note however, that even a mild derepression of many targets can have severe phenotypical consequencesly, especially if the targets are functionally linked. For example, in mice, deletion of *miR-128* results in fatal epilepsy due de-repression of several components of the MAPK pathway, leading to a significant increase in ERK2 phosphorylation [52]. Similarly, loss of *miR-205* results in neonatal lethality in mice with compromised epidermal and hair follicle growth [53], presumably by modulating the expression of multiple components of the PI(3)K signaling pathway. Finally, a recent study has implicated Drosophila's *miR-iab-8* in CNV patterning and fly fertility through the regulation of genes whose products act together within a protein complex [54]. Remarkably, there is evidence that even partial relief of miRNA-mediated repression can have phenotypic consequences. For example, hemizygous deletion of miR-17∼92 leads to severe developmental defects in mice and in humans [19].

In contrast to loss-of-function studies, ectopic expression often leads to supra-physiologic levels of the miRNA and stronger repression of its targets [23,33,34]. The magnitude of this repression can bring down to inconsequential levels the expression of genes that would otherwise remain functional even in the presence of the targeting miRNA. Often, these experiments also result in the expression of miRNAs in tissues or cells in which they would

normally be absent [55], leading to repression of messages that might not be their biological targets. Thus, despite their widespread use, the propensity to generate a high fraction of false-positive results constitutes a major caveat of miRNA overexpression experiments. This does not mean that such experiments are devoid of value. In fact, much of the knowledge we have accumulated over the years on miRNA biology has depended on them [27,44]. In addition, regardless of the physiologic relevance of these studies, overexpression of miRNA mimics may serve a therapeutic purpose [56-58] (Box 2).

Functional redundancy among family members

One remarkable aspect of miRNA genes is that a large number of them have obvious paralogs in the genome. Paralog miRNAs arise from both tandem and non-local gene duplication events, which give rise to either duplication of sequences in the same transcript —thus originating miRNA clusters—or on distant loci, typically on different chromosomes [59]. These miRNA 'copies' not only retain a high degree of sequence homology but also share the same seed-sequence and are thus by convention grouped into 'miRNA seed families' [60]. In *C. elegans*, about 60% of all miRNAs can be assigned to one of 23 families [61], a percentage significantly higher than that of protein-coding genes with known paralogs, which approximates 25% [62]. Similarly, about one-third of human miRNA genes and almost 40% of those in the mouse can be grouped into families based on sequence similarity [63,64].

Because paralog miRNAs share the same seed-sequence, they are expected to have similar affinities to messages. When expressed in the same cells, these related miRNAs can co-regulate targets, leading to higher levels of repression than those that could be achieved by each miRNA individually. In vertebrate multiciliated cells for example, the six miRNAs that comprise the conserved *miR-34/449* family—encoded by the *miR-34a*, *miR-34b/c*, and *miR-449a/b/c* loci—can coordinately repress *cp110* expression during ciliogenesis [65]. The existence of miRNAs with redundant functions means that in some instances several members of the family need to be deleted before a phenotypic consequence can be detected (see Table 1). Indeed, animals carrying targeted deletions of single genes of the *miR-34/449* family are viable and phenotypically normal [42,65,66], whereas deletion of all three loci leads to high postnatal mortality, with surviving animals displaying an array of phenotypes associated with defective ciliogenesis [65]. Analogously, partial functional overlap has been observed among the *miR-17∼92* cluster and its two paralogs: *miR-106a∼363* and *miR-106b∼25* [67].

This level of redundancy adds considerable complexity to gene knockout studies. For families as numerous as the *let-7* family for example—which in mice comprises 12 members distributed across 8 loci [68]—the effort involved in generating compound loss-of-function mutants effectively precludes such studies from being undertaken.

Chemically modified antisense oligonucleotides (Box 2) provide an alternative to genetic miRNA ablation [69,70], and can to some extent circumvent the difficulties posed by the existence of paralog genes. These antisense molecules inactivate miRNAs by binding with high affinity to their mature sequence, thus preventing interaction with targets. Typically,

antisense molecules are designed to have full complementarity to the mature miRNA [69,70], but because affinity remains very high even for 8-mers targeting the seed region, this strategy can be adapted to simultaneously inhibit the function of multiple members of the same family [16,71]. This approach has shown that global inhibition of the *let-7* family can prevent and treat impaired glucose tolerance in diet-induced obese mice [16]; this result is consistent with data gathered from *let-7* transgenic animals [16,72] and from animals carrying gain- or loss-of-function alleles of Lin28a or LIN28B, two proteins that specifically block *let-7* maturation [72,73].

Despite their clear utility, designing controls for experiments in which antisense oligos are used is challenging. A common approach is to deliver modified oligonucleotides with sequences that do not match any region in the genome, but these molecules often lead to unwanted effects when delivered systemically to animals. A recent study, for example, reported an increase in liver and spleen size as well as in the number of liver-associated macrophages upon injection of a control oligonucleotide in adult mice. These animals also displayed reduced number of white blood cells and a variety of alterations in blood chemistry [74]. In another example, injection of a control molecule in chick embryos resulted in a wide range of skeletal malformations including vertebral fusions, hemivertebrae, and split vertebrae [75]. Thus, while we can certainly learn a lot from studies with synthetic miRNA antagonists, the intrinsic limitations of this technology need to be carefully considered.

The question of functional redundancy among paralog miRNAs has perhaps been best tackled in *C. elegans*, where miRNA genes were systematically mutated to generate strains lacking all or most members of 15 of the 23 known miRNA families [61]. As in the cases discussed above, this approach uncovered high levels of redundancy among some paralogs: for example, although deletion of individual components of the *miR-35* and *miR-51* families did not cause phenotypical abnormalities, mutants for these families died as embryos or early larvae. Similarly, mutants for the *miR-58* family showed a variety of phenotypic abnormalities, which were absent in mutants for its individual miRNAs [20]. This is perhaps not unexpected. What is surprising is that mutant animals for the remaining 12 families appeared largely normal even when subjected to a broad panel of phenotypical characterizations [61]. This suggests, that at least in *C. elegans*, most miRNA families might not be essential for animal development or viability.

miRNAs and biological robustness

Despite the frequent lack of phenotypes in miRNA knockout animals, the strong evolutionary conservation of many miRNAs indicates that they must confer some selective advantage, regardless of our ability to detect it. An important aspect to consider is that miRNAs do not act in isolation. In fact, a common theme among miRNAs is their positioning within gene regulatory networks, in particular within feedback and feedforward loops [76] (Figure 1A). Moreover, though the vast majority of 3′UTRs have a single conserved match for a particular seed, they typically have more than four conserved miRNA binding sites in total, thus allowing for combinatorial and overlapping regulation [1,4]. Redundant components and regulatory loops are two common strategies used to achieve

canalization [77], i.e., the stabilization of biological outcomes in spite of genetic, environmental, and stochastic perturbations. This has led to idea that miRNAs play a crucial role in ensuring that small changes arising from such perturbations do not have a detrimental impact on animal development or homeostasis [47,78,79].

In support of this view several miRNA knockout animals display phenotypes only in response to genetic or environmental stresses (Table 1). Under normal conditions, for example, the *miR-143/145* cluster can be deleted without apparent consequences to intestinal architecture or epithelial turnover rate. However, *miR-143/145*-null mice fail to regenerate the intestinal epithelium upon injury, at least in part due to impaired IGF signaling [80], and a similar role for this cluster in the response to blood vessel injury has been reported [81-83]. In another example, mice lacking *miR-208* showed no overt cardiac defects under physiologic conditions, but cardiac remodeling was profoundly impaired in response to various stresses [84].

miRNA-dependent phenotypes can also be uncovered by mutations in other genomic loci. In *C. elegans*, for example, 25 out of 31 miRNA mutations resulted in phenotypic abnormalities only in animals from sensitized backgrounds [85]. In mice, *miR-34*'s role as a tumor suppressor is only uncovered when this miRNA family is co-deleted with *trp53* [42,86], an upstream regulator with whom it establishes a coherent feedforward loop that regulates MET expression [86,87]. Finally, it should be noted that a large fraction of miRNA knockout models show phenotypic traits with incomplete penetrance (Table 1), one of the hallmarks of impaired canalization [88-90].

Mechanisms of Canalization by miRNAs

As mentioned in the previous section, a possible mechanism by which miRNA-mediated regulation may confer phenotypic robustness is by buffering against cell-to-cell variability arising from stochastic fluctuations in gene transcription. These fluctuations occur because the biological processes that determine mRNA levels—such as binding of the RNA polymerase to the promoter and mRNA degradation—are inherently noisy, occurring at different times and different rates even among cells from a clonal population [91]. This noise is ultimately amplified by translation, which can give rise to even larger inter-cellular variability [92,93]. Though in many instances organisms can use such variability to control processes like lineage decision [94], noise must generally be buffered to ensure robust animal development. A failure to do so can result in pronounced phenotypic variation among genetically identical individuals [89].

One strategy to manage noise is to couple high rates of transcription with inefficient translation [92,95]. miRNAs limit translation largely by reducing mRNA stability and may therefore provide a simple mechanism to reduce overall fluctuations in protein synthesis. This might be of particular importance when subtle differences in gene expression can determine distinct cellular fates (Figure 1B). In Drosophila's larval wing imaginal disks, differentiation of sensory organ precursors (SOP) requires tight regulation of *senseless* (*sens*). While high levels of this transcription factor promote proneural gene expression in cells destined to become SOPs [96], low levels of Sens in non-SOP cells repress proneural

genes [97]. *Sens* is a direct target of *miR-9*, and in the absence of this miRNA about 33% of larvae develop extra sensory organs [98], presumably because fluctuations in Sens levels inappropriately trigger ectopic cell fate decisions [99]. Interestingly, recent experiments suggest that *miR-9* can also minimize the impact of genomic diversity on sensory organ differentiation, thus providing an example of genetic buffering by a miRNA [100].

Integration of miRNAs in incoherent feedforward loops (IFFL) (Figure 1A) provides another mechanism to minimize unwanted oscillations in gene expression. This type of motif is particularly useful because it can counteract the effects of fluctuations in upstream components of the network. In *C. elegans*, for example, an IFFL involving both *lin-4* and its target *lin-14* ensures that variation in *lin-14* mRNA levels are stabilized by synchronous oscillations in *lin-4* [101]. The presence of miRNAs in IFFLs has also been associated with canalization mechanisms in vertebrates. In *Zebrafish*, for example, an IFFL involving miR-430 plays a significant role in controlling unwanted fluctuations in chemokine receptor signaling, which seems to be important for robust germ cell migration [102].

miRNAs have also been implicated in the mechanisms that buffer gene expression against environmental perturbations. Arguably the best example comes from work in *Drosophila*, in which *miR-7* is involved in cell fate decisions that result in photoreceptor differentiation [103]. Before differentiation into photoreceptors is triggered, progenitor cells in the larval eye imaginal disk are maintained in an undifferentiated state by Yan [104], a direct target of *miR-7* [105]. In these cells, Yan binds to the promoter of *miR-7* and inhibits its transcription [105], thus establishing a negative feedback loop that reinforces its own expression (Figure 1A, 1C). Yan also inhibits *miR-7* indirectly through two other repressors, creating a coherent feedforward loop (CFFL) that is interlocked with the feedback loop [103]. This CFFL buffers *miR-7's* expression against fluctuations in Yan, ensuring that miR-7 is only turned on in response to a persistent decrease in *yan* expression (Figure 1A, 1C). Differentiation of progenitors into photoreceptors is triggered by signaling through the EGF receptor [106], which transiently degrades Yan and establishes a new coherent FFL involving *miR-7* and Pnt-P1 [103]. This loop ensures that *yan* is not inappropriately turned on in cells that have committed to the photoreceptor lineage.

Interestingly, under uniform laboratory conditions, regulation of Yan expression is robust enough to ensure normal eye development even in the absence of miR-7 [103,105]. In a classic example of impaired canalization, however, mutant *miR-7* flies fail to withstand fluctuations in environmental temperature and display abnormal patterning of sensory organs due to deregulated Yan expression [103].

Concluding Remarks

Over the past two decades we have witnessed a flourishing of studies aimed at defining the biological functions of miRNAs. Converging evidence from computational, biochemical, and genetic experiments have greatly expanded our understanding of their mechanism of action and biological properties. The picture emerging from these studies suggests that miRNAs occupy a very unique position in the hierarchy of gene regulators. By contrast to conventional transcription factors, in most cases miRNAs do not appear to act as master

regulators of gene expression. Rather, their mechanism of action allows them to act as fine tuners of transcriptional programs, as components of complex network motifs, and as “post-transcriptional buffers” to confer robustness to transcriptional programs in the face of environmental and genetic variability.

Although investigating individual miRNA-mRNA interactions can and has been useful in some instances, moving forward it will be essential to resist the temptation to reduce the biological functions of individual miRNAs to repression of one or a few “key targets.” Rather, new computational and systems biology approaches will be needed to fully appreciate the intricacy, beauty, and multifaceted roles of gene regulation by small non-coding RNAs.

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Box 1

Mechanisms of miRNA-mediated gene repression

Although it is well understood that miRNAs repress gene expression by recruiting the RISC silencing complex to target mRNAs, the molecular details underlying this repression are not entirely clear [107]. RISC binding to targets has been proposed to elicit both repression of translation and mRNA deadenylation and decay (Figure I). However, the mechanistic basis behind these modes of repression, their relative contribution to the overall action of RISC and the order by which they operate remain a matter of debate.

The core of the RISC complex is comprised of two components: an Argonaute protein (Ago1-Ago4 in mammals) and a GW182 protein (TNRC6A-C in mammals). While Ago proteins bind to miRNAs and are thus involved in target recognition, GW182 proteins seem to act as a molecular platform to which effector complexes bind to mediate target repression (Figure I, top panel). GW182 can directly bind PABP [108], a poly(A)-tail binding protein involved in the regulation of translation [109], but with a controversial role in miRNA-mediated gene silencing [107]. GW182 also binds (directly or through PABP) to deadenylation complexes [110,111]. Of these, CCR4-NOT has been shown to be required for miRNA-mediated deadenylation [110,112]. Finally, GW182 has been shown to bind to EDD, a protein that associates with the DEAD box helicase RCK/p54, which in turn enhances mRNA decapping and represses cap-dependent translation [113].

But what is the prevalent mechanism of miRNA-mediated repression? Recentgenome wide attempts to clarify this question have examined the influence ofendogenous and exogenous miRNAs [23,114,115] on mRNA levels (measuredby RNA-seq of polyadenylated mRNA; Figure I, middle panel) and translationefficiency (measured by sequencing of ribosome protected fragments or RPFs;Figure I, bottom panel). These studies suggest that the predominant mode ofmiRNA-mediated repression maybe context-dependent. In human cells and latezebrafish embryos the majority of RISC-mediated repression can be attributed toa reduction in the levels of mRNA, with only a minor fraction stemming fromreduced translation efficiency [23,115]. In early stages of zebrafish development (2h and 4h post-fertilization) however, miRNAs seem to predominantly reducetranslation of their targets, while causing no detectable changes in the levels oftheir mRNAs [114,115]. These results are not necessarily in conflict, since arecent study suggests that the prevalence of translational inhibition in earlyzebrafish embryos is caused by a strong coupling of poly(A)-tail length andefficiency of translation in this system [115].

Box 2

miRNA-based therapeutic strategies

Deregulation of miRNA activity has been frequently implicated in the development and progression of human diseases [116], and this observation has driven much of the research in the field. The discovery that *in vivo* delivery of oligonucleotides—that mimic or inhibit the activity of specific miRNAs—can have therapeutic effects in animal models has suggested that analogous approaches might be applicable in the clinic as well [117].

Several miRNA-based therapeutic strategies are currently under development, and these can broadly be divided into: (i) miRNA replacement strategies, which attempt to mimic the activity of specific miRNAs by delivering small double-stranded RNA molecules that resemble miRNA duplexes. MRX34 for example, is a mimic of the *miR-34* family [56], which is currently in Phase I of clinical trials to test its safety for patients with primary liver cancer or liver metastasis (NCT01829971); (ii) miRNA targeting strategies, which rely on antisense oligonucleotides (ASO) that bind to endogenous miRNAs to prevent their interaction with targets. One such molecule, designed to inhibit *miR-122* [118], has also reached clinical trials (Phase II) and is being evaluated for its long-term safety and efficacy in patients with chronicle HCV infection (NCT02031133).

An important aspect to keep in mind when thinking about delivery of oligonucleotides to patients or animal models is that they are generally poorly suited for *in vivo* applications [117]:

- They are substrates of serum nucleases, making them unstable;
- They are unable to penetrate the cell membrane;
- They have poor tissue distribution when delivered systemically.

These obstacles can be partially overcome by encapsulation in lyposomes or polymer-based nanoparticles and through addition of chemical modifications [117]. Conjugation of cholesterol groups to the 3′ end of the oligonucleotide, for example, improves both entry of the oligo in the cell and its distribution across tissues. Modifications like 2′-O-methyl (2′-OMe), 2′-O-methyoxyethyl (2′-MOE), and locked nucleic acid (LNA), on the other hand, improve the oligonucleotide's resistance to exonucleases. Of note, for miRNA-targeting strategies, LNA modifications have the additional advantage of increasing the affinity of the oligo to its complementary miRNA, leading to more efficient inhibition. Substituting the phosphodiester bond by a phosphorothioate bond can further increase stability of the oligonucleotides, but this modification decreases miRNA-binding affinity.

Highlights

- MicroRNA activity is essential for animal development and viability.
- Deletion of individual miRNAs often leads to subtle or no phenotypic consequences.
- miRNAs can have high levels of redundancy and are often placed in regulatory loops.
- miRNAs can buffer gene expression against internal and external perturbations.

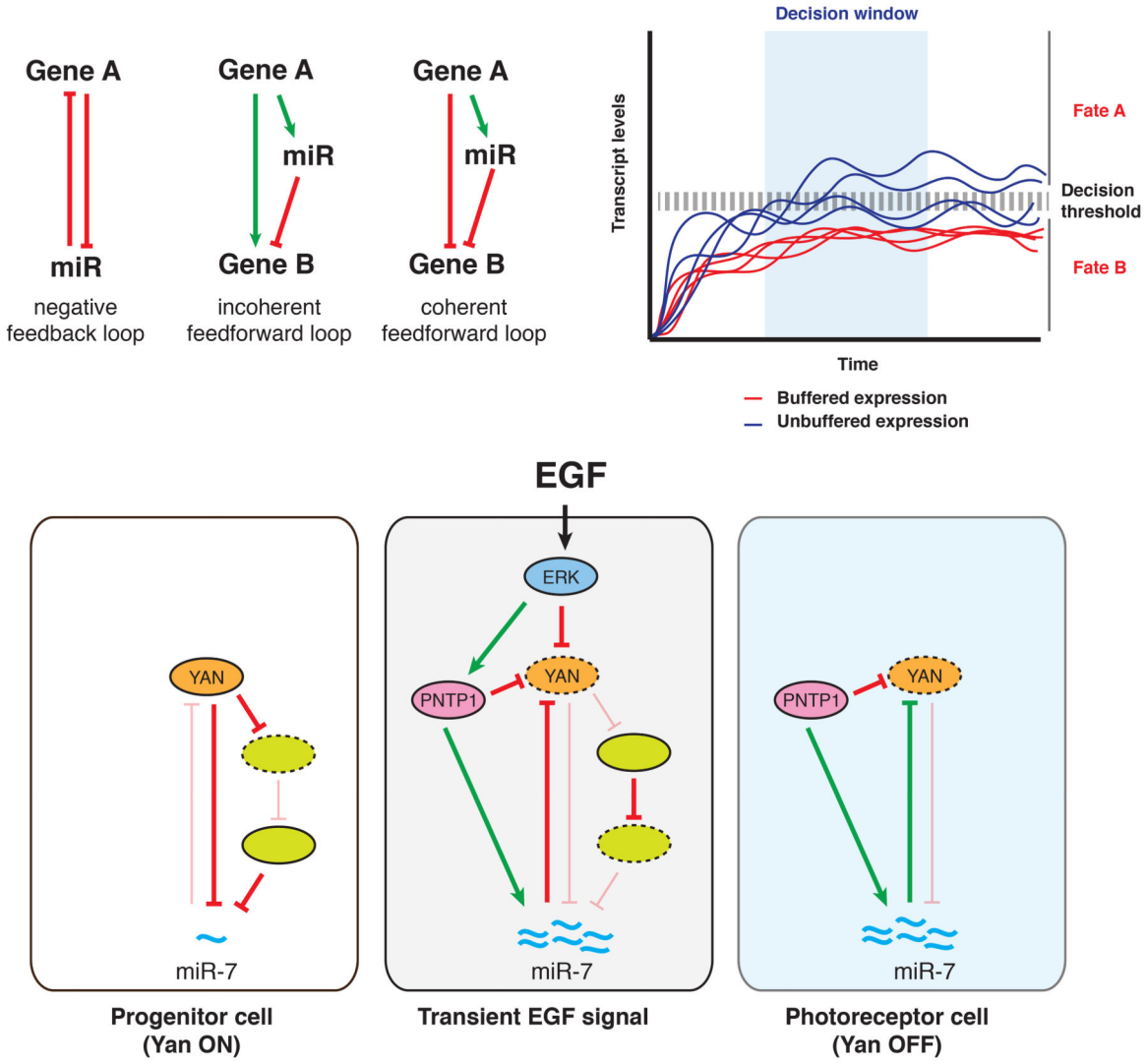

Figure 1. miRNAs participate in mechanisms of biological robustness

(A)miRNAs are common features of network motifs, some of which are represented here. **(B)** Regulation of transcripts by miRNAs can be important to ensure robust cell fate decisions by keeping expression levels of a particular gene below a decision threshold (red lines). In the absence of a miRNA, the expression of the gene becomes unbuffered (blue lines), and can lead to ectopic cell fate decisions in a fraction of the population due to stochastic fluctuations in transcript levels. **(C)** Schematic representation of *miR-7*'s role in the differentiation of photoreceptor cells in Drosophila. Panel (B) and (C) of this figure are inspired by illustrations from [89] and [142], respectively.

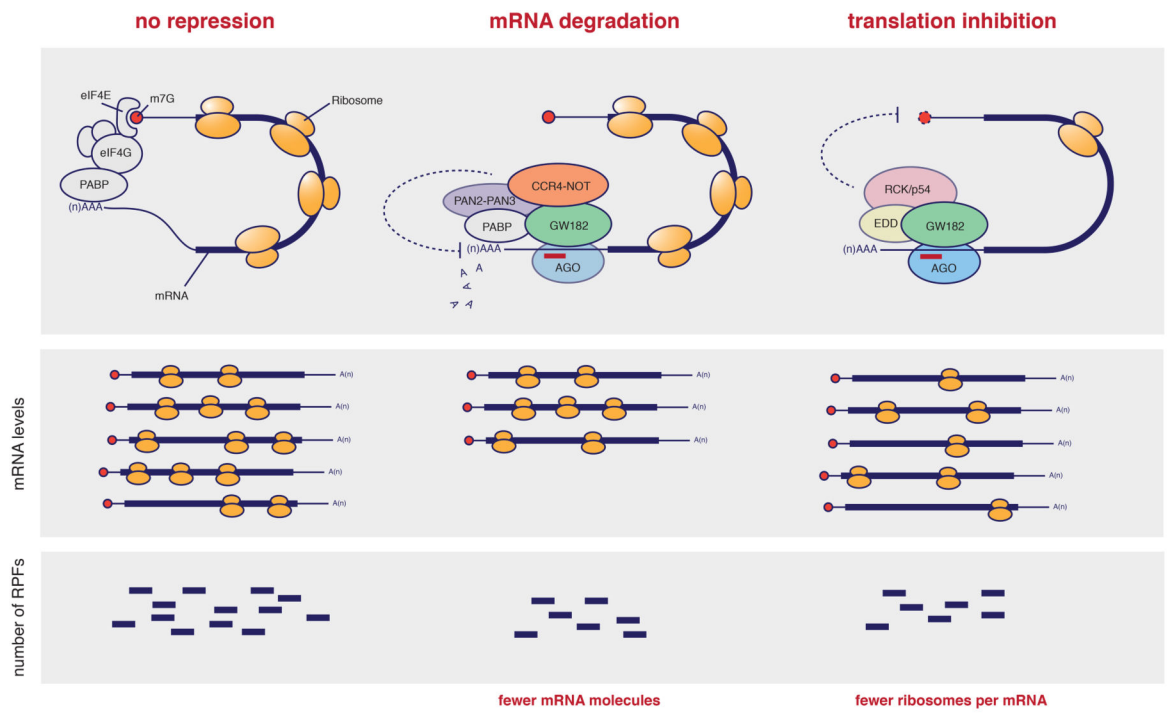

Figure I. Overview of protein complexes implicated in miRNA-mediated gene silencing and their effect on mRNA levels and translation efficiency

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Table 1

miRNA-knockout phenotypes in mice

miRNA gene	Knockout phenotype	Phenotype penetrance	Evidence of functional redundancy with family members	Phenotypes in response to external or internal perturbations	References
miR-155	immunodeficiency and increased lung remodeling	increased lung remodeling in ~56% of *miR-155*$^{-/-}$ animals	NA	NA	[119]
miR-17∼92	perinatal lethality with heart, lung, B cell and skeletal defects	100%	co-deletion of *miR-17∼92* with *miR-106∼25* aggravates developmental phenotypes of *miR-17∼92* single deletion	NA	[67], [19]
miR-106a∼363	no obvious phenotype	NA	co-deletion of *miR-17∼92* with *miR-106∼25* and *miR-106a∼363* aggravates developmental phenotypes of *miR-17∼92* single deletion	NA	[67]
miR-106b∼25	no obvious phenotype	NA	co-deletion of *miR-17∼92* with *miR-106∼25* aggravates developmental phenotypes of *miR-17∼92* single deletion	NA	[67]
miR-15a/16-1	B cell lymphoproliferative disorders	~24%	NA	NA	[120]
miR-144/451	impaired late erythroblast maturation	not specified. assumed to be 100%	NA	NA	[121]
miR-150	B1 cell expansion and increased humoral immune response	not specified. assumed to be 100%	NA	NA	[122]
miR-223	expanded granulocyte compartment	not specified. assumed to be 100%	NA	NA	[123]
miR-1-2	lethality at weaning with heart defects	~50%	NA	NA	[124]
miR-34a	no obvious phenotype	NA	co-deletion of *miR-34* and *miR-449* leads to defects in cilliogenesis. Lethal for 60% of animlals. Surviving adults are infertile	loss of miR-34 family members aggravates prostate neoplastic lesions caused by inactivation of *trp53; loss of miR-34a improves cardiac function during aging and in response to stress*.	[42],[65], [86] [125]
miR-34b/c	no obvious phenotype	NA	co-deletion of *miR-34* and *miR-449* leads to defects in cilliogenesis. Lethal for 60% of animlals. Surviving adults are infertile	loss of *miR-34* family members aggravates prostate neoplastic lesions caused by inactivation of *trp53*	[42],[65], [86]
miR-449	no obvious phenotype	NA	co-deletion of *miR-34* and *miR-449* leads to defects in cilliogenesis.	NA	[66], [65]

miRNA gene	Knockout phenotype	Phenotype penetrance	Evidence of functional redundancy with family members	Phenotypes in response to external or internal perturbations	References
			Lethal for 60% of animlals. Surviving adults are infertile Lethal for 60% of animlals. Surviving adults are infertile		
miR-290∼295	developmental delays; embryonic development outside yolk sac; embryonic lethality; germ cell deficiency in surviving adults	lethality in 75% of animals	NA	NA	[126]
miR-10a	no obvious phenotype	NA	NA	NA	[127]
miR-208a	no obvious phenotype, but minor cardiac conduction defects reported	80%	NA	reduced cardiac hypertrophy in response to stress and hypothyroidism	[84], [128]
miR-208b	no obvious phenotype	NA	decrease in type I myofibers in soleus muscle when *miR-499* is co-deleted	NA	[128]
miR-499	no obvious phenotype	NA	decrease in type I myofibers in soleus muscle when *miR-208b* is co-deleted	NA	[128]
miR-133a-1	no obvious phenotype	NA	50% perinatal lethality with ventricular septal defects when *miR-133a-2* is co-deleted. Cardiomyopathy and heart failure in adult compound animals.	NA	[129]
miR-133a-2	no obvious phenotype	NA	50% perinatal lethality with ventricular septal defects when *miR-133a-1* is odeleted. Cardiomyopathy and heart failure in adult compound animals.	NA	[129]
miR-126	embryonic/perinatal lethality with angiogenesis defects	40-50%	NA	adults are prone to myocardial rupture following myocardial infraction	[130], [131]
miR-143/145	no obvious phenotype; but smooth muscle cells display abnormal morphology	NA	NA	miR-143/145$^{-/-}$ have impaired responses to vascular and intestinal injuries.	[132], [83], [80]
miR-375	animals develop hyperglycemia; increased total pancreatic alpha-cells, decreased pancreatic beta-cell mass	not specified. assumed to be 100%	NA	NA	[133]
miR-140	mild skeletal phenotype: short stature, decreased body weight and craniofacial abnormalities	not specified. assumed to be 100%	NA	NA	[134]
miR-182	no obvious phenotype	NA	NA	NA	[135]
miR-132/212	minor synaptic transmission and plasticity defects in the neocortex	not specified. assumed to be 100%	NA	NA	[136]

miRNA gene	Knockout phenotype	Phenotype penetrance	Evidence of functional redundancy with family members	Phenotypes in response to external or internal perturbations	References
miR-22	no obvious phenotype	NA	NA	minor defects in cardiac function during acute and chronic hemodynamic stress	[137]
miR-21	no obvious phenotype	NA	NA	NA	[138]
miR-29ab1	reduced lifespan; thymic involution around 9-12 weeks of age	Not specified. assumed to be 100%	NA	NA	[139], [140]
miR-29b2c	no obvious phenotype	NA	NA	NA	[139]
miR-128	increased motor activity and fatal epilepsy	Not specified. assumed to be 100%	NA	NA	[52]
miR-205	neonatal lethality with compromised epidermal and hair follicle growth	Not specified. assumed to be 100%	NA	NA	[53]
miR-200b	NA	NA	Co-deletion of mir-200b and miR-429 results in female infertility	NA	[141]
miR-429	NA	NA	Co-deletion of mir-200b and miR-429 results in female infertility	NA	[141]

NA, not available or—to our knowledge—not addressed; grey rows highlight miRNA knockout models with no obvious phenotypes.