microRNA-mediated gene regulation and the resilience of multicellular animals

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Abbreviations: GRN – gene regulatory network; miRISC – microRNA induced silencing complex; TDMD – Target Dependent MicroRNA Degradation

ABSTRACT

MicroRNAs are small RNAs that enable parts of the genome to regulate the other parts of the genome by RNA::RNA complementarity. Genes that encode microRNAs function as trans-acting regulators of hundreds of other genes, primarily by inhibiting the production of protein from mRNAs to which the microRNAs can bind by base pairing. MicroRNAs and their Argonaute partner proteins constitute a regulatory complex (the miRISC) that exhibits astonishing regulatory versatility. microRNAs have been shown to perform diverse roles in genetic regulatory networks (GRNs) – to control developmental switches, to dampen gene expression noise, to coordinate multigene functional modules, and more broadly, to confer robustness and resilience to developmental and homeostatic processes. Genetic analysis reveals that the function of particular microRNAs can be conditional, such that the microRNA is required under particular environmental or physiological conditions, but relatively dispensable under other conditions. The diversity and versatility of microRNA function in animal systems reflects the many ways that miRISC can be regulated by cellular signaling pathways, and the structure-function interplay among microRNA, target, and Argonaute.

EVOLUTIONARY ORIGINS OF microRNAS

The partnership between Argonaute family proteins and small (approximately 20-21 nucleotides) nucleic acid guides is evolutionarily ancient, being present in eukaryotes, archaea and bacteria, where broadly speaking, Argonaute::guide complexes function to exert immunity against foreign nucleic acid [1]. MicroRNAs represent an adaptation of RNA interference (RNAi) phenomena associated with the Ago sub-class of Argonautes. An important distinction between 'traditional' RNAi and microRNA activity is that in RNAi, Argonaute uses guide RNAs derived from foreign (for example, viral) dsRNA, whilst for microRNAs, the guide RNA is derived from endogenous genes (microRNA genes) that evolved to regulate other genes through RNA::RNA complementary base-pairing. As such, microRNA-encoding genes are found in the genomes of essentially all plants and animals [2]. Remarkably, the genomes of multicellular animals contain hundreds of microRNA genes, many of which are evolutionarily conserved, indicating that microRNA-mediated gene regulation is deeply embedded in metazoan genetic regulatory networks. Accordingly, microRNAs have been shown to have roles in essentially all aspects of cellular and organismal physiology in experimental organisms, and the evolution of microRNAs is thought to contribute to the evolution of complexity in animal lineages [2].

Another distinction between RNAi and microRNAs, particularly in animals, is in the fate of the target RNA that is engaged by the Argonaute::guide complex. RNAi is characterized by an extensive pairing configuration between the guide and target RNA that enables the slicer activity of Argonaute to cleave the target and trigger rapid target degradation. By contrast, most animal microRNAs recognize their targets through discontinuous pairing configurations that preclude slicing, and thereby enable the Argonaute::microRNA complex to elicit more nuanced repression of the target mRNA, including various modes and degrees of translational repression, and/or mRNA destabilization [3]. The prevalent configuration of discontinuous pairing by microRNAs to targets is via nucleotides g2-g8 of the microRNA (the 'seed' sequence), with absence of pairing at the slicing-critical nucleotides g9 and g10. Seed pairing is often combined with additional base pairing in the 3' region of the microRNA, involving various numbers of nucleotides beyond g10 [4,5]. Non-seed pairing can critically determine relative site occupancy by a microRNA, affecting potency and specificity of targeting [4-8]. Moreover, particular configurations of 3' pairing can affect the conformation of Argonaute in the miRISC::target ternary complex, and thereby confer distinct outcomes, notably target dependent microRNA degradation (TDMD; see below) [9].

microRNA VERSATILITY: MANY TARGETS, DIVERSE OUTCOMES

Computational predictions [10], *in vitro* biochemical studies [4], and sequence analysis of *in vivo* Argonaute::microRNA::target complexes [11,12] reveal that each animal microRNA can engage scores, or in some cases hundreds, of distinct target genes through base-pairing to mRNA 3′ untranslated (3′ UTR) sequences. A major challenge to understanding the function of a given microRNA in a given biological context is to determine which one(s) of the many targets of a microRNA are phenocritical. In experimental genetic systems, this question is approached by comparing the phenotypes resulting from deletion of the microRNA gene to the phenotypes (if any) resulting from deletion of the corresponding complementary sites in specific target gene(s). The results of such studies point to the capacity of microRNAs to engage in diverse modes of action within genetic regulatory networks.

DEVELOPMENTAL SWITCHES

In certain cases, striking phenotypes result from deletion of a single microRNA gene, and those phenotypes can be further attributed to the disregulation of a single target gene. Indeed, the first microRNA-target regulatory motif to be identified, involving the *C. elegans lin-4* microRNA and the LIN-14 protein-coding mRNA target, revealed itself through the identical developmental timing phenotypes for mutations of the *lin-4* locus that affect the microRNA, and mutations that affect the *lin-4*-complementary sites in the *lin-14* 3′ UTR [13] [14]. This situation represents an example of a 'genetic switch', wherein the action of a single regulatory molecule (in this case the *lin-4* microRNA) is a critical determinant of the level of activity an effector molecule (in this case the LIN-14 transcription factor) that in turn acts as a cell fate determinant. By repressing the expression of LIN-14 protein as larval development progresses, *lin-4* controls the proper timing of early larval stage-specific cell fate transitions [13]. A similar developmental switch, with a single critical microRNA (*let-7*), and a single critical target (*lin-41*) controls aspects of the larval to adult transition in *C. elegans* [15,16].

Genetic criteria for switch-like microRNA function include that the microRNA gene *loss-of-function (lf)* phenotypes can be replicated by deletion of that microRNA's complementary sequences in the 3′ UTR of a single gene. Additional genetic criteria for identifying cases of simple genetic switches includes whether knockdown of the candidate target is sufficient to suppress the phenotype of the microRNA loss-of-function. Examples include the *C. elegans lin-4-lin-14* motif, where loss-of-function (lf) mutations of *lin-14* suppress *lin-4(lf)* [17], and also the *let-7-lin-41* motif where the developmental timing phenotypes of *let-7(lf)* are suppressed by *lin-41(lf)* [15].

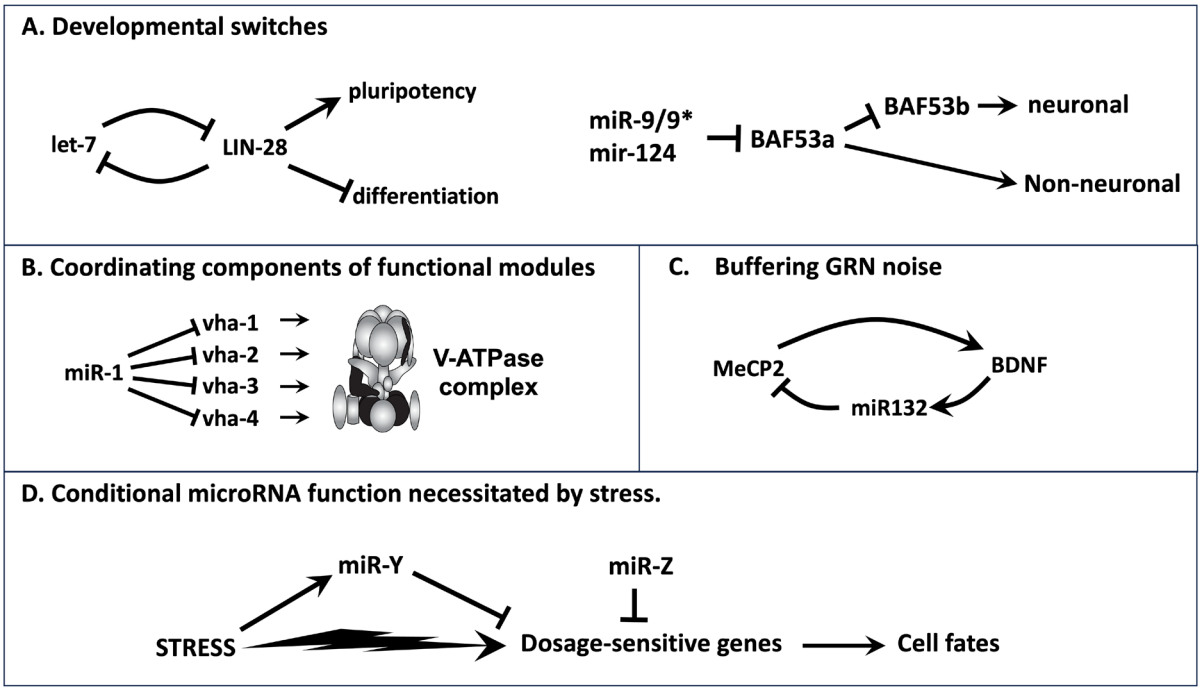

Figure 1. Context-dependent functional modes of action of microRNAs in animal cells. (**A**) MicroRNAs can be critical components of developmental cell fate switches, such as the phylogenetically conserved double negative feedback loop between the differentiation associated tumor suppressor let-7 microRNA and the pluripotency factor LIN-28 [86,87]. In another example, vertebrate neurogenesis is associated with expression of miR-9 and miR-124, which repress BAF53a, a non-neuronal component of ATP-dependent chromatin complexes, which in turn represses the homologous neuron-specific BAF53b. This microRNA-driven switch is so powerful that ectopic expression of miR-9/mirR-9* and miR-124 is sufficient to directly reprogram human fibroblasts to neurons [88]. (**B**) MicroRNAs can exert stabilizing activity to gene regulatory motifs. For example, mammalian methyl CpG-binding protein 2 (MeCP2) is an acutely dosage-sensitive determinant of neuronal development. Homeostasis of MeCP2 dosage is conferred by a feedback loop wherein MeCP2 acts through BDNF to induce the neuronal miRNA miR-132, which in turn represses MeCP2, thereby buffering against deleterious fluctuations in MeCP2 level (Klein *et a*l., 2007). (**C**) MicroRNAs can help coordinate stoichiometry among the products of multiple functionally related mRNAs. In this example, mirR-1 targets multiple components of the V-ATPase complex, and mutations that disrupt the repression of these targets by miR-1 result in strong phenotypes that can be traced to discordant V-ATPase function due to defects in complex assembly [30]. (**D**) MicroRNA mutant phenotypes can be modified by environmental or physiological conditions, consistent with accentuated requirement for of the function of those microRNAs by particular stresses. These stress-conditional functions suggest that the corresponding microRNAs confer the proper levels of dosage-sensitive developmental determinants that otherwise would be dysregulated by stress signals.

Switch-like function of a microRNA acting through a single phenocritical target also emerge in the context of human disease, for example as for the *let-7* tumor suppressor microRNA and its HMGA2 target. In certain contexts, either loss of the microRNA or loss of the *let-7* sites from the HMGA2 3′ UTR can drive oncogenesis [18,19].

A noteworthy evolutionarily conserved switch-like microRNA-target motif is the direct reciprocal regulatory loop between the RNA binding protein LIN-28 by the *let-7* microRNA (Fig. 1A). In invertebrates and vertebrates, *let-7* family microRNAs directly bind the *lin-28* 3′ UTR and repress LIN-28 production, whilst LIN-28 protein binds to *let-7* family microRNAs to inhibit their biogenesis [20]. This mutual negative feedback loop appears to be incorporated into diverse contexts in animals, including the regulation developmental cell fate timing in *C. elegans* [21], and the control of transitions between pluripotency and differentiation in mammalian systems [22,23].

Individual or a small number of microRNAs can exert a powerful switch-like reinforcement of cell type specification. In an early study [24], it was found that forced expression of a cell type-specific microRNA in Hela cells caused the transcriptome of the cells to shift to a pattern more like the cell type that normally expresses the microRNA. Expression of the neuron-specific microRNA miR-124, or the muscle-specific microRNA miR-1, resulted in the downregulation of populations of non-neuronal transcripts, or non-muscle transcripts, respectively, consistent with a role for these microRNAs in reinforcing each of their home cell types.

A remarkably potent effect of cell-type specific microRNAs on cell fate is exhibited by the finding that forced expression of the neuronal-specific microRNAs *miR-9/9** and *mi-124* in primary cultures of human fibroblasts causes the cells to exit the cell cycle and trans-differentiate into neurons [25]. This direct reprogramming of fibroblasts to neurons by microRNAs reflects instructive roles of miR-9/9* and mirR-124 in normal neurogenesis, where they target subunits of BAF chromatin remodeling complexes to effect a switch from non-neuronal to neuron-specific BAF complexes [26] (Fig. 1A).

COORDINATION OF FUNCTIONALLY RELATED GENE EXPRESSION MODULES

The high connectivity of microRNA-target interactions within gene regulatory networks suggests a mode of action where a microRNA could coordinate the expression of multiple genes that are themselves functionally related, such as genes encoding enzymes in biochemical pathways or members of multiprotein complexes (Fig. 1B).

The regulation by a microRNA of multiple genes that function in the same pathway can have surprisingly profound outcomes. One example is microRNA regulation of glycosylation in certain mammalian cells [27]. For example, through systems analysis of gene expression and cell surface glycosylation for mammalian cells, combined with experimental perturbation of specific microRNAs, a number of cases were identified where microRNA-mediated regulation of particular glycosylation enzymes controls the glycosylation complexion of the cell surface [28]. Such findings suggest that even a single microRNA, by regulating multiple glycosylation enzymes, could tune the information content of the cell surface and thereby affect cell-cell interactions, a scenario that is born out in various contexts of human disease and animal development [27]. Genetic studies using *C. elegans* identified *mir-79* as a critical regulator of migration and axon outgrowth for a particular neuron, the HSN [29]. *mir-79* loss-of-function resulted in defects in HSN cell migration and axon guidance similar to those of other mutants that disrupt proteoglycan homeostasis. These *mir-79* phenotypes were attributed to disregulation of two predicted *mir-79* targets, *sqv-5* and *sqv-7*, which encode proteoglycan biosynthetic enzymes [29].

microRNAs have been shown to play fundamental roles in regulating multiple components of multiprotein complexes. A well-studied case is for muscle-specific microRNA miR-1 (Fig. 1B), which is predicted to target transcripts encoding many of the 15 subunits of the vacuolar adenosine triphosphatase (V-ATPase) complex in *C. elegans*, *Drosophila*, zebrafish, and mammals, including humans [30]. In both *C. elegans* and *Drosophila*, *mir-1* loss-of-function was shown to result in defective mitochondrial and vacuolar function, protein aggregation, and other with proteostasis related phenotypes, presumably caused by stress from non-stoichiometric accumulation of V-ATPase subunits. Disruption of V-ATPase homeostasis as the basis for *mir-1(lf)* phenotypes was validated by epistasis: In both flies and worms, the *mir-1(lf)* phenotypes were suppressed by knocking down V-ATPase subunit targets, and in *C. elegans*, the *mir-1(lf)* phenotypes could be replicated by mutation of miR-1 binding sites in the 3′ UTRs of predicted V-ATPase component targets [30]. The conservation of miR-1 microRNA sequence from worms to humans, and the deep conservation of targeting by miR-1 of multiple V-ATPase components underscores the fundamental importance of this regulatory module throughout the evolution of animal muscles.

SUPPRESSION OF GENE EXPRESSION NOISE

Numerous investigations in diverse animal models support the idea that microRNAs contribute to buffering gene regulatory networks (GRNs) against noise, such as transcriptional bursts, and other stochastic processes that can causer variation in gene expression [31]. There is compelling experimental and theoretical support for noise-buffering roles for microRNAs [31-35]. microRNAs are often found to function within regulatory motifs that include feedback or feedforward loops (Fig. 1C) that can buffer outcomes against variation in input [36,37]. It has been argued that, by acting as repressors of gene expression, microRNAs can stabilize the otherwise noisy temporal dynamics of dosage-sensitive developmental factors, and thereby ensure robust temporal boundaries for developmental systems [38]. A "sharpening of boundaries" role for microRNAs also emerges in the spatial regulation of gene expression, for example, where expression of a microRNA and its target are sharply anti-correlated between cell types or across physical domains in the developing animal [32].

BIOLOGICAL ROBUSTNESS AND CONTEXT-DEPENDENT FUNCTION OF microRNAS

microRNA functions in intact organisms are interrogated using mutations that disrupt microRNA expression or sequence. The first microRNAs to be identified, *lin-4* and *let-7* of *C. elegans*, were found through the cloning of genes whose loss of function results in distinct developmental phenotypes [14,39]. Likewise, for a number of mouse microRNAs, single gene knockout can also cause developmental phenotypes, including embryonic or neonatal lethality [40]. However, for many microRNAs, loss-of-function mutation of an individual microRNA gene results in no apparent phenotypes [40,41]. This can be attributed in part to redundancy among genes encoding microRNAs of the same seed sequence [42,43]. However, for several microRNA seed families in *C. elegans*, simultaneous knockout of the entire family nevertheless did not result in apparent phenotypes [43].

CONDITIONAL PHENOTYPES OF MICRORNA MUTANTS

Absence of detectable phenotypes for a loss-of-function mutant under standard laboratory conditions does not necessarily mean that the gene in question is utterly dispensable – rather that the gene may function under other conditions. Indeed, novel phenotypes could be uncovered for many *C. elegans* microRNA mutants by genetically compromising cell signaling pathways [44], indicating that in many cases the functions of microRNAs may depend on physiological or genetic context.

A striking indicator of the contextual character of microRNA function is that loss-of-function mutants of microRNA genes can display emergent developmental or physiological defects when subjected to a biological challenge, such as injury or other stresses. "Stresses" in this context refer to contingencies that the animal has evolved to successfully tolerate such that wild type animals are unperturbed. These would include temperature fluctuations across the animal's normal range, exposure to pathogens or toxins that are endemic to the animals normal environment, or nutritional challenge [31,45-54]. The observation of a stress-dependent conditional phenotype for a particular microRNA mutant is interpreted to indicate that the microRNA is required more critically under stressed conditions compared to unstressed conditions, and that the microRNA normally functions to support the stress-robustness of the animal (Fig. 1D).

One of the first prominent demonstrations of conditional microRNA phenotypes was for *Drosophila miR-7(lf)* mutants; absence of miR-7 in the developing eye of the fly caused no apparent abnormalities unless the animal was subjected to cycles of temperature fluctuations during eye development [45]. The temperature fluctuations employed in these experiments – between 18°C and 31°C – are within the fly's normal thermotolerant range, and have no effect on wild type eye development. It appears that a function of miR-7 within the genetic circuitry of eye photoreceptor cell type specification is to stabilize cell fate choices in the context of changing temperature.

A similar case of microRNA mutant phenotypes aggravated by alternating temperature is the *C. elegans* miR-86 (the homolog of human miR-29) and miR-34, which function redundantly to control a temporal-spatial program of gonadal morphogenesis [44,49]. Simultaneous loss-of-function of the *mir-34* and *mir-86* genes results in very weak gonadal morphogenesis defects when larvae develop their whole lives at a uniform temperature (either 15°C or 25°C), but the phenotype of these *mir-34(lf); mir-86(lf)* doubly-mutant animals is dramatically aggravated for larvae that developed under an alternating temperature regimen (for example 15-minute cycles between 15°C and 25°C) [49]. The temperature-aggravated phenotype could be suppressed by knockdown of genes predicted to be co-targeted by miR-34 and miR-86, including *cdc-42* (encoding a GTPase) and *pat-3* (beta-integrin), suggesting that the level of expression of CDC-42 and PAT-3 is critical for proper gonadal development, and that *mir-34* and *mir-86* are required to buffer CDC-42 and PAT-3 levels during the stress of unstable temperature.

The evolutionarily conserved muscle-specific microRNA miR-1, discussed above as an example of microRNA function in coordinating the expression of components of a multi-protein complex, also can exhibit conditional phenotypes. Although the cellular phenotypes of *C. elegans* and *Drosophila mir-1(lf)* mutant larvae are striking, and include muscle degradation [30,55], these phenotypes can be conditional, especially in the case of *Drosophila*. After completing embryogenesis, the newly-hatched *mir-1(lf)* larvae exhibit normal motility and no apparent muscle cellular defects; larvae on a sugar-only diet (which does not trigger postembryonic development) are motile and viable for days. However, upon being fed a growth-stimulating diet, the *mir-1(lf)* larvae rapidly develop dramatic muscle degenerative phenotypes [55], indicating that although miR-1 microRNA is not essential for baseline muscle cell fate specification and differentiation, miR-1 is nevertheless critical for enabling muscle cells to grow without self-destructing.

A remarkable example of the principle of conditional microRNA function is the ability of certain *Drosophila* mutants that are defective in microRNA biogenesis machinery, and hence dramatically depleted of microRNA, to traverse the entirety of development from embryo to adult [38]. Importantly, this nearly microRNA-less development is only possible for a fortunate subset of larvae that are metabolically depressed and developing exceedingly slowly. This result suggests that, broadly speaking, microRNAs provide a stabilizing layer of post-transcriptional gene regulation that enables multicellular animals to successfully undergo rapid and energetically intensive development [38].

An example of mammalian microRNA mutants with conditional phenotypes is the knockout of the *mir-143/mir145* cluster in mouse [56]. miR-143 and miR-145 are expressed in mouse intestine, yet *mir-143/145* knockout animals exhibit normal intestinal development and function. Nevertheless, after injury to the intestinal epithelium, the normal process of regeneration of damaged tissue was dramatically impaired in the *mir-143/145* knockout. Therefore, miR-143 and miR-145 are dispensable for basal intestinal development,

but are critically required for regeneration after injury. Interestingly, miR-143 and miR-145 are expressed exclusively in the mesenchymal cells of the mouse intestine, yet they are required for regeneration of the overlying epithelial layer, which indicates a role for these microRNAs in coordinating regenerative signals from smooth muscle and myofibroblasts to epithelium [56].

It should be noted that the dependence of a microRNA mutant phenotype on particular conditions does not necessarily reflect a corresponding conditional regulation of the abundance of the microRNA. For example, the levels of miR-143 and miR-145 microRNAs in intestine were not reported to change in response to intestinal injury, so their roles specifically in regeneration after injury does not seem to reflect an enhanced expression in response to injury, but perhaps more consistent with a 'sentinel' role in uninjured intestine – standing ready to coordinate the regenerative response in case of injury. At the same time, in principle, the activity of a microRNA could be modulated in response to signals without necessarily affecting its level. These considerations emphasize the importance of understanding in molecular detail the regulatory mechanisms that can affect microRNA activity as well as those that affect microRNA abundance.

REGULATION OF microRNA EXPRESSION AND FUNCTION

In experimental systems where microRNA expression has been well studied, most microRNAs exhibit patterns of expression that vary (often strikingly so) by tissue, cell type, and/or developmental stage. Current understanding of molecular mechanisms underlying the regulation of microRNAs in animal cells has been reviewed elsewhere [57-59]. Perhaps not surprisingly, the spatial and temporal pattern of expression of a microRNA can reflect various modes of regulation, including regulation of the rate of transcription of the microRNA, regulation of transcript processing, stability, trafficking, Argonaute binding, and target engagement (Fig. 2).

SPECIFIC REGULATION OF INDIVIDUAL microRNAS.

The level of a given microRNA can be reflect multiple modes of transcriptional and post-transcriptional regulation of the transcription, processing, and turnover of the microRNA. For example, the temporal pattern of accumulation of let-7 microRNA in *C. elegans* involves developmental regulation of *let-7* transcription [60], combined with at least two LIN-28-regulated steps in *let-7* transcript processing [21]. The regulation of let-7 biogenesis by LIN-28 is highly specific to let-7, reflecting the direct interaction of LIN-28 to binding motifs in the *let-7* primary transcript [21,61].

Mature microRNAs are generally relatively stable, with half-lives on the order of tens of hours, or even longer [62], but turnover rates can vary for different microRNAs co-expressed in the same cells [57]. It is clear from a number of studies that microRNAs can be selectively destabilized as a function of contextual factors such as cell type [63,64], cellular physiology [65], or cell cycle progression [66].

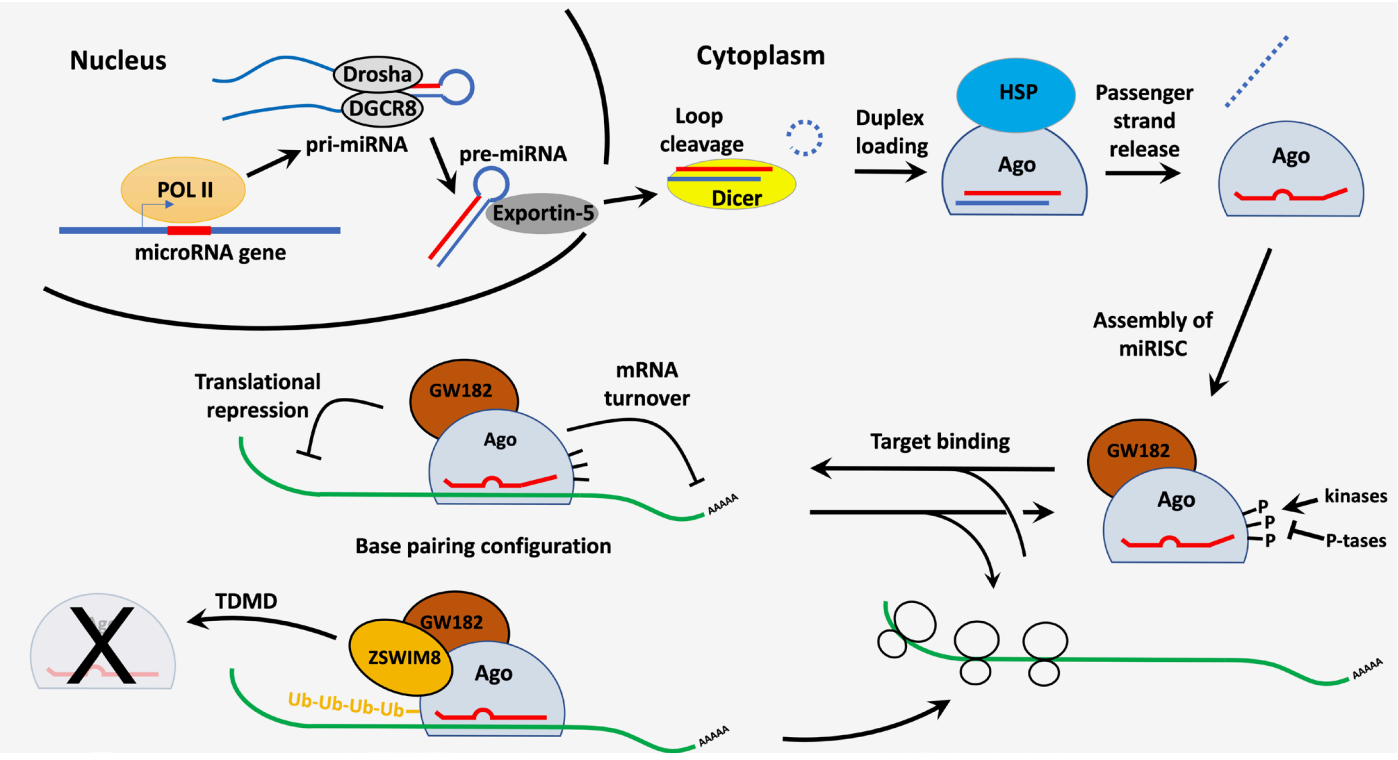

Figure 2. Diverse modes of regulation of microRNA expression and/or function. Essentially every known step in microRNA biogenesis, miRISC assembly, target binding, and target regulation has been shown to be regulatable by cellular context or physiological conditions. Regulation can be highly specific, as for TDMD, where turnover of Argonaute and microRNA is triggered by specific pairing configurations between the microRNA and particular targets, which signals ubiquitination of Argonaute by ZSWIM8 [71,72]. Alternatively, regulation can be simultaneously applied to diverse microRNAs, such as wherein phosphorylation of Argonaute at certain sites can broadly modulate microRNA-target affinities [81-84, 89]. Areas of interest to the field include learning more about how cellular signaling pathways are linked to the regulation of microRNA biogenesis, activity, and turnover, and acquiring a better understanding of how the structure of specific microRNA::target interactions can affect miRISC conformation and thereby govern the nature and potency of target regulation.

For many instances, the mechanisms by which the stability of certain microRNAs are regulated are not well understood; however recent findings have identified mechanisms that contribute to microRNA turnover triggered by target engagement [9,67-71]. Target Dependent MicroRNA Degradation (TDMD) involves the recognition of particular Argonaute configurations associated with microRNA::target base pairing by an E3 ligase ZSWIM8 which then mediates degradation of both the Argonaute and the microRNA [71,72]. In this fashion, specific targets can negatively regulate specific microRNAs. TDMD has been shown to function in normal development, as TDMD-is required for the developmental downregulation of the *mir-35* family microRNAs in *C. elegans* [73], and TDMD-defective *Drosophila* experience de-repression of certain microRNAs and suffer embryonic lethality [74]. TDMD is also essential for normal regulation of microRNA expression and embryonic development in mammals [75].

The canonical microRNA-target pairing configuration that triggers TDMD is one where pairing to target extends to the 3′ end of the microRNA, resulting in release of the 3′ end from the PAZ domain, which causes a conformational switch in Argonaute that is recognized by ZSWIM8 [9,71], but the rules for TDMD are not completely understood; for example, the *C. elegans mir-35* family microRNAs in are developmentally downregulated by a ZSWIM8-dependent mechanism that appears to be independent of 3′ pairing [73]. This finding highlights the importance of determining precisely what structural and/or sequence characteristics of the miRISC::target interactions can elicit TDMD.

More broadly, we need a better understanding of how specific microRNA-target base pairing configurations can shape the structure of miRISC and determine regulatory outcomes for target and/or microRNA (Fig. 2). It is clear that base pairing configuration between target and microRNA, including seed pairing in combination with certain patterns of 3′ non-seed pairing, confers target specificity for seed family isoforms [4,5,8,76,77]. Less is known about how 3′ non-seed pairing could qualitatively affect regulatory outcome, such as translational repression and or mRNA turnover (Fig. 2).

Besides the regulation of the abundance of a microRNA, it is also apparent that the activity of a microRNA can be regulated, independently of its abundance. An excellent example of distinct outcomes exerted by the same microRNA in distinct contexts is for *C. elegans* mirR-228, which is expressed in both the soma and germline of the worm, but elicits distinct outcomes for its targets in the two settings [78]. In the soma, translational repression of targets by miR-228 is accompanied by target mRNA degradation, whilst in the germline the opposite occurs; translationally repressed target mRNAs are stabilized [78]. This example illustrates how the same microRNA can regulate the same targets in two settings by different modes. Interestingly, in this case the germline mode of miR-228 action would seem to be reversible, since the translationally repressed target mRNAs are stabilized, whilst in the soma the same interaction results in a relatively more irreversible mRNA turnover scenario.

GLOBAL REGULATION OF microRNAs

One of the first reports of a global regulation of populations of diverse microRNAs was of confluence-dependent microRNA biogenesis in cultured cells [79]. The levels of most or all mature microRNAs were observed to be dramatically up-regulated as cultured cells reached confluency, and the effect could be attributed to increased efficiency of processing of microRNA gene transcripts into mature microRNA. This implied that the microRNA biogenesis machinery can be regulated by cellular signals, a conclusion borne out by other studies [80]. For example, mutation of a phosphorylation site on *C. elegans* ALG-1 prevents proper loading of microRNA duplexes into ALG-1, indicating that upstream kinase/phosphatase signaling could contribute to context-dependent miRISC maturation [81].

Post-translational modifications of Argonaute can also affect target binding. In *C. elegans* and in mammalian cells, phosphorylation of Argonaute at a cluster of conserved serine/threonine residues affects the affinity of miRISC binding to targets [81-84]. Disruption of the cycle of phosphorylation and dephosphorylation by mutation of the responsible kinases or phosphatases, or by mutating the phosphorylation sites on Argonaute, has functional consequences. Interestingly, although phosphorylation of Argonaute at these sites inhibits target binding, a non-phosphorylatable Ago2 mutant exhibited reduced microRNA activity. This was attributed to profligate binding of miRISC to ectopic targets, reducing the amount of miRISC available for proper regulation of normal targets [83]. A fascinating aspect of these phenomena is that target binding by unphosphorylated Ago seems to trigger phosphorylation, and hence target release [83,84]. One implication here would be that the affinity of miRISC for target could be tuned by an ongoing kinase/phosphatase cycle. These findings point to a potentially powerful node for regulation of the potency of microRNA-target interactions by kinases and phosphatases whose activity is coupled to upstream signals, and which could perhaps act either locally on specific miRISC targets, or globally, affecting microRNA activity broadly.

A striking example of the apparent global regulation of miRISC binding to mRNA targets comes from studies of resting cells in intact tissues, where the majority of microRNA appears to be in relatively low molecular weight complexes (LMW-RISC) of a size consistent with miRISC free from bound target; by contrast, in activated T cells, or tumor cells, or cells growing in culture, the majority of microRNA is found in high molecular weight complexes (HMW-RISC) consistent with miRISC bound to targets [85]. Although a role for mTOR-dependent signaling in the transition between LMW-RISC and HMW-RISC was found – apparently through enhancement of the miRISC effector protein GW182 [85], it is not clear to what extent direct phosphorylation of Argonaute by mTOR or other pathways could be involved in this particular context. These provocative finding suggests that at steady state in intact tissues, cells may hold their microRNAs in ready reserve, enabling cells

to dynamically engage microRNA regulation upon embarking on demanding changes in cell state, such as proliferation and/or differentiation. This scenario harkens to the role of miR-1 in *Drosophila* larval muscle specifically after the muscle is called upon to grow [55].

PERSPECTIVE

Many aspects of human disease pathology can be considered to reflect a breaching of mechanisms that have evolved to confer homeostatic equilibrium and resilience against everyday stresses. Therefore, the study of how microRNAs contribute to homeostasis and biological robustness is motivated by the hope to alleviate suffering through deepening the understanding of disease mechanisms. At the same time, the inverse of disease – the incredible fidelity of animal homeostatic processes, minute by minute, day by day, year by year – is astonishing, and so a sense of wonder also drives interest in this area.

There are a number of opportunities for advancing mechanistic understanding of how microRNAs contribute to biological homeostasis in animals. For a microRNA whose function is required primarily under certain conditions and not under other conditions, an important question is whether the level and/or activity of the microRNA is regulated by those conditions; if so, what cellular signals regulate the microRNA? Regulation of microRNA abundance has been relatively well studied, in part because the assays are straightforward, whilst less is known about situations where a microRNA does not appear to change in level between conditions, but nevertheless exhibits conditional function. There is surely a lot to learn about how microRNAs can buffer against stress by functioning as static sentinals (Fig. 1D), or by being regulated not by modulation of microRNA abundance but by control of target binding or effector activity (Fig. 2). An ongoing challenge for the field is to better understand how cell signaling pathways interface with the microRNA biogenesis machinery and miRISC components to regulate microRNA-mediated regulation in specific cellular and physiological contexts. Moreover, an area for further investigation is how AGO:microRNA:target conformation, together with post-translational modifications of miRISC components, can affect miRISC composition, conformation, stability, and regulatory outcomes.

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