Seminars in Cell & Developmental Biology 21 (2010) 728–737

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

The microRNAs of *Caenorhabditis elegans*

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ARTICLE INFO

Article history:
Available online 15 July 2010

Keywords:
microRNA
C. elegans
Development
Heterochrony
Posttransciptional gene regulation

ABSTRACT

The soil nematode, *Caenorhabditis elegans*, occupies a central place in the short history of microRNA (miRNA) research. The converse is also true: miRNAs have emerged as key regulatory components in the life cycle of the worm, as well as numerous other organisms. Since the landmark discovery in 1993 of the first miRNA gene, lin-4, several other miRNAs have been characterized in detail in *C. elegans* and shown to participate in diverse biological processes. Moreover, the worm has provided, by virtue of its ease of genetic manipulation and amenability to high-throughput methods, an ideal platform for elucidating many general and conserved aspects of miRNA biology, namely mechanisms of biogenesis, target recognition, gene silencing, and regulation thereof. In this review, we summarize both the contribution of miRNAs to *C. elegans* physiology and development, as well as the contribution of *C. elegans* research to our understanding of general features of miRNA biology.

Contents

1. Introduction

Over the past decade, microRNAs (miRNAs) have emerged as pervasive regulators of gene expression in metazoans. Potentially any gene, in principle, could be a target of miRNA-mediated silencing, and indeed, miRNAs have been shown to regulate extremely diverse biological processes, from skin cell differentiation [1], to the timing of flowering in plants [2], to the response to osmotic stress [3] or DNA damage [4], to name but a few examples.

The miRNA biogenesis pathway is summarized in Fig. 1. Generally, miRNAs are derived from capped and polyadenylated Pol II transcripts [5]. Such primary miRNA transcripts are sequentially processed by two RNA endonucleases, Drosha in the nucleus followed by Dicer in the cytoplasm, into their mature form [6]. Mature miRNAs are then incorporated into protein complexes [7], within which they direct silencing of target miRNAs through the dual mechanisms of mRNA degradation [8] and translational repression [9]. There is still considerable dispute, however, over the mechanism of miRNA-mediated translational repression, with con-

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doi:10.1016/j.semcdb.2010.07.001

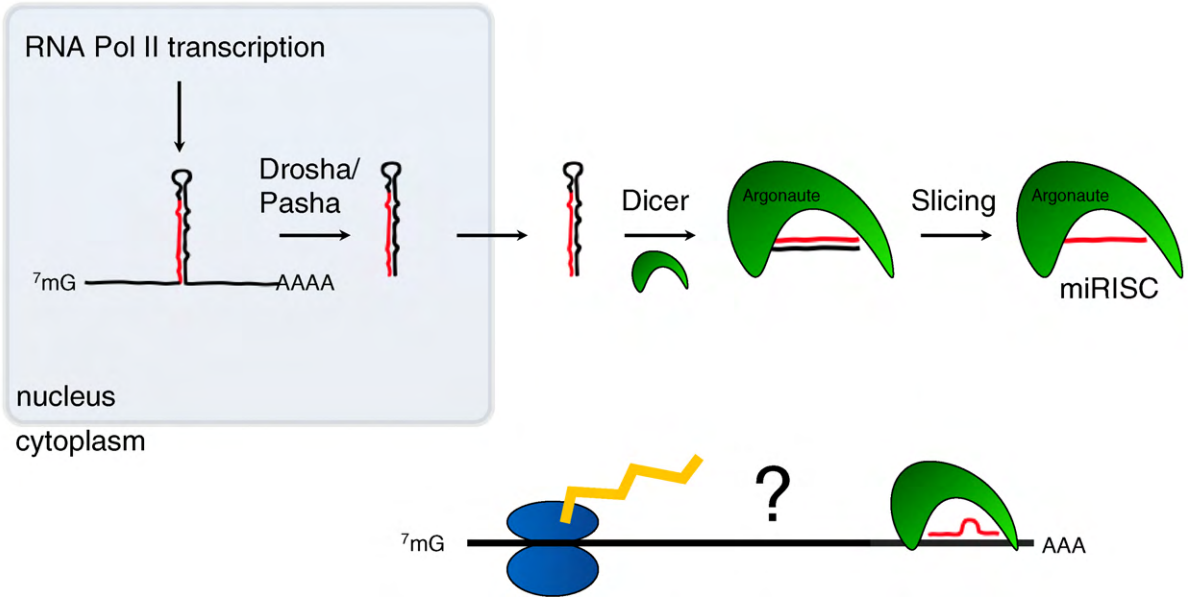

Fig. 1. Schematic of microRNA (miRNA) biogenesis and function. The mechanism of translational repression is currently not well understood.

flicting data supporting repression at both initiation [9–13] and post-initiation steps [14–16].

As the anatomically simplest laboratory animal in which miRNAs occur, *Caenorhabditis elegans* has been a genetic workhorse of miRNA research ever since the discovery of the first miRNA nearly 20 years ago. In this review, we summarize the various functions performed by miRNAs during *C. elegans* development as well as the discoveries in *C. elegans* that have contributed to our understanding of general features of miRNA biology.

2. Functions of miRNAs I: the heterochronic pathway

MicroRNAs owe their discovery to the genetic analysis of developmental timing mutants in *C. elegans*, carried out by the laboratories of Victor Ambros and Gary Ruvkun [17,18]. Study of the developmental timing pathway, in turn, depended on earlier pioneering work by Sulston and Horvitz [19,20] in Sydney Brenner's Lab at the MRC-LMB in Cambridge to trace the single fertilized egg through every cell division, cell death, cell fusion and cell migration to the final position of every cell in the adult animal, simply by observing development under a compound light microscope equipped with Nomarski optics. Importantly, this cell lineage turned out to be completely invariant from animal to animal, with every developmental event occurring at a precise moment in time and space. Hence, genetic analysis of development was made possible, by screening for mutants exhibiting changes to this normally invariant cell lineage [21,22]. Such mutants could, for example, exhibit complete loss of particular lineages, owing to the absence of a blast cell or its failure to divide. Conversely, certain mutants exhibited proliferative lineages, where an abnormally high number of progeny cells are generated, usually by a series of symmetrical divisions.

An unusual subclass of lineage mutants was identified, however, in which patterns of cell division were normal, but were displaced along the temporal axis for several lineages [23] (Fig. 2a). Such mutants were termed heterochronic, by analogy to the homeotic mutants of nematodes and insects, which are conversely characterized by spatial transformations in cell fates. In *C. elegans*, the timing of developmental events is measured with reference to the four larval molts that occur between hatching and adulthood, demarcating the four larval stages, termed L1, L2, L3, and L4. The heterochronic phenotype, which can either be precocious or retarded, thus describes a temporal shift of one or more cell lineages relative to the independent beat of the larval molts.

Although the heterochronic pathway regulates the timing of many events in postembryonic development, it is the seam cell lineage that is most commonly used as a readout for the heterochronic phenotype. Seam cells, named for the way they appear to stitch the animal together, are epidermal cells organized into a single row on both the left and right surface of the animal that undergo stage specific patterns of cell division and differentiation [19] (Fig. 2b). The seam cells possess a stem cell-like character. At each larval stage, they undergo a division with the posterior daughter retaining the capacity to divide while the anterior daughter differentiates by fusing to the hyp7 hypodermal syncytium, except during L2 when the division is proliferative and both daughters retain the capacity to divide. This stem cell pattern of division continues until the L4 molt, at which point the seam cells exit the cell cycle, fuse to form a syncytium, and synthesize longitudinal cuticular ridges termed alae, which serve as markers of adult onset (Fig. 2c).

2.1. Early days: lin-4

The first heterochronic mutant to be described was also the first miRNA, *lin-4*, although it would be more than a decade until it was recognized as such [24]. Many but not all cell types in *lin-4* mutant animals reiterate lineage patterns characteristic of the L1 stage at all subsequent stages, thus delaying indefinitely the onset of adult characteristics. The seam cells of *lin-4* animals exhibit L1-like patterns of division at all larval stages, without ever ceasing their division to fuse and produce alae (Fig. 2a). That this phenotype arises from a loss of function mutation suggests that reiterations are a natural part of the underlying structure of development and that mutations that unmask this latent potentiality may play important roles in the evolution of novel traits.

A clue as to the molecular function of *lin-4* came with the description of another mutant, *lin-14*, that exhibited the opposite heterochronic phenotype of *lin-4*, that is, precocious expression of fates characteristic of the L2 stage one stage early, in L1 [23]. For example, seam cells underwent a proliferative division in L1, one stage earlier than normal, thus skipping the L1 wild type pattern of division and fusion to hyp7 (Fig. 2a). Correspondingly, L3 and

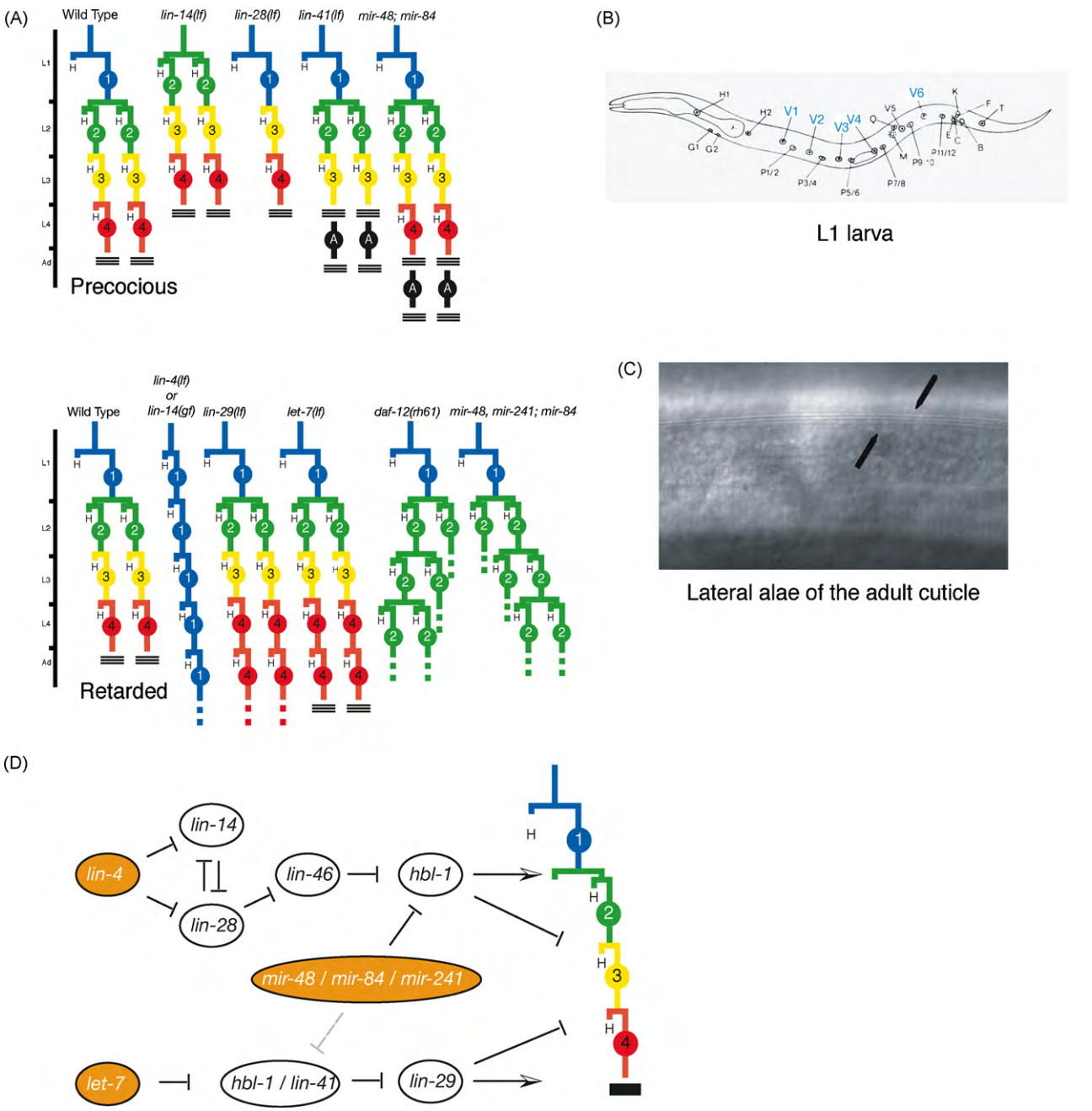

Fig. 2. Abnormal seam cell development in heterochronic mutants. (A) Seam cell (lateral hypodermal V1–V4 cell) lineage of the wild type and various precocious and retarded heterochronic mutants. Cell lineages are diagrammed according to [19]. Numbers indicate that the given cell exhibits the characteristic division pattern of the numbered larval stage. "H" represents fusion to the hyp7 hypodermal syncytium. Exit from the cell cycle and cessation of division is represented by the triple horizontal bars. (B) Drawing of L1 stage animal with seam cells labeled (H1, H2, V1–6, T). (C) Nomarski-DIC image of wild type adult animal with alae in focus (arrowheads). (D) Genetic pathway diagram of the heterochronic pathway. miRNAs highlighted in orange.

L4 were moved up a stage, with seam cells exiting the cell cycle, fusing, and producing alae at the L3 molt. The complementarity of *lin-4* and *lin-14* recessive phenotypes suggested perhaps their gene products had opposing activities in the same genetic pathway.

Furthermore, a semidominant, gain of function allele of *lin-14* was isolated that displayed reiterations in L1 fates nearly identical to what had been observed for *lin-4* mutant animals [23,25] (Fig. 2a). Hence, *lin-14* behaved as a developmental switch, with excess gene activity contributing to repetitions of L1 fates and loss of gene activity leading to L1 being skipped altogether. Gene dosage experiments in which the semidominant *lin-14* allele was put in trans to the null, wild type, or semidominant *lin-14* allele led to increasing phenotypic severity, suggesting that the retarded phenotype of the semidominant allele was caused by elevated levels of *lin-14* [25]. Indeed, cloning of the *lin-14* gene and subsequent antibody staining of the protein product revealed that while wild type LIN-14 was expressed in a temporal gradient, peaking at hatching and greatly diminished by the end of L1 and essentially gone in L2, the semidominant mutant version of LIN-14 persisted throughout larval development into adulthood [26]. This indicated that the reiterations of L1 fates in the semidominant mutant resulted from a failure to downregulate *lin-14* at the end of L1. Consistent with this, temperature shift experiments with a temperature sensitive version of the semidominant allele demonstrated that expression of LIN-14 beyond L1 is sufficient to cause reiteration of the L1 fate in L2 [25].

lin-4 was the natural candidate to coordinate the temporally graded expression of LIN-14. Genetic epistasis analysis showed that a wild type copy of *lin-14* was required to mediate the *lin-4* heterochronic phenotype, indicating *lin-4* lay upstream of *lin-14* [27]. Moreover, the temporally graded protein expression of LIN-14 was eliminated in *lin-4* mutant animals. Instead, LIN-14 persisted into adulthood, mirroring the effect of the semidominant mutant and thus demonstrating a key role for *lin-4* in downregulating *lin-14* at the end of L1 [28]. The major breakthrough, however, came with the cloning of *lin-4*, and the realization that it encoded not a protein, but rather a tiny RNA, only 22 nucleotides in length [17]. Hypothesizing that a regulatory RNA might act via Watson–Crick base pairing to sequences in a target gene, Ruvkun and Ambros [17,18] scanned the *lin-14* gene for sequences complementary to the lin-4 RNA and found several, all located in the 3′UTR of the *lin-14* mRNA. Remarkably, it turned out that it was these complementary sites that were in fact deleted in the semidominant *lin-14* alleles [18]. Ruvkun et al. [18] also showed that the regulation of *lin-14* was posttranscriptional, as LIN-14 protein was downregulated between L1 and L2, but the levels of mRNA remained constant. This finally led to the model that *lin-4* RNA becomes activated towards the end of L1 and binds to complementary sequences in the 3′UTR of *lin-14* to mediate translational repression and ensure a proper transition from L1 to L2.

2.2. Coming of age: let-7

Further study of the heterochronic pathway in *C. elegans* revealed that *lin-4* is only one of several miRNAs that play a role in the temporal control of development. Whereas *lin-4* activity is required for the transition from L1 to L2, another miRNA gene, *let-7*, controls the transition from L4 to adult [29]. *let-7* mutant animals undergo normal development until the L4 molt, however the transition to adulthood is not executed. Seam cells continue dividing, no alae is produced, and even the molting cycle continues, such that a fifth, or supernumerary molt, occurs (Fig. 2a). This defect is at least partially explained by the failure to downregulate the *let-7* target *lin-41* [30]. Much like the genetic relationship between *lin-4* and *lin-14*, *lin-41* mutants display the opposite phenotype of *let-7* (precocious seam cell fusion in L4), are epistatic to mutations in *let-7*, and LIN-41 protein expression is temporally graded so that it is greatly diminished at the end of L4 in a manner that is dependent on *let-7* activity.

Unlike *lin-4*, the discovery of *let-7* had a major impact on the study of miRNAs in other organisms, as its sequence and temporal expression pattern was quickly recognized to be conserved in a multitude of species, spanning ecdysozoa, lophotrochozoa, echinodermata, and chordates [31]. It was not long after this discovery that cloning efforts identified hundreds more miRNAs in fruit flies, mice and humans [32,33]. Interestingly, while it was not detected initially, a *lin-4* ortholog, *mir-125*, was ultimately discovered in *Drosophila* and moreover, shown to be required for larval progression [33,34].

Squeezing their way into this increasingly crowded picture are three more miRNAs: *mir-48*, *mir-84* and *mir-241*, which exhibit sequence similarity to each other and to *let-7* at their 5′ end, in particular nucleotides 2–8, and are thus referred to as let-7 family miRNAs (let-7fam) [35,36]. The significance of 5′ sequence similarity is that this is the region of the miRNA that mediates target recognition [37]; hence miRNAs that share the same 5′ sequence (termed the "seed") can regulate the same set of targets, and therefore may act redundantly to coordinate a particular process. Indeed, prior to the accumulation of mature let-7, the let-7fam miRNAs are coexpressed beginning in L2 and act redundantly to coordinate the L2 to L3 transition [35]. While *mir-48* exhibits a weakly penetrant heterochronic phenotype on its own, the combined knockout of all three let-7fam miRNAs results in reiterations of L2 fates, most notably the proliferative division of seam cell in L2 is reiterated in L3, thus leading to increased seam cell numbers later in development (Fig. 2a). This phenotype is mediated by the direct target *hbl-1*, the *C. elegans* ortholog of the *Drosophila* transcription factor, *hunchback*. Interestingly, *hunchback* seems to play an analogous role in *Drosophila*, integrating spatial cues to drive tissue differentiation through specific gene expression patterns at precise positions in the embryo [38]. In *C. elegans*, *hbl-1* mutants are precocious, skipping the L2 proliferative seam cell division, and capable of suppressing the let-7fam retarded heterochronic phenotype [39,40]. Moreover, the multiple let-7 complementary sites in the *hbl-1* 3′UTR are necessary and sufficient to recapitulate the temporally graded expression of HBL-1 when fused to a GFP reporter, in a manner dependent on the presence of the let-7fam miRNAs [35]. In sum, miRNAs play key roles to coordinate progression through each stage of larval development.

2.3. Posttranscriptional regulation of miRNA activity: lin-28

Much has been learned about the various modes of transcriptional and posttranscriptional regulation of miRNA expression through the study of the heterochronic pathway. One interesting aspect of the heterochronic miRNAs is how individual miRNA–target regulatory cassettes are linked together to produce the sequential larval progression characteristic of *C. elegans* development. *lin-4* downregulation of *lin-14* is largely complete by the end of L1, yet the effect of *lin-4* mutation continues to be felt much later, in the disabling of *let-7* activity and the failure to progress to adulthood. What connects *lin-4* and *lin-14* to *let-7*?

Genetically, another heterochronic gene, *lin-28*, is at the crossroads between early and late development. *lin-28* null mutants are precocious, expressing adult characteristics as early as the L2 molt [23]. Hence, *lin-28* represses the adult fate and, like *lin-14*, must be switched off to permit the larval to adult transition. In fact, the role of *lin-14* in delaying adult onset can be partially explained by its effect on *lin-28*, as the retarded phenotype of the *lin-14* semidominant, *lin-4*-insensitive mutant requires a wild type copy of *lin-28* [27]. Consistent with this, *lin-28* is epistatic to *lin-4* [27]. More recently LIN-14 has been showed to be a transcription factor [41], and this has led to the proposal that one of its functions in the heterochronic pathway is to promote transcription of *lin-28* to repress the adult fate in young larvae [42].

While the downregulation of *lin-14* reduces *lin-28* expression, multiple other regulatory pathways converge on *lin-28* and are collectively required to switch it off [42,43] (Fig. 2d). First, it is clear that *lin-28* is a miRNA target. The *lin-28* 3′UTR contains a single complementary site for *lin-4* and mutation of this site leads to ectopic expression throughout development as well as retarded heterochronic defects [44]. In addition, *lin-28* has a *let-7* site. Given the timing of *lin-28* disappearance, it seems plausible that this site would be accessed by the let-7fam miRNAs. This hypothesis is consistent with phenotypic data from a specific gain of function mutation in the nuclear hormone receptor *daf-12*, known to modulate transcription of the let-7fam miRNAs [45,46]. This mutation, which prevents ligand binding and thus locks DAF-12 into a constitutively repressive transcriptional complex, leads to derepression of *lin-28* and retarded heterochronic defects, possibly due to the loss of let-7fam miRNA-mediated silencing [42,43]. Moreover, this derepression of *lin-28* is mediated entirely through its 3′UTR [42]. However, levels of *lin-28* do not appear to be affected in the let-7fam triple mutant [35], suggesting perhaps a let-7fam-independent effect of *daf-12* on *lin-28*. Another possible role for this *let-7* site in the 3′UTR of *lin-28* is that it is regulated by let-7 itself, discussed in more detail below. Finally, acting in parallel to miRNA regulation is a protein, LIN-66, with an unknown molecular

function but required for *lin-28* silencing through the latter's 3′UTR [42].

Why is it so important to switch off *lin-28* that multiple regulatory inputs must act cooperatively to do the job? The major breakthrough for understanding the role of *lin-28* in repressing adult fates actually came from work outside the *C. elegans* field, by several groups investigating the nature of *let-7* posttranscriptional regulation in mammalian embryonic stem (ES) cells [47–50]. In this system, the *let-7* gene is transcribed, but not processed, accumulating as non-functional pri-miRNA. Only upon differentiation to embryoid bodies is let-7 processed to its mature form. Through biochemical characterization of the repressive pre-let-7 complex in ES cells, lin-28 was identified, and by RNAi knockdown shown to be required to selectively block processing of let-7 into its mature form. Indeed, Lin-28 protein expression is complementary to mature let-7 accumulation, diminishing and ultimately disappearing upon differentiation to embryoid bodies. Remarkably, expression of LIN-28 along with three other pluripotency factors is sufficient to reprogram human somatic cell nuclei to an undifferentiated state, demonstrating the importance of the LIN-28:*let-7* interaction for maintaining stemness [51]. Further work showed that the repression of *let-7* processing is achieved by polyuridylation of the *let-7* precursor by a terminal uridylyl transferase, recruited by LIN-28, which destabilizes the *let-7* precursor by an unknown mechanism [48–50].

Genetic analysis in *C. elegans* has confirmed that LIN-28 acts in a similar fashion to selectively block let-7 processing at the Dicer step during early larval development [52]. Similar to its expression in ES cells, *C. elegans* pri-let-7 is detectable from the L1 stage but the mature form only begins to accumulate in L3, peaking in L4. Precocious *lin-28* mutant animals accumulate *let-7* from L2. Further work demonstrated that LIN-28 repression of processing is mediated by recruitment of a poly(U) polymerase, PUP-2, which catalyzes destabilizing polyuridylation of the let-7 precursor, diverting it away from Dicer processing to a degradation pathway [52]. In sum, LIN-28 and let-7 form an anciently conserved regulatory switch, conserved from nematodes to humans, controlling the progression of development from early to late temporal states.

2.4. Identification of novel miRNA pathway genes

The suite of heterochronic defects represents the strongest, most easily observable postembryonic phenotype associated with complete loss of miRNA function in *C. elegans*. Animals with mutations in the core miRNA biogenesis and effector complex machinery, drosha [53], pasha, dicer [54], and the miRNA effector argonautes alg-1 and alg-2 [55], are viable (but sterile), and display the retarded heterochronic defects associated with loss of *lin-4* or *let-7*. In such animals, embryonic requirement of miRNA activity, of which there are documented cases, are concealed by maternal provision of the wild type allele from heterozygous mothers. The heterochronic phenotype therefore presents a readout for the identification of novel components of the miRNA biogenesis or silencing machinery.

Several components of *C. elegans* miRISC have been discovered with this approach. The GW182 proteins, AIN-1 and AIN-2, are present in distinct RISC complexes with alg-1 and alg-2, and are redundantly required for miRNA silencing activity [56,57]. In particular, a strong effect on let-7fam silencing of *hbl-1* during the L2 to L3 transition was observed, resulting in persistent expression of HBL-1 and reiteration of the proliferative seam cell division in *ain-1*; *ain-2* double mutants [57]. AIN-1 and AIN-2 contribute to gene silencing through the dual mechanisms of translational repression at the initiation stage and mRNA degradation [58]. In the latter case, the GW182 proteins were shown to recruit ALG-1 to P bodies, discrete cytoplasmic foci where mRNAs are decapped and degraded by 5′–3′ exonuclease activity. Importantly, this role of the GW182 proteins in the miRNA pathway is conserved to *Drosophila*, underscoring the evolutionary importance of this function [59,60].

A similar study identified NHL-2 as another component of miRNA effector complexes [61]. The *nhl-2* phenotype resembles that of *ain-1*, and NHL-2 protein localizes to P bodies and coimmunoprecipitates with components of miRISC, although this physical interaction is eliminated by RNase treatment, suggesting NHL-2 may bind distal to the core RISC complex on a translationally repressed mRNA. From a yeast two-hybrid screen, a DEAD-box helicase, CGH-1, was identified as an interacting partner of NHL-2. *cgh-1* mutants exhibit mild heterochronic defects, consistent with the idea that these two proteins coordinate some function to ensure robust miRNA activity, but the molecular means by which they achieve this is currently unknown.

A useful genetic tool for the identification of novel miRNA pathway genes has been hypomorphic alleles of *let-7*, which express mature let-7 at lower levels than wild type. Such a sensitized background can then be used to look for enhancers or suppressors, to identify genes that positively or negatively modulate the miRNA pathway, respectively. The suppressor approach identified an exonuclease, XRN-2, that accelerates the turnover of mature let-7 molecules, thus demonstrating that miRNAs are not as stable as previously thought [62]. Complementing this work, another study performed a genome wide RNAi screen for enhancers of a *let-7* hypomorphic allele and identified a set of intriguing candidate genes, several of which disrupted *lin-14* silencing as well, consistent with a general role in the miRNA pathway [63]. However, the molecular characterization of these genes in the miRNA pathway remains an open challenge.

3. Functions of miRNAs II: left/right neuronal asymmetry

The great majority of miRNA research in *C. elegans* has focused on only the five heterochronic miRNAs, comprising just two miRNA families. However, *C. elegans* encodes more than 100 miRNAs, so there is undoubtedly more fascinating biology yet to be unraveled [64] (Table 1). One other well characterized model of miRNA function during development, however, is the role of the *lsy-6* miRNA in establishing bilateral asymmetry of the *C. elegans* nervous system [65] (Fig. 3).

Consistent with their general body plan, the anatomical structure of the nervous system of bilateria is generally bilaterally symmetric [66]. In contrast to this anatomical symmetry, however, is a subtle lateralization of neuronal function, reflected by expression of laterally specific markers, and it is a major question as to how cells can develop this asymmetry despite beginning life in highly similar microenvironments. As a model system for this phenomenon, Hobert and colleagues have studied the bilateral taste receptor neurons in *C. elegans*, ASEL and ASER, which despite morphological symmetry express unique guanylyl cyclase receptors (GCRs), which correlates with the ability of these neurons to respond to distinct chemosensory cues.

3.1. Specification of the ASEL fate by lsy-6

Like the original discovery of miRNAs in the heterochronic pathway, the identification of the *lsy-6* miRNA in coordinating this process was quite accidental, coming out of a forward genetic screen for mutants in which the lateralization of GCR expression was lost [65]. *lsy-6* mutant animals exhibit the "two ASER" phenotype, in which both neurons express the ASER-specific marker *gcy-5* along with the concomitant loss of the ASEL-specific marker *gcy-7*. *lsy-6* is expressed specifically in ASEL, along with a few other

Table 1
C. elegans miRNA families, with indicated conservation in other species.

Seed	*C. elegans*	*C. briggsae*	*D. melanogaster*	*D. rerio*	Mammal
CCCUGA	lin-4/237	√	√	√	√
UUUGUA	lsy-6	√	×	×	×
GAGGUA	let-7/48/84/241/793–795	√	√	√	√
GGAAUG	miR-1/796	√	√	√	√
AUCACA	mIR-2/43/250/797	√	√		
GGCAGU	miR-34	√	√	√	√
CACCGG	miR-35–42	√	×	×	×
GACUAG	miR-44/45/61/247	√	√	×	×
GUCAUG	miR-46/47	√	√	×	×
AGCACC	miR-49/83	√	√	√	√
GAUAUG	miR-50/62/90	√	×	√	√
ACCCGU	miR-51–56	√	√	√	√
ACCCUG	miR-57	√	×	√	√
GAGAUC	miR-58/80–82	√	√	×	×
CGAAUC	miR-59	√	×	×	×
AUUAUG	miR-60	√	×	×	×
AUGACA	miR-63–66/229	√	×	×	×
CACAAC	miR-67	√	√	√	×
AAUACG	miR-70	√	×	×	×
GAAAGA	miR-71	√	×	×	×
GGCAAG	miR-72–74	√	√	×	√
UAAAGC	miR-75/79	√	√	×	×
UCGUUG	miR-76	√	×	×	×
UCAUCA	miR-77	√	×	×	×
GGAGGC	miR-78	×	×	×	×
ACAAAG	miR-85	√	×	×	×
AAGUGA	miR-86/785	√	×	×	×
UGAGCA	miR-87/233	√	√	×	×
AAGGCA	miR-124	√	√	√	√
AUGGCA	miR-228	√	×	√	√
UAUUAG	miR-230	√	×	×	×
AAGCUC	miR-231/787	√	×	×	×
AAAUGC	miR-232/357	√	√	×	×
UAUUGC	miR-234	√	×	√	√
AUUGCA	miR-235	√	√	√	√
AAUACU	miR-236	√	√	√	√
UUGUAC	miR-238/239a/b	√	√	×	×
ACUGGC	miR-240	√	×	√	√
UGCGUA	miR-242	√	×	×	×
GGUACG	miR-243	×	×	×	×
CUUUGG	miR-244	√	√	√	√
UUGGUC	miR-245	√	×	√	×
UACAUG	miR-246	√	×	×	×
UACACG	miR-248.1	×	×	×	×
ACACGU	miR-248.2	√	×	×	×
CACAGG	miR-249	√	×	×	×
UAAGUA	miR-251/252	√	×	×	×
UAGUAG	miR-253	√	×	×	×
GCAAAU	miR-254	√	×	×	×
AACUGA	miR-255	√	×	×	×
AAUCUC	miR-259	√	√	√	√
UUGUUU	miR-355	√	×	×	×
UUGGUA	miR-358	√	×	×	×
CACUGG	miR-359	√	√	×	×
AUCAUC	miR-392	√	×	×	×
GGCACA	miR-784	√	×	×	×
AAUGCC	miR-786	√	×	√	√
CCGCUU	miR-788	√	×	×	×
CCCUGC	miR-789-1/-2	√	×	×	×
UUGGCA	miR-790/791	√	√	√	√
UGAAAU	miR-792	√	×	√	×
AAGCCU	miR-798	×	×	×	×
GAACCC	miR-799	×	×	×	×
AAACUC	miR-800	×	×	×	×

Table is compiled with data from miRBase (http://www.mirbase.org/) [101–104].

neurons, and misexpression of *lsy-6* in both ASEL and ASER using a heterologous promoter led to the opposite "two ASEL" phenotype, demonstrating that *lsy-6* is both necessary and sufficient to specify the ASEL fate. This specification is accomplished through the targeting of the *cog-1* transcription factor, which normally acts in ASER to repress the ASEL-specific transcription factor *lim-6*, which in turn represses *gcy-5*.

3.2. Specification of the ASER fate by mir-273

This *lsy-6*:*cog-1* interaction specifying the ASEL fate is matched by a second miRNA:target interaction operating in ASER and required to establish its fate. Evidence suggests the ASER-specific miRNA miR-273 represses the normally ASEL-specific transcription factor *die-1*, a positive regulator of *lim-6* [67], although it should be

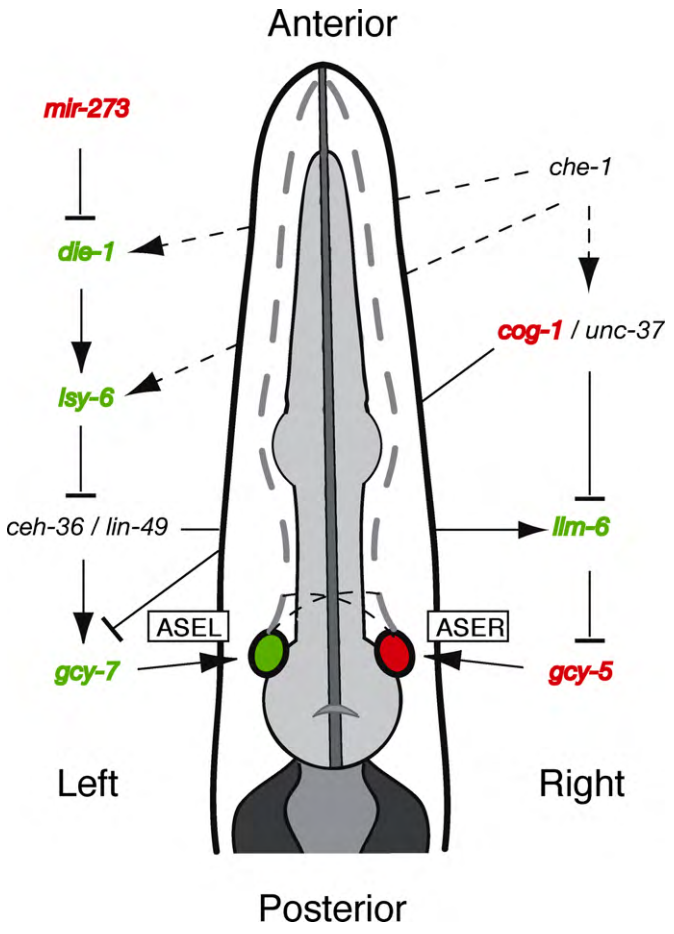

Fig. 3. Summary of left/right specification of ASE neuron. Genes required for ASEL fate are in green, genes required for ASER fate are in red.

noted that sequencing efforts have thus far failed to detect miR-273 from whole worm lysates [64]. Remarkably, both miRNA–target interactions are integrated into a complex double-negative feedback loop, as DIE-1 and COG-1 are required to positively regulate the transcription of *lsy-6* in ASEL and *mir-273* in ASER, respectively [67,68]. Such a circuit constitutes a highly sensitive generator of bistable cell fates, as an imbalance in any one component of the circuit will be amplified through positive feedback and lead inexorably to one of the two fates. For example, a slight increase in *lsy-6* expression would alleviate *die-1* silencing, via the silencing of *cog-1* and resultant loss of *mir-273* expression, and therefore promote *lsy-6*'s own expression. Indeed, both ASE neurons appear to pass through a hybrid state on their way to being specified, in which both ASER and ASEL markers are coexpressed [68]. Despite the understanding of the roles of miRNAs in this system to generate and maintain neuronal asymmetry, it is still unknown how the initial symmetry embodied by the hybrid state is broken, although it has been shown to require an anterior–posterior asymmetry present as early as the four-cell embryo [69].

3.3. Components of regulatory networks: miRNAs and double-negative feedback

The *lsy-6-mir-273* double-negative feedback loop represents a complex example of a fairly commonplace motif in gene regulatory networks, crucially important for developmental decision-making. Double-negative feedback, in which two regulatory components downregulate one another, can guarantee (depending on the downstream regulatory potential of the components involved) that a given biological system may only occupy one state at a particular point in time and space. This feature is critical to give structural integrity to development, to overcome the fluctuations and variability inherent in biological systems and the environments they occupy, and to provide a reasonably invariant blueprint upon which natural selection can act.

miRNAs, as abundant and widespread negative regulators of gene expression, are prime candidates to operate within the negative feedback structure and play key roles in developmental transitions. We have already seen the importance of *let-7* in the larval to adult transition, but it is likely that this transition is guaranteed and made irreversible by negative feedback of *let-7* on *lin-28*, through the *let-7* site in the *lin-28* 3′UTR, as noted above. Indeed, this negative feedback loop has been confirmed in a neural stem cell model, in which both let-7 and the mouse *lin-4* ortholog, *mir-125*, are released from Lin-28 inhibition upon neural differentiation and cooperate to downregulate Lin-28 [70]. The targeting of *lin-41* by *let-7* in the heterochronic pathway may also be a form of double-negative feedback, as the murine ortholog of *lin-41* was recently shown to be a stem cell specific E3 ubiquitin ligase and mediates polyubiquitylation of the core miRISC component Ago2 [71]. Finally, the nuclear hormone receptor *daf-12*, which under conditions of environmental stress represses transcription of the let-7fam miRNAs and promotes entry into the developmentally arrested dauer phase, also seems to be targeted by the let-7fam miRNAs in L3 [46]. This may partly explain why dauer entry is only possible up until the L2 molt. In addition to work in *C. elegans*, several examples of negative feedback involving miRNAs have been described in other organisms [72–74].

Extending these results for other mRNAs in *C. elegans*, Walhout and colleagues sought to place each intergenic miRNA into a transcriptional network by using yeast one-hybrid (Y1H) screening to identify transcription factor:miRNA promoter interactions [75]. Then, using miRNA target prediction, 23 potential feedback loops were identified, double the number expected by chance. These loops await *in vivo* confirmation, but they suggest the widespread importance and dynamism of miRNAs in coordinating developmental changes and provide an entry point to investigate the *in vivo* function of the many miRNAs that remain uncharacterized.

3.4. Components of regulatory networks: target recognition

Understanding the biological function of miRNAs by placing them within regulatory networks not only involves determination of the transcriptional inputs into their expression but also requires accurate target identification (Table 2). Indeed, this latter approach would seem to be easier, since miRNAs recognize their targets through Watson–Crick base pairing to their 5′ end (the "seed"), suggesting it might be possible for simple computational identification of miRNA targets. Using genome alignments of *C. elegans* with closely related nematode species, bioinformaticians have done precisely this, predicting miRNA targets by identifying conserved seed matches [76].

Unfortunately, this straightforward *in silico* approach falls short of identifying functional targets *in vivo*. Making use of their internally controlled ASER/ASEL reporter assay developed to prove that *cog-1* was a bona fide *lsy-6* target, Didiano and Hobert swapped in 3′UTRs of computationally predicted targets into their assay and asked whether they conferred *lsy-6*-dependent silencing of a GFP transgene in ASEL but not in ASER [77]. Unlike the *cog-1* 3′UTR, 0/13 tested predictions were repressed. Even more, the *cog-1* site itself was insufficient to confer regulation if taken out of context and placed in the normally unregulated *unc-54* 3′UTR, suggesting that the surrounding sequence context is critical to confer functionality to a potential miRNA site. Further studies analyzing

Table 2
Validated miRNA:target interactions in *C. elegans*.

microRNA	Target(s)	Suppression?[a]	Sensor/antibody?[b]	Mutated site?[c]	References
lin-4	*lin-14*	Y	Y	Y	[18]
	lin-28	Y	Y	Y	[44]
let-7	*lin-41*	Y	Y	Y	[30]
	let-60	Y	Y	N	[99]
	daf-12	Y	Y	N	[100]
mir-48/84/241	*hbl-1*	Y	Y	N	[35]
	daf-12	N	Y	N	[46]
lsy-6	*cog-1*	Y	Y	Y	[65]
mir-273	*die-1*	N	N	Y	[67]
mir-1	*unc-29*	N	Y	N	[93]
	unc-63	N	Y	N	[93]
	mef-2	Y	Y	N	[93]
mir-61	*vav-1*	N	N	Y	[94]
mir-51-6	*cdh-3*	N	Y	Y	[98]

[a] Loss of function mutation or RNAi knockdown in predicted target suppresses miRNA mutant phenotype.
[b] Observed increased expression of protein or GFP transgene containing target 3′UTR in miRNA mutant animals relative to wild type.
[c] Observed increased expression of sensor transgene when predicted miRNA target site was mutated.

the *lsy-6*:*cog-1* [78] interaction or the *let-7*:*lin-41* [79] interaction have reached similar conclusions regarding the importance of a permissive sequence context and the insufficiency of a single conserved seed match to confer silencing. Such additional sequence may bind an additional factor needed to cooperate with the miRNA machinery for robust repression, or alternatively, may simply alter the secondary structure of the 3′UTR such that the site becomes exposed to the miRNA. Consistent with the latter model, the accessibility, as predicted by RNA folding software, of a *let-7* site moved to various positions in the *lin-41* 3′UTR correlates with sensitivity to *let-7* regulation, and this feature has been used to design a more accurate target prediction algorithm for general use [80].

Another approach to finding miRNA targets is to not rely on prediction at all, but to use high-throughput methods to empirically identify which genes are regulated by a given miRNA and then determine a posteriori what shared features of these targets may be functionally important for silencing. This can be done biochemically by immunoprecipitating miRISC and identifying bound miRNAs and mRNA by pyrosequencing [57]. Alternatively, one could simply compare total mRNA expression profiles in wild type versus a mutant for a particular miRNA and look for genes that are upregulated in the mutant [81]. Although miRNA:target interactions were originally thought not to affect mRNA stability, many subsequent studies in *C. elegans* and other species have disputed this [8,82–84]. Importantly, a large-scale quantitative study from mice analyzing proteome and transcriptome changes in a miRNA mutant found that mRNA destabilization constituted the major component of miRNA-mediated repression, vindicating the use of microarrays and other mRNA profiling methods to identify miRNA targets [85]. However, unlike the biochemical approach, profiling cannot distinguish between direct or indirect targets of miRNA activity and thus still relies upon prediction software to make this distinction.

What has come out of such empirical studies is that, while not perfect, target prediction generally works. Predicted targets are enriched in immunoprecipitated miRISC complexes, or in the class of genes that are derepressed in a miRNA mutant [57,81]. What is perhaps surprising, however, is that these experimental approaches confirm the very widespread changes in gene expression previously implied by the high number of targets predicted by software, typically in the hundreds. This is hard to reconcile with the genetic data in *C. elegans*, in which the best-characterized miRNAs exert profound, switch-like effects on only one or a few targets. Moreover, the quantified changes to target expression measured by such methods are curiously slight, typically less than twofold [81]. This then poses the question to what extent are these broad but slight changes in gene expression biologically significant? Indeed, the fold changes induced by miRNA mutation are less than intraindividual variation in protein expression, yet this variation is well tolerated by natural populations [86,87]. Moreover, few animal genes are haploinsufficient [88], suggesting that biological systems are robust and can tolerate twofold changes in expression because most genes are integrated into complex networks that buffer natural fluctuations.

One intriguing hypothesis that would explain the evolutionary conservation of this widespread, low-level target repression is that these interactions function to regulate the miRNA itself, and not the other way around [86]. Indeed, the use of miRNA “sponges”, transcripts driven by strong promoters containing tandemly repeated target sites for a miRNA of interest, as competitive inhibitors of miRNA activity in cell-based systems and whole organisms suggests that it is indeed possible to calibrate miRNA function by adjusting the number of target sites it can access [89–91]. Consistent with this model, the efficacy of target knockdown following miRNA or siRNA transfection into cultured cells correlates with the total concentration of available target transcripts [92]. Given these results, it is possible to interpret the large number of miRNA targets not as reflecting the biology of the miRNA, but rather as a collective means of miRNA regulation. Under this interpretation, the biologically relevant targets are therefore those that are particularly sensitive to a twofold decrease in protein expression. Indeed, some of the best-characterized miRNA targets in *C. elegans*, including *lin-14*, *lin-41*, and *cog-1* are very sensitive to dosage [86].

The preceding discussion underscores the importance of careful genetic analysis for understanding miRNA biology and biology in general. Only a genetic experiment can determine whether or not an organism is sensitive to a slight change in the expression of a given gene. Perhaps this is why the best examples of miRNA–target interactions were initially identified by suppression or phenocopy of the miRNA mutant phenotype, while attempts to identify similar switch-like targets by mRNA profiling have been largely unsuccessful. Although a slow process, genetics provides our most effective tool for unraveling miRNA biology and future work on miRNA knockout mutants promises to enrich our understanding of how miRNAs function during development.

4. Functions of miRNAs III: other miRNAs of *C. elegans*

At the moment, only a few of the 115 *C. elegans* miRNAs have ascribed functions. In addition to those mentioned above, the muscle-specific miRNA *mir-1* has been shown to modulate acetylcholine secretion and receptor function at neuromuscular junctions [93], while *mir-61*, when misexpressed, can transform the fates of particular vulval precusor cells, suggesting a possible role in vulval fate specification [94].

In order to expand our understanding of the function of other miRNAs in *C. elegans*, two important resources have been developed: a comprehensive library of miRNA knockout strains [95] and an atlas of miRNA spatiotemporal expression patterns using promoter-GFP fusion transgenes [96]. Somewhat disappointingly, superficial examination of the miRNA single gene deletion mutants led to the observation that most miRNAs are not individually essential for development or viability [95]. One explanation for this may be that many miRNAs act redundantly within families that share the same seed sequence, and thus only removal of the entire family would reveal a phenotype, as is the case for the *let-7* family. Indeed, complete knockouts of the *mir-35* or *mir-51* families are embryonic lethal, with the latter family also exhibiting a specific failure to attach the pharynx to the mouth [97,98]. However, the majority of complete family knockouts (12/15) do not exhibit any gross abnormalities, suggesting more subtle biological roles for the majority of *C. elegans* miRNAs [97].

5. Conclusions

The large number of still uncharacterized miRNAs presents an open challenge to *C. elegans* biologists. Despite the availability of knockout mutants for nearly every miRNA for over 4 years, the lack of described abnormal phenotypes makes this a difficult start for genetic analysis. *C. elegans* biologists who study a particular biological process could exploit the wealth of miRNA resources at their disposal, including mutants, expression data, lists of predicted targets and transcription factor:miRNA promoter interaction data, to develop testable hypotheses that a particular miRNA is involved in a biological process of interest. If the experience with the heterochronic miRNAs is any indication, there is much fascinating biology waiting to be uncovered.

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