Recent Molecular Genetic Explorations of *Caenorhabditis elegans* MicroRNAs

Victor Ambros*,[1] and Gary Ruvkun†,‡
*Program in Molecular Medicine; University of Massachusetts Medical School, Worchester, Massachusetts 01605, †Department of Genetics, Harvard University, Boston, Massachusetts, and ‡Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

ABSTRACT MicroRNAs are small, noncoding RNAs that regulate gene expression at the post-transcriptional level in essentially all aspects of *Caenorhabditis elegans* biology. More than 140 genes that encode microRNAs in *C. elegans* regulate development, behavior, metabolism, and responses to physiological and environmental changes. Genetic analysis of *C. elegans* microRNA genes continues to enhance our fundamental understanding of how microRNAs are integrated into broader gene regulatory networks to control diverse biological processes, including growth, cell division, cell fate determination, behavior, longevity, and stress responses. As many of these microRNA sequences and the related processing machinery are conserved over nearly a billion years of animal phylogeny, the assignment of their functions via worm genetics may inform the functions of their orthologs in other animals, including humans. *In vivo* investigations are especially important for microRNAs because *in silico* extrapolation of their functions using mRNA target prediction programs can easily assign microRNAs to incorrect genetic pathways. At this mezzanine level of microRNA bioinformatic sophistication, genetic analysis continues to be the gold standard for pathway assignments.

KEYWORDS *Caenorhabditis elegans*; microRNA; Argonaute; miRISC; mutant phenotypes; WormBook

TABLE OF CONTENTS

doi: https://doi.org/10.1534/genetics.118.300291
Manuscript received February 21, 2017; accepted for publication April 30, 2018.
[1]Corresponding author: University of Massachusetts, 373 Plantation St., Biotech II - Suite #306, Worchester, MA 01605. E-mail: victor.ambros@umassmed.edu

Overview

HERE, we discuss the current understanding of how microRNAs function in *Caenorhabditis elegans*. While striving to be as comprehensive as possible, we will emphasize the contexts in which research using *C. elegans* has provided unique insight into evolutionarily conserved aspects of microRNA biology. We will also highlight where worm microRNA research motivates interesting, unanswered questions and potentially fertile opportunities for future research.

Genetic Analysis of *C. elegans* MicroRNA Function

Much of what is known about microRNA function in *C. elegans* is derived from studies of microRNA gene mutants (Table 1, Table 2, and Table 3). Forward genetic screens identified *lin-4* and *let-7* based on the developmental abnormalities caused by single-gene knockout mutations (Chalfie *et al.* 1981; Ferguson and Horvitz 1985; Reinhart *et al.* 2000). Discovery of *lin-4* and *let-7* mutations with visible phenotypes enabled the identification of the gene products of *lin-4* (Lee *et al.* 1993) and *let-7* (Reinhart *et al.* 2000) as microRNAs: short, 21–22 nt RNAs processed from longer hairpin precursors. Classical genetic analysis (rather than the more promiscuous genome-scale mRNA target prediction programs) was also used to assign these microRNA genes to genetic pathways. Phenotype suppression genetics or epistasis analysis enabled the discovery of protein-coding mRNA targets of these microRNAs (Ambros 1989; Reinhart *et al.* 2000; Slack *et al.* 2000). These genetically discovered target mRNAs bore complementarity to the upstream microRNA (Lee *et al.* 1993; Wightman *et al.* 1993) and were regulated at the level of translation (Wightman *et al.* 1993; Olsen and Ambros 1999; Stadler *et al.* 2012) or mRNA stability (Bagga *et al.* 2005). Dozens of other microRNA genes in *C. elegans* were subsequently identified by cDNA cloning (Lau *et al.* 2001; Lee and Ambros 2001). Their functions were tested by generating strains that were singly or multiply mutant for these microRNAs (Miska *et al.* 2007). These reverse genetics studies led to the realization that microRNAs, with *lin-4* and *let-7* being notable exceptions, often function redundantly with members of the same microRNA family (Abbott *et al.* 2005) or other microRNA families (Brenner *et al.* 2010).

The findings from *C. elegans* genetics studies suggest a classification of microRNAs into two broad functional classes. One class includes *lin-4* and *let-7*, which control developmental switches, where a single major microRNA regulates the expression of a single major target. Single-gene mutations in these microRNAs cause visible phenotypes. The second class encompasses most of the other *C. elegans* microRNAs and exerts redundant and/or conditional functions in the context of developmental or physiological robustness. These microRNAs generally act in conjunction with other microRNAs and can act on multiple targets.

Heterochronic microRNAs and larval development

The first microRNAs to be identified were the products of the *C. elegans* genes *lin-4* (Lee *et al.* 1993) and *let-7* (Reinhart *et al.* 2000). These microRNAs emerged from classical Mendelian genetic analysis of strains that had relatively rare recessive mutations, and exhibited visible defects in egg laying or developmental timing (or heterochrony) (Chalfie *et al.* 1981; Ambros and Horvitz 1984, 1987). For example, *lin-4*(*e912*) was identified by its unusual adult morphology and egg-laying defects in homozygous, mutant hermaphrodites. The primary targets of *lin-4* and *let-7* were identified as the protein-coding genes *lin-14* and *lin-41*, respectively, by genetic epistasis and by examining their roles in developmental timing (Ambros 1989). For example, *lin-14* loss-of-function (*lf*)

Table 1 Genetically-defined functions of *C. elegans* microRNA genes

Conserved family	MicroRNA	Function	Target(s)	References
mir-125	*lin-4*	Developmental timing	*lin-14*; *lin-28*	Chalfie *et al.* (1981)[a] Ambros (1989)[a] Lee *et al.* (1993); Moss *et al.* (1997)
		Postdauer developmental timing	*hbl-1*	Karp and Ambros (2012)[a]
		Dauer formation	*lin-14*	Liu and Ambros (1989)[a]
		Vulva fate patterning	*lin-14*	Li and Greenwald (2010)[a]
		HSN axon extension	*lin-14*; *lin-28*	Olsson-Carter and Slack (2010)[a]
		Axon guidance	*lin-14*	Zou *et al.* (2012)[a]
		Life span	*lin-14*	Boehm and Slack (2005)[a]
		Energy homeostasis	*lin-14*	Dowen *et al.* (2016)[a]
	mir-237	Radiation sensitivity	*jun-1*	Metheetrairut *et al.* (2017)[a]
let-7 family	*let-7*	Developmental timing	*lin-41*; *hbl-1*; *daf-12*	Reinhart *et al.* (2000)[a], Slack *et al.* (2000); Abrahante *et al.* (2003), Lin *et al.* (2003); Grosshans *et al.* (2005)
		Hypodermal cell fate, vulva integrity	*opt-2*; *prmt-1*; *T27D12.1*; *lin-41*	Reinhart *et al.* (2000)[a], Hunter *et al.* (2013); Hunter *et al.* (2013); Hunter *et al.* (2013); Slack *et al.* (2000), Ecsedi *et al.* (2015)
		Axon regenerative capacity	*lin-41*	Zou *et al.* (2013)[a]
		Nucleolar size	*ncl-1*	Yi *et al.* (2015)[a]
		Life span	*akt-1/2*	Ren and Ambros (2015)[a], D. Wang *et al.* (2017)
		Survival on *P. aeruginosa*	*sdz-24*	Ren and Ambros (2015)[a], Zhi *et al.* (2017)
		Energy homeostasis	*lin-41*	Dowen *et al.* (2016)[a]
	mir-84	Motor neuron connectivity	*hbl-1*	Thompson-Peer *et al.* (2012)[a]
	let-7, *mir-84*	Molting cycle exit	*nhr-23*; *nhr-25*	Hayes *et al.* (2006)[a]
		Vulva integrity	*let-60*	Johnson *et al.* (2005)[a]
	mir-48, *mir-84*, *mir-241*	Developmental timing	*hbl-1*; *daf-12*	Abbott *et al.* (2005)[a]; Hammell *et al.* (2009a)
		Dauer formation	*daf-12*; *hbl-1*	Hammell *et al.* (2009a)[a]; Karp and Ambros (2011)
		Life span		Ren and Ambros (2015)[a]
		Survival on *P. aeruginosa*	*skn-1*	Liu *et al.* (2013)[a], Ren and Ambros (2015)
	lsy-6	ASE left/right specification	*cog-1*	Johnston and Hobert (2003)[a]

Where there is more than one target and more than one reference, references are listed in the order of the targets in the preceding column. HSN, hermaphrodite-specific neuron.
[a]Denotes the reference(s) that first reported the function.

mutations cause precocious expression of L2 and later cell fates, which is in contrast to the reiterated L1 phenotype of *lin-4*(*lf*). Importantly, in double mutants, *lin-14*(*lf*) suppresses *lin-4*(*lf*) phenotypes, consistent with a role of *lin-4* in repression of *lin-14* activity to control transitions from L1 to later cell fates. Similarly, *lin-41*(*lf*) causes precocious adult fates, while *let-7*(*lf*) causes reiteration of the L4 and delay of adult fates. Moreover, *lin-41*(*lf*) is epistatic to *let-7*(*lf*), consistent with negative regulation of *lin-41* by *let-7* (Slack *et al.* 2000).

The identification of *lin-14* as the direct target of *lin-4* originally emerged from the discovery of evolutionarily conserved base-pairing complementarity between *lin-4* and *lin-14* 3′-UTR sequences (Lee *et al.* 1993; Wightman *et al.* 1993). Likewise, there are conserved sites complementary to *lin-4* in the 3′-UTR of another heterochronic gene target, *lin-28* (Moss *et al.* 1997), and sites complementary to *let-7* in the 3′-UTR of its direct target *lin-41* (Slack *et al.* 2000). The pattern of predicted base pairing of *lin-4* and *let-7* to their respective targets is characterized by conserved complementarity of the 5′ nucleotides of the microRNA. In particular, nucleotides 2–8, now named the "seed" region, demonstrate significant conservation, with incomplete, variable pairing in the more 3′ regions of the microRNA, especially in the case of *let-7* and its mRNA targets. This foreshadowed the principle of seed-pairing that is now recognized as an organizing principle of animal microRNA function and evolution.

The realization that the *let-7* microRNA sequence is deeply conserved across animal phylogeny, including in humans, (Pasquinelli *et al.* 2000) triggered a search for other microRNAs in *C. elegans*, (Lau *et al.* 2001; Lee and Ambros 2001) in *Drosophila*, and in mammalian cells (Lagos-Quintana *et al.* 2001). The advent of protocols for the specific prospecting of 20–25-nt RNAs and deep sequencing technologies suited for short-insert libraries enabled the rapid expansion of microRNAs from a *C. elegans* cottage industry to a global effort, encompassing essentially all plant and animal experimental systems. It soon became clear that, in addition to *let-7*, many microRNAs are evolutionarily conserved, with highly conserved seed regions that define families of microRNA genes of common evolutionary origin. It also suggested that the seed region comprises a functional domain of microRNAs that is

Table 2 Genetically-defined functions of *C. elegans* microRNA genes

Conserved family	MicroRNA	Function	Target(s)	References
mir-1	*mir-1*	Gonadal morphogenesis		Brenner *et al.* (2010)[a]
		Synaptic function	*unc-29*; *unc-63*; *mef-2*	Simon *et al.* (2008)[a]
mir-34	*mir-34*	Dauer formation	*daf-16*	Isik *et al.* (2016)[a]
		Gonadal morphogenesis	*cdc-42*; *pat-3*	Burke *et al.* (2015)[a]
		Life span	*atg-9*	Yang *et al.* (2013)[a]
		Heat and oxidative stress resistance		Yang *et al.* (2013)[a]
nc	*mir-35-42*	DNA damage response		Kato *et al.* (2009)[a]
		Embryonic development		Alvarez-Saavedra and Horvitz (2010)[a]
		Developmental apoptosis	*egl-1*	Sherrard *et al.* (2017)[a]
		Fecundity	*sup-26*	McJunkin and Ambros (2014)[a]
		Sex determination	*nhl-2*; *sup-26*	McJunkin and Ambros (2017)[a]
		Embryonic hypoxic stress resistance	*sup-26*	Kagias and Pocock (2015)[a]
mir-100	*mir-51-56*	Pharyngeal development	*cdh-3*	Shaw *et al.* (2010)[a], Alvarez-Saavedra and Horvitz (2010)[a]
		Regulation of microRNA activity		Brenner *et al.* (2012)[a]
nc	*mir-57*	Posterior patterning	*nob-1*	Zhao *et al.* (2010)[a]
		Embryonic viability		Brenner *et al.* (2012)[a]
mir-58 family (bantam in *Drosophila*)	*mir-58*, *mir-80-82*, *mir-1834*, *mir-2209*	Developmental apoptosis	*egl-1*	Sherrard *et al.* (2017)[a]
		Dauer formation	*daf-1*; *daf-4*; *sta-1*	Alvarez-Saavedra and Horvitz (2010)[a], de Lucas *et al.* (2015); de Lucas *et al.* (2015); Lozano *et al.* (2016)
		Body size	*dbl-1*; *sma-6*; *daf-4*;	Alvarez-Saavedra and Horvitz (2010)[a]; de Lucas *et al.* (2015); de Lucas *et al.* (2015)
		Timing of egg laying		Alvarez-Saavedra and Horvitz (2010)[a]
		Locomotion		Alvarez-Saavedra and Horvitz (2010)[a]
		Life span	*cbp-1*	Vora *et al.* (2013)[a]
		Tissue specificity of immune response	*pmk-2*	Pagano *et al.* (2015)[a]
nc	*mir-59*	Embryonic viability		Brenner *et al.* (2012)[a]
		Adult viability		Brenner *et al.* (2012)[a]
		Gonadal morphogenesis		Brenner *et al.* (2012)[a]

Where there is more than one target and more than one reference, references are listed in the order of the targets in the preceding column.
[a]Denotes the reference(s) that first reported the function.
In the first column, nc denotes microRNAs that are not members of well conserved seed families.

primarily responsible for the specificity of microRNA–target recognition. Although certain microRNAs, exemplified by *let-7* (Pasquinelli *et al.* 2000), are well conserved over their entire ~22 nt length, other conserved microRNAs, such as *lin-4* (*mir-125* in other animals) preserve only the seed region. This suggests that certain microRNAs have been under more complex evolutionary constraints than others. However, the nature of these constraints is still not understood.

lin-4 and *let-7* regulate a range of stage-specific developmental events across diverse tissues, and the phenotypes of *lin-4* or *let-7* mutants include altered timing of expression of stage-specific genes (Liu *et al.* 1995; Slack *et al.* 2000). GFP reporters driven by the promotor of the adult-specific collagen gene *col-19* have been used to screen for heterochronic mutants, and to quantify the expression of precocious and retarded hypodermal adult fates (Abrahante *et al.* 2003). In addition, stage-specific expression of yolk proteins and other energy carriers by the intestine, and their transport to the germline upon the initiation of adulthood, is of particular significance to reproduction. This program of intertissue transport of energy reserves from the soma to the germline is regulated by *lin-4* and *let-7*, acting via downstream heterochronic genes in the hypodermis (Dowen *et al.* 2016).

Heterochronic microRNA pathways impact development of the vulva; for example, *lin-4* is required for the proper expression of the Vulval Precursor Cell (VPC) fate in the L2 stage (Chalfie *et al.* 1981; Euling and Ambros 1996), for the specification of certain VPC progeny cell fates (Li and Greenwald 2010), and *let-7* is critical for the proper morphogenesis and structural integrity of the vulva (Johnson *et al.* 2005; Ecsedi *et al.* 2015).

Additional microRNAs function within the complex signaling networks that regulate vulval cell fate specification; for example, *lin-12*/Notch signaling in presumptive vulval secondary cells triggers the expression of *mir-61*, which in turn represses *vav-1*, a Vav oncogene ortholog that opposes *lin-12* activity (Yoo and Greenwald 2005). Thus, *mir-61* functions in a feedback loop with *lin-12* and *vav-1* to reinforce the specification of secondary vulval fates.

Functional redundancy within microRNA seed families

The assignment of mRNA targets to microRNAs identified by deep sequencing of animal small RNAs has been haunted by the hundreds of potential targets predicted by computational approaches (Lewis *et al.* 2005; Agarwal *et al.* 2015). The loops and base mismatches characteristic of genetically discovered and validated microRNA–mRNA interactions (Wightman

Table 3 Genetically-defined functions of *C. elegans* microRNA genes

Conserved family	MicroRNA	Function	Target(s)	References
nc	*mir-60*	Oxidative stress	*zip-10*	Kato *et al.* (2016)[a]
nc	*mir-61*	Vulva development	*vav-1*	Yoo and Greenwald (2005)[a]
	mir-64-66, mir-229	Heat stress		Nehammer *et al.* (2015)[a]
nc	*mir-67*	Avoidance of *P. aeruginosa*	*sax-7*	Ma *et al.* (2017)[a]
	mir-70	Survival on *P. aeruginosa*		Kudlow *et al.* (2012)[a]
mir-71	*mir-71*	L1 diapause survival	*age-1*; *unc-31*	Zhang *et al.* (2011)[a]
		Post L1 diapause developmental timing	*hbl-1*; *lin-42*	Zhang *et al.* (2011)[a]
		AWC left/right specification	*tir-1*	Hsieh *et al.* (2012)[a]
		Life span		de Lencastre *et al.* (2010)[a], Boulias and Horvitz (2012)
		Heat stress		Nehammer *et al.* (2015)[a]
nc	*mir-73-74*	Adult viability		Brenner *et al.* (2010)[a]
nc	*mir-79*	Neuronal migration	*sqv-5*; *sqv-7*	Pedersen *et al.* (2013)[a]
mir-29	*mir-83*	Gonadal morphogenesis	*cdc-42*; *pat-3*	Brenner *et al.* (2010)[a], Burke *et al.* (2015); Burke *et al.* (2015)
mir-124	*mir-124*	Dauer formation		Than *et al.* (2013)[a]
		Gonadal morphogenesis		Brenner *et al.* (2010)[a]
nc	*mir-228*	Embryonic viability		Brenner *et al.* (2010)[a]
nc	*mir-233*	Survival on *P. aeruginosa*	*sca-1*	Dai *et al.* (2015)[a]
nc	*mir-234*	Dauer formation		Than *et al.* (2013)[a]
mir-92	*mir-235*	Adult viability		Brenner *et al.* (2010)[a]
		L1 diapause arrest	*nhr-91*	Kasuga *et al.* (2013)[a]
nc	*mir-238*	Nicotine signaling	*acr-19*	Rauthan *et al.* (2017)[a]
nc		Life span		de Lencastre *et al.* (2010)[a]
nc	*mir-239*	Life span		de Lencastre *et al.* (2010)[a]
nc	*mir-246*	Life span		de Lencastre *et al.* (2010)[a]
nc	*mir-251*, *mir-252*	Survival on *P. aeruginosa*		Kudlow *et al.* (2012)[a]
nc	*mir-259*	Gonadal morphogenesis		Brenner *et al.* (2010)[a]
	mir-273	ASE left/right specification	*die-1*	Chang *et al.* (2004)[a]
mir-365	*mir-786*	Defecation cycle length	*elo-2*	Miska *et al.* (2007)[a], Kemp *et al.* (2012)
	mir-791	CO_2 sensing	*akap-1*[a]; *cah-3*[b]	Drexel *et al.* (2016)[a]

Where there is more than one target and more than one reference, references are listed in the order of the targets in the preceding column.
[a]Denotes the reference(s) that first reported the function.
In the first column, nc denotes microRNAs that are not members of well conserved seed families.

et al. 1993; Ha *et al.* 1996; Slack *et al.* 2000; Ecsedi *et al.* 2015) confound the accurate prediction of animal microRNA targets. By contrast, plant microRNAs, which generally perfectly base pair along their entire 21–24 nt to target mRNAs, can be easily assigned to particular mRNA targets, and hence to particular pathways (Rhoades *et al.* 2002). The genome-wide identification of *C. elegans* microRNAs, many of which, like *lin-4* and *let-7*, are also evolutionarily conserved, suggested that the functions of these microRNAs have been under strong selection for the billion-year history of animals. It was assumed that such conserved microRNAs were likely to have conserved functions that could be revealed by genetic analysis in *C. elegans*. Surprisingly, most microRNA single-gene mutants, including for those that are conserved in phylogeny, displayed no readily evident phenotypes (Miska *et al.* 2007). Therefore, *lin-4* and *let-7* were essentially the only *C. elegans* microRNA genes for which single-gene mutations caused visible phenotypes, which partially explains why only these two microRNA genes had been previously cloned from genetically identified loci [although the nonconserved *lsy-6* microRNA and its target mRNA *cog-1* did emerge from genetic analysis of neural development (Johnston and Hobert 2003)].

For some single-microRNA gene mutants, the lack of visible phenotypes can be attributed to genetic redundancy among microRNAs of the same seed family. In a systematic genetic analysis of 15 of the 23 microRNA families in *C. elegans* (Alvarez-Saavedra and Horvitz 2010), mutant strains were generated that lacked most or all members of a given microRNA seed family. For 12 of these families, full family knockout caused no strong observable synthetic phenotypes. For two families, the *mir-35* family (*mir-35-42*) and the *mir-51* family (*mir-51-56*), synthetic embryonic arrest phenotypes resulted from knockout of the entire family, and for the *mir-58* family (*mir-58.1*, *-58.2*, *-80, -81, -82, -1834, -2209a, -2209b,* and *2209c*), deletion of multiple paralogs caused a complex syndrome of morphological and behavioral defects (Alvarez-Saavedra and Horvitz 2010).

Similarly, animals multiply-mutant for the *let-7* paralogs (*mir-48*, *mir-84*, and *mir-241*) exhibit heterochronic phenotypes characterized by repetition of the L2 cell fate programs (Abbott *et al.* 2005). By examining other combinations of mutations in the *let-7* family microRNAs, other developmental timing functions for this family emerged. These functions include the regulation of the timing of exit from the L4-to-adult molt by the action of *mir-84* and *let-7* on their targets,

the nuclear hormone receptor transcription factors (TFs) *nhr-23* and *nhr-25* (Hayes *et al.* 2006).

Thus, among a sample of 15 microRNA families in *C. elegans*, four families (*let-7*, *mir-35*, *mir-51*, and *mir-58*) are associated with phenotypes resulting from the deletion of multiple members of the family. What about the other 11 of these families, for which complete genetic deletion of all members of the family did not uncover detectable phenotypes (Alvarez-Saavedra and Horvitz 2010)? Perhaps these could represent microRNAs whose functions depend on particular physiological or stress conditions (see *Longevity, stress responses, and stress robustness* below), and/or they may function redundantly with microRNAs of other families (see *Sensitized backgrounds uncover cryptic microRNA functions* below).

Sensitized backgrounds uncover cryptic microRNA functions

One explanation for the apparent lack of visible phenotypes for microRNA gene deletion mutants, besides the functional redundancy among microRNAs of the same family discussed above, emerged from studies designed to uncover otherwise cryptic microRNA functions using sensitized genetic backgrounds (Brenner *et al.* 2010). A significant finding from this study is that many *C. elegans* microRNAs functionally interact with microRNAs of other seed families. For example, for at least six microRNAs of distinct seed families, single-gene knockout caused gonad migration defects in an *alg-1*(*0*) background, where microRNA activity was broadly compromised, owing to loss of one of the two microRNA-specific Argonautes (ALG-1 and ALG-2) (Brenner *et al.* 2010). This suggests that these microRNAs may functionally interact with each other and/or with other microRNAs in regulating pathways related to the program of gonadal morphogenesis. The roles of microRNAs in gonadal morphogenesis was not previously appreciated. Based on the findings that deletion of either *mir-34* or *mir-83* (the *C. elegans* ortholog of mammalian miR-29) could impact this phenotype in the *alg-1*(*0*)-sensitized background (Brenner *et al.* 2010), common targets of *mir-34* and *mir-83* were identified (Burke *et al.* 2015). Interestingly, these include conserved components of cell migration and cell adhesion, *pat-3*/integrin and *cdc-42*.

Synergy between unrelated microRNA families is perhaps not unexpected, considering that the 3′-UTRs of mRNAs often have multiple microRNA complementary sites. Distinct microRNA families can even interact negatively; *mir-52* loss-of-function results in suppression of the phenotypes of *let-7* family mutants (Brenner *et al.* 2012). It is not clear whether the apparent opposition between *mir-52* and *let-7* microRNAs is direct, for example by competition for overlapping target sites, or indirect, for example via impacting separate but opposing pathways.

Longevity, stress responses, and stress robustness

Another explanation for the apparent lack of visible phenotypes for microRNA gene deletion mutants, besides functional redundancy among microRNAs of the same family or redundancy across families, emerged from experiments designed to stress mutant animals in an effort to uncover conditional functions for the microRNAs. Investigators speculated that some microRNA mutations might yield conditional phenotypes revealed only by subjecting mutant animals to the appropriate stress regimen.

Perhaps nothing is as stressful as aging. The first microRNA found to function in longevity was *lin-4*, which acts via its major downstream heterochronic gene target *lin-14* to promote normal life span, at least in part by engaging the *daf-16* and *hsf-1* transcriptional programs (Boehm and Slack 2005). Similarly, *let-7* family microRNAs seem to be integrated into pathways affecting fertility and longevity (Ren and Ambros 2015; D. Wang *et al.* 2017).

Evidence that other microRNAs could function in regulating life span came from sensitized genetic backgrounds, including *pash-1*(*ts*) mutants (carrying a weak mutation in the microRNA maturation factor gene *pash-1* that affects all microRNAs) shifted to the nonpermissive temperature during adulthood (Lehrbach *et al.* 2012), or from animals depleted for *alg-1* specifically during adulthood (Kato *et al.* 2011), where life span was shortened, presumably due to the compromised microRNA activity in these mutants (it should be noted that a standard caveat applies regarding shortened-life span phenotypes, wherein the genetic lesion may not identify a regulator of longevity *per se*, but rather could partially disable a pathway essential for robust health.)

Candidates for specific microRNAs that could control adult life span were identified by profiling microRNAs during adulthood to identify those whose levels change with age (Ibáñez-Ventoso *et al.* 2006; de Lencastre *et al.* 2010). Examples of specific microRNA genes where deletion mutations impact life span include *mir-71*, *mir-238*, *mir-239.1*, *mir-239.2*, and *mir-246* (de Lencastre *et al.* 2010). An independent systematic survey of microRNA mutants for life span defects, coupled with mosaic analysis tests for cell autonomy, identified a strong role for *mir-71* function in neurons in regulating normal adult life span (Boulias and Horvitz 2012).

A classic mode of regulating longevity is by dietary restriction (DR). One such DR model is the *C. elegans* mutant *eat-2*(*ad1116*), which is defective in eating. Profiling of microRNAs in *eat-2*(*ad1116*) adults compared to wild-type uncovered sets of microRNAs whose expression, and hence function, could be linked to DR-regulated longevity (Pandit *et al.* 2014). In another study, deletion of the microRNA *mir-80* induced DR-like phenotypes, including extended longevity via its regulation of *cbp-1*/CREB-binding protein mRNA translation (Vora *et al.* 2013).

mir-34 is an evolutionarily conserved microRNA with multiple functions in *C. elegans*. These functions include regulation of life span (Yang *et al.* 2013), and conferring robustness against physiological and developmental challenges, including dauer formation (Isik *et al.* 2016). Roles for *mir-34* in dauer formation were revealed by examination of the morphology and measuring the survival capacity *of mir-34* mutant larvae. In this context, an interesting DAF-16-*mir-34*

feedback loop appears to mediate robustness of the dauer larva program (Isik *et al.* 2016).

mir-34 plays an evolutionarily conserved function in DNA damage responses. Similar to mammalian cells, where *mir-34* is upregulated in response to radiation-induced DNA damage, *C. elegans mir-34* is induced after irradiation; however, unlike in mammalian cells where irradiation induction of *mir-34* requires p53 (Rokavec *et al.* 2014), *C. elegans mir-34* induction is independent of *cep-1* (which is considered to be a functional p53 ortholog despite relatively weak sequence homology). Even without p53 involvement, the *mir-34* mutant *C. elegans* displays abnormal survival of somatic and germline cells after irradiation, consistent with *mir-34* functioning to regulate apoptotic and nonapoptotic cell death, possibly in parallel to *cep-1/p53* (Kato *et al.* 2009). Another radiation sensitivity phenotype was found for mutants of *mir-237*, the only other member of the *lin-4* family in *C. elegans* (Metheetrairut *et al.* 2017).

A role for *mir-34* in developmental robustness against stress emerged from studies of genetic interactions between *mir-34* and *mir-83* (see *Sensitized backgrounds uncover cryptic microRNA functions*). The relatively mild penetrance of gonad migration defects in *mir-34*; *mir-83* double mutants was dramatically increased by cycling the temperature of developing larvae between temperatures within the worm's normal temperature range (for example 15 and 25°C). Constant temperature throughout development did not affect the *mir-34*; *mir-83* phenotype, indicating that this mutant appears to be sensitive specifically to changing environmental temperature, suggesting that *mir-34* functions with *mir-83* to maintain the robustness of gonadal migratory morphogenesis against the stress of unstable temperature (Burke *et al.* 2015).

Certain *C. elegans* microRNA mutants were tested in the context of heat stress and functions were identified for several microRNAs, including *mir-71*, as regulators of the heat stress response (Nehammer *et al.* 2015). Worms subjected to stress caused by benzo-α-pyrene (Wu *et al.* 2015) or graphene oxide (Wu *et al.* 2014) exhibited altered expression of certain sets of microRNAs and, in the latter case, worms with mutations in the genes for some of these microRNAs exhibited altered tolerance to graphene oxide stress. Likewise, *mir-35-41* mutant embryos were found to exhibit hypersensitivity to hypoxia stress (Kagias and Pocock 2015) and *mir-60* mutants exhibit a dysregulated adaptive response to oxidative stress (Kato *et al.* 2016).

Studies of the response of *C. elegans* to pathogen stress have uncovered roles for microRNAs in regulating innate immune pathways. Using a sensitized genetic background, phenotypic evidence emerged for the involvement of microRNAs in regulating the *C. elegans* antibacterial pathogen response, and the characterization of microRNAs identified by co-immunoprecipitation (co-IP) with the microRNA-Induced Silencing Complex (miRISC) identified candidate pathogen-responsive microRNAs (Kudlow *et al.* 2012). Mutants of either *miR-70* or *miR-251/miR-252* showed enhanced survival on *Pseudomonas aeruginosa* compared to wild-type worms, indicating a negative regulation of immune responses by these microRNAs (Kudlow *et al.* 2012). *mir-233* mutants are more sensitive to infection than wild-type worms, apparently through dysregulation of the unfolded protein response (Dai *et al.* 2015). *mir-67* mutants exhibited reduced pathogen avoidance behavior, apparently from the derepression of the *mir-67* target, *sax-7* (Ma *et al.* 2017). Mutations in the microRNA and small interfering RNA (siRNA) maturation factor *dcr-1*/Dicer confer sensitivity in *C. elegans* to the *Bacillus thuringiensis* pathogen, suggesting that microRNAs mediate immunity to the Cry toxins of *B. thuringiensis* (Iatsenko *et al.* 2013).

The heterochronic microRNAs, whose functions are primarily the control of developmental cell fates (*Heterochronic microRNAs and larval development*), have also been found to affect stress responses. *let-7* family microRNA mutants were found to exhibit either positive or negative effects on resistance to *P. aeruginosa*, suggesting a delicate temporal modulation of innate immune pathways in the worm (Ren and Ambros 2015). The activity of *let-7* in modulating the innate immune response to *P. aeruginosa* infection was shown to occur in the intestine, via regulation of SDZ-24-mediated signaling (Zhi *et al.* 2017). Other candidate targets of *let-7* and *let-7* family microRNAs for innate immune modulation may include components of the *pmk-1*/p38 innate immune pathway (Ren and Ambros 2015). No doubt the heterochronic microRNAs will likely be found to have additional roles in the modulation of various aspects of cellular physiology; one example is the regulation of ribosome biogenesis through the repression of *ncl-1* by *let-7* (Yi *et al.* 2015).

L1 diapause and dauer larva arrest

L1 larvae that hatch in the absence of food enter a developmentally arrested diapause stage that can survive for many days, and then reinitiate postembryonic development upon encountering food. A screen for microRNA gene mutations that perturb the ability of newly hatched larvae to enter the L1 diapause identified *mir-235*, the *C. elegans* homolog of the mammalian oncogenesis-associated microRNA *mir-92* (Kasuga *et al.* 2013). *mir-235* mutants fail to properly arrest development when hatched in the absence of food. *mir-235* expression is regulated by insulin/IGF signaling, such that *mir-235* is elevated during L1 diapause and declines upon feeding. *mir-235* seems to act in several major tissues of L1 larvae to inhibit postembryonic developmental programs in the absence of food. Loss of *mir-235* causes increased expression of its target *nhr-91*, a nuclear hormone receptor gene.

mir-71 was identified as being critical for L1 diapause animals to properly develop after feeding (Zhang *et al.* 2011). Interestingly, *mir-71* mutants that did recover from L1 starvation often displayed retarded VPC divisions, similar to mutants that are defective in the regulation of *lin-14* and other heterochronic genes. These results indicate that *mir-71* contributes to the regulation of heterochronic pathway genes, perhaps in a fashion that is coupled to the stress of starvation and L1 diapause. In this context, it is noteworthy

that several of the key heterochronic gene mRNAs, including *lin-42* and *hbl-1*, contain *mir-71* complementary sites in their 3′-UTRs.

mir-58 family microRNAs are redundantly required for dauer larva formation (Alvarez-Saavedra and Horvitz 2010). Other microRNAs were placed in dauer larva formation genetic pathways using a combination of genetic and biochemical strategies. Sensitized genetic backgrounds designed to compromise microRNA activity in the nervous system yielded phenotypic evidence for multiple dauer-regulating microRNAs (Than *et al.* 2013). Tissue-specific immunoprecipitation (IP) of miRISC identified the neuronally expressed microRNAs *mir-80/81*, *mir-124*, and *mir-234*, whose mutant phenotypes were subsequently determined to include effects on dauer formation, likely through multiple targets in the dauer regulatory pathways (Than *et al.* 2013). Similarly, *mir-58* family microRNAs regulate specific target genes in the TGF-β dauer as well as TGF-β body size regulatory cascades, including *dbl-1*, *daf-1*, *daf-4*, *sma-6* (de Lucas *et al.* 2015), and *sta-1* (Lozano *et al.* 2016).

Heterochronic microRNAs also regulate dauer formation. *lin-4* mutants are completely unable to form dauer larva due to the overexpression of *lin-14*, which is a potent regulator of the timing of dauer formation (Liu and Ambros 1989). *lin-14* activity in the L1 stage prevents early dauer formation and, accordingly, the downregulation of *lin-14* by *lin-4* is critical for dauer formation to be permitted at the normal time, at the end of the L2 stage. *let-7* family microRNAs also affect the decision to undergo dauer formation by modulating the levels of DAF-12 and HBL-1 proteins, suggesting that the upregulation of *let-7* family microRNAs during the L2 stage may modulate the temporal response of the dauer entry program according to environmental signals (Hammell *et al.* 2009a; Karp and Ambros 2011).

In animals that develop through the dauer larval stage, microRNA pathways are reprogrammed in interesting ways. The temporal profile of expression of certain microRNAs is altered in L2 animals entering the dauer stage ("L2D" larvae), and in L3 and L4 animals developing after dauer arrest ("postdauer" larvae), compared to continuously developing larvae (Karp *et al.* 2011). Moreover, the relative functional contributions of *lin-4* and *let-7* family microRNAs to developmental cell fate specification are altered for postdauer development compared to continuous development (Karp and Ambros 2012).

Embryonic development

The *mir-35-42* family of microRNAs are maternally contributed to the early embryo, expressed in the zygote shortly after fertilization (Wu *et al.* 2010), and contribute redundantly to embryonic development and viability. The precise nature of the essential functions of *mir-35-42* are unknown and appear to be complex (Alvarez-Saavedra and Horvitz 2010). *mir-35* family mutants exhibit diverse pleiotropic phenotypes at embryonic and postembryonic stages, suggesting functions for these microRNAs in multiple pathways. Among the characterized early embryonic functions of *mir-35-42* is a role in sex determination, wherein these microRNAs act by regulating a set of RNA-binding protein targets to prevent the premature expression of the male developmental program in XX embryos (McJunkin and Ambros 2017). In this capacity, *mir-35-42* serves as a sort of "timer" to delay sex determination until after the proper reading of the zygotic X/A ratio. It is possible that some of the essential functions of *mir-35-42* in the early embryo could include analogous roles in preventing premature expression of other, "late" developmental programs. The fact that the *mir-35-42* family microRNAs are downregulated during mid embryogenesis is consistent with the model that they may broadly control early-to-late developmental transitions in the embryo.

The *mir-35* family microRNAs also act, together with *mir-58/bantam* microRNAs, to prevent inappropriate expression of the EGL-1 proapoptotic protein in certain embryonic cell lineages. In particular, these two microRNA families cooperate to target the *egl-1* mRNA in the mothers of cells programmed to die, thereby preventing precocious apoptosis (Sherrard *et al.* 2017).

Another abundant microRNA family expressed in the worm embryo is the *mir-51* family, the worm homolog of the deeply conserved *miR-100*. The *mir-51* family functions redundantly with the *mir-35* family to regulate embryonic viability (Alvarez-Saavedra and Horvitz 2010) and pharyngeal morphogenesis (Shaw *et al.* 2010). The pleiotropic phenotypes of mutants of the *mir-35* family or the *mir-51* family indicate that these abundant early embryo microRNAs are engaged with multiple essential developmental pathways. Interestingly, the *mir-35* family microRNAs are relatively specific for the early embryo and are relatively nematode-specific compared to the *mir-51* family, which are abundant in *C. elegans* larvae as well as in embryos, and are broadly conserved evolutionarily. *mir-51/mir-100* may function in diverse and conserved genetic regulatory contexts, while *mir-35-42* may be adapted for coping with gene regulatory challenges that are more particular to nematodes.

Germline development

C. elegans germline development and gametogenesis appear to be impacted by microRNA genes, although there is clearly much more to be learned about germline functions of microRNAs in the worm. Characterization of the phenotypes resulting from the depletion of ALG-1 and ALG-2 from the somatic distal tip cells (DTCs) suggests cell nonautonomous roles for microRNAs in processes where signals from the DTCs regulate the germline cell cycle and proliferation (Bukhari *et al.* 2012). Whether or not microRNAs expressed within the germline function cell-autonomously is less clear. Small RNA cDNA sequencing has identified over a dozen microRNAs that are enriched in the germline, including prominently the *mir-35-42* family (McEwen *et al.* 2016). Although it is clear that the maternal contribution of *mir-35-42* can affect embryonic viability (Alvarez-Saavedra and Horvitz 2010), it is not yet established whether these roles for

maternally expressed *mir-35-42* include the repression of targets within the maternal germline itself or only after deposition in the embryo. Among the postembryonic phenotypes of *mir-35-42* mutants are defects in hermaphrodite fecundity, owing at least in part to impaired spermatogenesis (McJunkin and Ambros 2014). This function of *mir-35-42* could be the result of a combination of germline and/or somatic gonad activity of these microRNAs.

An apparent direct function for microRNAs within the germline is suggested from the phenotype of loss-of-function mutants for ALG-5, a microRNA-associated Argonaute that is expressed primarily in the germline of hermaphrodites. *alg-5*(*lf*) mutants exhibit reduced fertility and a precocious developmental switch from spermatogenesis to oogenesis (Brown *et al.* 2017).

Neural development and behavior

One of the first *C. elegans* microRNAs that was found to affect behavior is *mir-786*, deletion mutations of which display abnormally long intestinal defecation cycles (Miska *et al.* 2007). Detailed genetic analysis showed that *mir-786* regulates the expression of the fatty acid elongase, *elo-2*, in intestinal cells, and thereby ensures the proper rhythmic behavior of those cells in their role as pacemakers for the defecation cycle (Kemp *et al.* 2012).

mir-1 is an evolutionarily conserved muscle-expressed microRNA whose function in *C. elegans* was not apparent at first, as *mir-1* was among those microRNAs for which mutants had no apparent defects. *mir-1* mutant phenotypes identified from genetically sensitized screens (Brenner *et al.* 2010) have not yet been investigated in depth, but a window into *mir-1* function in *C. elegans* was opened by challenging *mir-1* mutants pharmacologically (Simon *et al.* 2008). *mir-1* mutants show altered acetylcholine sensitivity and, based on that phenotype, roles for *mir-1* were uncovered in controlling muscle–neuronal signaling at the neuromuscular junction. Another microRNA implicated in the regulation of neuromuscular signaling is *mir-238*; upregulation of the nicotinic acetylcholine receptor *acr-19* during chronic exposure of *C. elegans* to nicotine was traced to a downregulation of *mir-23*8, which was found to directly target *acr-19* (Rauthan *et al.* 2017).

The formation of specific neurons and neuronal connections is coordinated with positional and temporal information in the developing worm. Certain microRNAs, including *mir-54* and *mir-56*, have been implicated in the regulation of the Hox gene *egl-5* in the context of specifying the posterior pattern of male sensory rays (Zhang and Emmons 2009). Neuronal development in response to temporal cues is exemplified by the hermaphrodite-specific neuron (HSN), which extends its axon in the L4 stage. The developmental timing microRNA *lin-4* is critical for specifying the timing of HSN axon outgrowth through the developmental downregulation of two targets, *lin-14* and *lin-28*, which inhibit HSN differentiation (Olsson-Carter and Slack 2010).

Another role for *lin-4* in controlling the timing of steps in the outgrowth and migratory behavior of axons occurs for the anterior ventral microtubule (AVM) neurons, where *lin-4* acts cell autonomously in AVM neurons to promote the proper formation of AVM connections, apparently by repressing its target LIN-14 and thereby terminating AVM axon migration (Zou *et al.* 2012). Another microRNA affecting neuronal migration is *mir-79*, which functions in the epidermis to control the properties of the extracellular matrix (Pedersen *et al.* 2013). MicroRNAs can also regulate the capacity of neurons to regenerate after injury, as exemplified by a role for *let-7* in the developmental decline of AVM axon regeneration (Zou *et al.* 2013).

Mutants of the *let-7* family microRNA *mir-84* display defects in the stage-specific rewiring of the dorsal D (DD) motor neuron in the L1 larval stage, due to dysregulation of heterochronic genes including *hbl-1*, an apparent *mir-84* target (Thompson-Peer *et al.* 2012). The heterochronic gene *lin-14* also controls the timing of DD rewiring (Hallam and Jin 1998), although curiously it is not clear whether microRNAs that could target *lin-14* (which include the *let-7* family as well as *lin-4*) may act via *lin-14* to participate in regulating the stage-specificity of DD rewiring.

Developmental decisions between alternative neuronal subtype fates often occur in response to the activity of developmental signals. In many cases, these decisions involve precise, yet subtle, distinctions in gene activity. One such situation is the stochastic left/right specialization of the two AWC neurons. *nsy-4* and *nsy-5* signals act stochastically to inhibit calcium signaling asymmetrically in the pair of AWC precursor cells to produce asymmetric alternative fates, AWC(OFF) and AWC(ON). However, the mechanism of coupling *nsy-4* and *nsy-5* to asymmetric calcium signaling, and hence cell fate, is not understood. *mir-71* was identified genetically as an integral post-transcriptional switch for specifying distinct left *vs.* right AWC fates (Hsieh *et al.* 2012). *mir-71* acts as a repressor of TIR-1/Sarm1, a critical calcium signaling component, to promote the AWC(ON) identity. Tests of epistasis and cell autonomy indicate that *nsy-4* and *nsy-5* promote *mir-71* activity in one AWC, possibly by stabilizing mature *mir-71*, to promote the AWC(ON) fate (Hsieh *et al.* 2012). Similarly, the *lsy-6* and *mir-273* microRNAs are deployed asymmetrically in the left ASE (ASEL) *vs*. the right ASE (ASER) neurons (Johnston and Hobert 2005; Cochella and Hobert 2012), and control a bimodal developmental switch that specifies the distinct chemosensory properties of ASEL and ASER (Johnston and Hobert 2003; Chang *et al.* 2004).

A conceptually novel perspective on microRNAs in neuronal specialization emerges from studies of the functions of microRNAs expressed at high levels in a very limited set of neurons in *C. elegans*. *mir-791* was found to be expressed exclusively in certain CO_2-sensing neurons, and was shown to confer the CO_2-sensing functionality of these neurons by repressing two otherwise broadly expressed genes (Drexel *et al.* 2016). This mode of action, where a microRNA expressed specifically in a particular cell modulates the level of otherwise broadly expressed (even essential) genes, could underlie the elaboration of neuronal diversity in more complex nervous systems.

MicroRNAs can also function to sharpen neuronal *vs.* non-neuronal gene expression patterns. For example, the *mir-58* microRNA family functions to restrict the expression of *pmk-2*/p38 to the nervous system, where it is coexpressed with its ortholog *pmk-1*. Consequently, *pmk-1* and *pmk-2* function together and redundantly in the nervous system to control pathogen avoidance behavior, while *pmk-1* functions on its own in the intestine to guard against pathogen infection (Pagano *et al.* 2015).

Regulation of the Biogenesis, Stability, and Activity of MicroRNAs

C. elegans research has led to many of the advances in our understanding of the expression and regulation of microRNA genes, how mature microRNAs are generated from primary transcripts of microRNA genes, and how the activity of a microRNA is regulated after biogenesis (Figure 1). Forward genetic screens (for example, Ding *et al.* 2005) and RNA interference (RNAi) screens [for example, Parry *et al.* (2007) and Rausch *et al.* (2015)] have enabled the identification of scores of genes encoding protein factors that positively or negatively contribute to microRNA activity.

Genetic identification of Dicer, Argonautes ALG-1/2, and microRNA effectors AIN-1/ 2

The Argonaute RDE-1 emerged from a genetic screen for RNAi-defective mutants and provided the first evidence that the Argonaute class of proteins are intimately associated small RNAs (Tabara *et al.* 1999). Using an RNAi screen for heterochronic phenotypes similar to those caused by mutations in the microRNAs *lin-4* or *let-7*, the Argonautes ALG-1 and ALG-2 (paralogs of RDE-1) were shown to be required for proper microRNA biogenesis and function (Grishok *et al.* 2001). The seminal discovery of the roles for specialized Argonautes in RNAi and microRNAs, together with the identification of microRNA-related phenotypes associated with a loss-of-function of *dcr-1*, the *C. elegans* gene encoding Dicer, cemented our understanding of the fundamental linkage between RNAi and microRNAs (Grishok *et al.* 2001).

There is intriguing evidence that ALG-1 and ALG-2 may not be the only *C. elegans* Argonautes that associate with microRNAs. Immunoprecipitation of epitope-tagged ALG-5 resulted in enrichment for a specific subset of germline microRNAs, indicating that the reduced fertility of *alg-5*(*lf*) hermaphrodites (see *Germline development*, above) may reflect a role for the ALG-5-associated miRISC in the germline (Brown *et al.* 2017). Similarly, HA-tagged RDE-1 was found to co-immunoprecipitate with a subset of microRNAs (Steiner *et al.* 2007; Corrêa *et al.* 2010), suggesting possible crossover between microRNA and RNAi pathways (see *mRNA translational repression and/or mRNA turnover*).

The AIN-1 and AIN-2 proteins were initially identified as suppressors of the *lin-31* multivulva (Muv) phenotype and found to have more general heterochronic phenotypes (Ding *et al.* 2005). AIN-1 and AIN-2 function redundantly, and depletion of both proteins causes pleiotropic phenotypes consistent with general impaired microRNA activity. AIN-1 and AIN-2 are degenerate orthologs of the conserved miRISC component and microRNA activity effector GW182 (Ding *et al.* 2005). Tagged AIN-1 or AIN-2 can be used to immunoprecipitate miRISC from worms, and this approach has been a powerful means of identifying other miRISC-associated proteins, for profiling microRNAs associated with AIN-1 or AIN-2 in particular cell types at particular developmental stages, and for profiling mRNA targets engaged by miRISC (Zhang *et al.* 2007, 2009).

Transcriptional regulation of microRNA gene expression

As is the case for animals in general, some microRNAs in *C. elegans* are produced from a dedicated noncoding primary transcript (and therefore likely from a dedicated promoter), and other microRNAs are processed from pre-mRNAs of coding genes, so that a microRNA gene can share transcriptional regulatory sequences with one or more protein-coding genes. It is also not uncommon for several microRNAs to be expressed from the same primary transcript. For many of the *C. elegans* microRNAs, transcriptional regulatory sequences have been characterized using fluorescence reporter transgenes (Martinez *et al.* 2008b), but for several other microRNAs, primary transcript configurations and expression parameters have not been characterized.

Interestingly, in many cases, *C. elegans* microRNAs are located in an intron and in the sense direction relative to the host protein-coding gene, but nevertheless appear to be expressed from a dedicated intronic promoter (Lee *et al.* 1993; Martinez *et al.* 2008b). Obvious exceptions include the mirtrons, which are microRNAs whose precursor hairpins are processed out of pre-mRNA transcripts by the spliceosomal machinery, bypassing the requirement for Drosha processing (Ruby *et al.* 2007; Chung *et al.* 2011). There are at least 13 mirtrons encoded in the *C. elegans* genome whose expression patterns have been confirmed (Chung *et al.* 2011). These *C. elegans* mirtrons have not been well-studied genetically, and no functions have been yet ascribed to them.

In some cases, the transcriptional regulation of microRNA gene expression is coupled to developmental signals. *let-7* expression is subject to complex transcriptional control (Kai *et al.* 2013), including temporal modulation by another heterochronic gene, *hbl-1* (Roush and Slack 2009). Similarly, the expression of the *let-7* family microRNAs *mir-48*, *mir-84*, and *mir-241* appears to be restrained by the heterochronic TF LIN-14, such that the midlarval events triggered by those particular microRNAs, particularly the downregulation of LIN-28, are restricted to stages after the downregulation of LIN-14 (Tsialikas *et al.* 2017).

The heterochronic gene *lin-42* encodes a Period homology protein (Jeon *et al.* 1999), and *lin-42*(*lf*) mutants exhibit precocious developmental timing phenotypes that appear to reflect the hyperactive transcription of certain microRNA genes, identifying LIN-42 as a transcription repressor of microRNAs that likely modulates their developmental

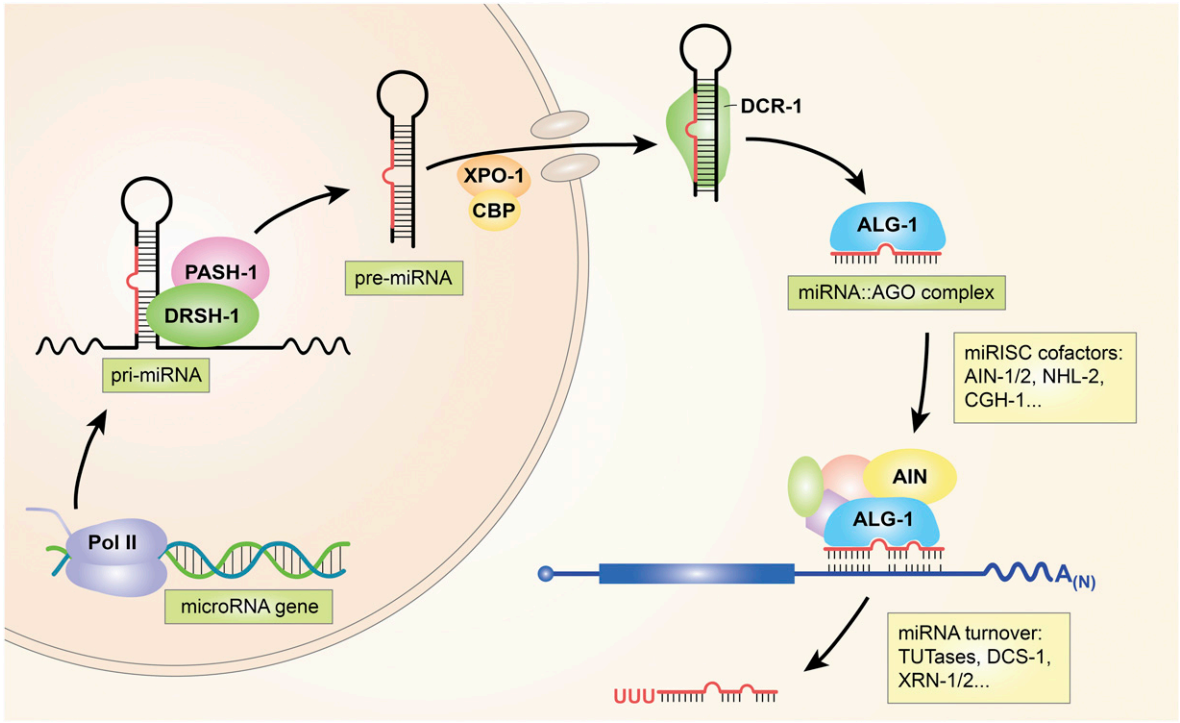

Figure 1 MicroRNA metabolism and function in *C. elegans*. Current understanding of major factors involved with various steps in the transcription and processing of microRNA primary transcripts (pri-miRNA) in the nucleus (left), export of the hairpin RNA microRNA precursor (pre-miRNA) through the nuclear pore to the cytoplasm (top), processing of the pre-miRNA by Dicer/DCR-1, and loading of the mature miRNA into a core microRNA-Induced Silencing Complex (miRISC) Argonaute protein (ALG-1). Additional factors assemble with miRISC, including the general miRISC effector protein AIN-1/2. The miRISC complex binds to target mRNAs via complementary sites in their 3′-UTRs and represses protein production from the target by various mechanisms, as discussed in the text. MicroRNAs eventually undergo downregulation through processes involving 3′ terminal uridyl modifications and degradation by the cellular RNA turnover machinery (figure courtesy of Gloria Ha). Pol II, RNA polymerase II: TUTase, terminal uridyl transferase.

expression (McCulloch and Rougvie 2014; Perales *et al.* 2014; Van Wynsberghe *et al.* 2014).

C. elegans is a good model for how microRNAs and TFs are organized into gene regulatory network motifs that provide feedback and/or feed forward functionality. A specific example is a motif consisting of the microRNA *mir-57* and the Hox gene *nob-1* (Zhao *et al.* 2010). *nob-1* activates *mir-57* expression in the posterior of the embryo, and *nob-1* mRNA is also a direct target of *mir-57*, producing a negative feedback loop between the microRNA and the Hox gene, perhaps to sharpen positional cues in the embryo. A broader, genome-wide analysis of predicted interactions of TFs with microRNA regulatory sequences, combined with microRNA target prediction, led to the construction of a genome-scale model of TF→microRNA interactions, as well as predicted microRNA→TF interactions. More than 20 microRNA←→TF-predicted composite feedback loops were identified in *C. elegans* (Martinez *et al.* 2008a). Such mutually direct regulatory motifs containing microRNAs and TFs could help to coordinate the regulation of microRNA and TF target repertoires.

An example of a rather complex microRNA←→TF feedback motif, which acts during early larval development to integrate environmental and developmental signals, consists of a set of *let-7*-family microRNAs and the DAF-12 nuclear hormone receptor (Bethke *et al.* 2009; Hammell *et al.* 2009a). These microRNAs directly regulate DAF-12 levels and, in turn, their levels are transcriptionally regulated by DAF-12, which directly activates (in the presence of ligand) or represses (in the absence of ligand) transcription of the microRNA genes.

Post-transcriptional regulation of microRNA biogenesis and turnover

Much remains to be learned about how the *C. elegans* core microRNA biogenesis machinery (Figure 1) can be regulated to control microRNA levels in response to signals. There is evidence that microRNA biogenesis can be regulated at the level of the microprocessor complex, which consists of DRSH-1/Drosha and PASH-1/DGCR8 (Denli *et al.* 2004; Lehrbach *et al.* 2012). For example, *trans*-splicing of the *let-7* primary transcript (pri-*let-7*) seems to modulate the processing of pri-*let-7* by microprocessor (Mondol *et al.* 2015).

After release of the microRNA precursor hairpin (by microprocessor activity, in the case of conventional microRNAs, or by the spliceosome, in the case of mirtrons), subsequent steps include nuclear export of the pre-microRNA, followed by further processing by Dicer to produce the mature microRNA (Figure 1). In *C. elegans*, nuclear export of the majority of pre-microRNAs appears to depend on the nuclear export receptor XPO-1 and components of the cap-binding complex (CBC)

(Büssing *et al.* 2010). Interestingly, the export of mirtrons seems to occur independently of XPO-1/CBC (Büssing *et al.* 2010). Little is known about potential modes of regulation of microRNA nuclear–cytoplasmic trafficking. There are indications that regulation of the Dicer (DCR-1) processing step can occur; for example, DCR-1 appears to be developmentally regulated by phosphorylation in oocytes, suggesting that some maternally deposited microRNAs may not be processed until fertilization (Drake *et al.* 2014).

Upon Dicer processing of the pre-microRNA, the mature microRNA is loaded into one of the principle miRISC Argonautes, ALG-1 and ALG-2 (Figure 1), or in rarer cases, into an alternative Argonaute such as ALG-5 (Brown *et al.* 2017) or RDE-1 (Steiner *et al.* 2007). Evidence that the Argonaute loading step can be regulated includes the observation that the developmental profiles of microRNAs associated with ALG-1 *vs.* ALG-2 differ (Vasquez-Rifo *et al.* 2012; Brown *et al.* 2017). Also, there is evidence that pre-microRNA hairpin structure can influence Argonaute loading specificity, such that certain microRNAs with precursors that have relatively few mismatches can be preferentially loaded into the (otherwise RNAi-specific) Argonaute RDE-1 (Steiner *et al.* 2007; Corrêa *et al.* 2010).

Evidence that Argonaute may actively participate in miRISC loading comes from studies of antimorphic alleles of ALG-1 that broadly impair the function of many microRNAs. ALG-1(anti) proteins show an increased association with Dicer and a decreased association with AIN-1/GW182, suggesting that these antimorphic mutations cause ALG-1 to stall in a microRNA loading state, prior to advancing to effector status. Tellingly, the *alg-1*(anti) mutants dramatically overaccumulate microRNA* ("star," *i.e.*, passenger) strands, suggesting that wild-type ALG-1 complexes recognize structural features of microRNAs in the context of the guide strand selection and passenger strand ejection steps of miRISC maturation (Zinovyeva *et al.* 2014, 2015).

Mechanisms involved in regulating the stability and degradation of microRNAs in *C. elegans* have been identified. The enzymes involved in these mechanisms include terminal uridyl transferase (Lehrbach *et al.* 2009), the decapping scavenger enzyme DCS-1 (Bossé *et al.* 2013), and the exonucleases XRN-1 and XRN-2 (Chatterjee and Grosshans 2009; Chatterjee *et al.* 2011; Miki *et al.* 2014). Interestingly, the degradation of microRNAs in worm lysates or *in vivo* can be modulated depending on the presence of target mRNA, consistent with the finding that microRNA homeostasis may be coupled to target recognition (Chatterjee and Grosshans 2009; Chatterjee *et al.* 2011).

There is evidence that the turnover of microRNAs in *C. elegans* could also be coupled to the turnover of miRISC protein components. The finding that microRNA-mediated gene regulation in *C. elegans* can be modulated by autophagy (Zhang and Zhang 2013) suggests that the degradation of miRISC components, including miRISC-bound microRNAs, could be a potent mechanism of controlling microRNA activity in response to signals that regulate autophagy. Similarly, genetic and biochemical evidence suggests that TEG-1, a conserved protein that can associate with miRISC (C. Wang *et al.* 2017), regulates the levels of miRISC proteins (particularly ALG-1 and VIG-1), and also regulates the levels of several microRNAs (C. Wang *et al.* 2017). *teg-1*(*lf*) mutants exhibit developmental defects consistent with reduced microRNA function, reinforcing the model that TEG-1 functions to stabilize miRISC complexes. Going forward, an interesting aspect of better understanding microRNA/miRISC turnover mechanisms will be to determine how signaling pathways may be coupled to the selective inactivation of miRISC complexes containing specific microRNAs.

Regulators of miRISC activity

RNAi screens for enhancers of microRNA-related phenotypes in *C. elegans* have contributed to the identifications of proteins that could link developmental or physiological signals to the regulation of microRNA activity, without necessarily affecting microRNA abundance (Parry *et al.* 2007; Rausch *et al.* 2015). Similarly, candidate microRNA regulatory cofactors have been identified among proteins found to be associated with miRISC in *C. elegans* and verified functionally by genetics or RNAi knockdown. In this manner, the miRISC-associated proteins NHL-2 (a TRIM-NHL protein) and CGH-1 (an RNA helicase domain protein) were found to function as positive cofactors for microRNAs (Hammell *et al.* 2009b). These results are consistent with NHL-2 and CGH-1 having evolutionarily conserved roles in modulating the efficacy of microRNA–target interactions *in vivo*.

C. elegans casein kinase II (CK2) promotes miRISC function. *kin-3* and *kin-10* encode subunits of CK2. *kin-10* is required for RNAi (Kim *et al.* 2005) and casein kinase subunits can be obtained via co-IP with AIN-1 (Alessi *et al.* 2015). Casein kinase inactivation causes developmental defects that phenocopy a loss of miRISC cofactors and enhance the loss of microRNA function in diverse cellular contexts. CK2 is dispensable for microRNA biogenesis and the stability of miRISC cofactors, but is required for miRISC target mRNA binding and silencing. The conserved DEAD-box RNA helicase, CGH-1/DDX6, is a key CK2 substrate within miRISC; CGH-1 phosphorylation is required for CGH-1 function in the microRNA pathway (Alessi *et al.* 2015).

Other candidate miRISC cofactors in *C. elegans* that were confirmed functionally by using sensitized genetic backgrounds include PUF-9 (Nolde *et al.* 2007) and poly(A)-binding protein (Hurschler *et al.* 2011). The latter finding corroborates the idea that miRISC can regulate mRNA translation and/or stability by affecting polyadenylation (Flamand *et al.* 2016). Also implicating microRNA function in translational control, a yeast two-hybrid screen for proteins that can interact with ALG-1 identified RACK1 (receptor for activated C-kinase), a protein known to interact with ribosomes. *rack-1* knockdown resulted in developmental phenotypes attributable to defects in microRNA activity, suggesting that RACK-1 may mediate interactions between miRISC and ribosomes, possibly in the context of microRNA repression of translation

(Jannot *et al.* 2011). The ribosome connection is further supported by reports that knockdown of ribosomal protein RPS-14 can modify *let-7* phenotypes (Chan and Slack 2009).

Staufen (STAU-1) is a double-stranded RNA-binding protein with known functions in the regulation of mRNA activity, including translation (Micklem *et al.* 2000). STAU-1 binds to multiple mRNAs in *C. elegans* (LeGendre *et al.* 2013), suggesting that, in principle, STAU-1 could functionally interact with microRNAs for cotargeted mRNAs. Indeed, *stau-1*(*lf*) can suppress phenotypes associated with the depletion of certain microRNAs in *C. elegans* without discernably affecting microRNA levels, indicating that STAU-1 may function as a negative regulator of microRNA activity (Ren *et al.* 2016).

Certain microRNA cofactors that have emerged from genetic enhancer screens point to an intimate relationship between microRNAs and vesicular sorting pathways. Components of the Golgi-Associated Retrograde Protein (GARP) complex have been functionally implicated with miRISC activity in *C. elegans*, suggesting a miRISC connection with membranes that may affect the abundance of GW182/AIN proteins and/or microRNAs (Vasquez-Rifo *et al.* 2013). ER pathways such as HMG-CoA reductase have also emerged as genetic enhancers of weak *let-7* mutations (Parry *et al.* 2007). These *C. elegans* findings are endorsed by genetic analysis of microRNA-defective mutations in *Arabidopsis* (Li *et al.* 2016). For example, the ER-associated mevalonate pathway of sterol and dolichol synthesis in protein glycosylation strongly regulates *let-7* activity in *C. elegans* as well as microRNA function in plants (Shi and Ruvkun 2012), and *Arabidopsis* microRNAs are strongly associated with ER-associated polysomes (Li *et al.* 2016). It is tantalizing to think that the target of one of the world's most prescribed class of drugs, the statins, may affect microRNA function in the regulation of secreted protein translation (Shi and Ruvkun 2012).

Reciprocal regulation between let-7 and LIN-28

lin-28 negatively regulates the accumulation of *let-7* mature microRNA in *C. elegans*. Mature *let-7* accumulates to dramatically elevated levels at abnormally early larval stages in *lin-28*(*lf*) mutants (Van Wynsberghe *et al.* 2011). Furthermore, biochemical evidence points to direct *in vivo* binding of LIN-28 to the *let-7* primary transcript in the nucleus at early larval stages, suggesting that LIN-28 inhibits processing of the *pri-let-7* transcript into the *let-7* precursor (Van Wynsberghe *et al.* 2011; Stefani *et al.* 2015). This situation reflects an apparently evolutionarily conserved, mutually antagonistic and direct relationship between *let-7* and *lin-28*, where LIN-28 binds to the *let-7* transcript, and *let-7* binds to *lin-28* mRNA. Interestingly, in *C. elegans*, LIN-28 binding seems to be downstream of the *let-7* hairpin (Stefani *et al.* 2015), indicating that the regulation of *let-7* biogenesis by LIN-28 in *C. elegans* may occur exclusively in the nucleus.

Feedback autoregulation of let-7 and lin-4

There is evidence that *lin-4* and *let-7* in *C. elegans* may offer fascinating opportunities to study the ways that a microRNA may feedback and regulate its own expression, perhaps even by interacting with its own primary transcript in the nucleus. *lin-4* complementary sites were identified upstream of the *lin-4* hairpin in the *lin-4* primary transcript, and tests using mutated transgenic reporters suggest that these sites (and, by implication, the base pairing of *lin-4*) could affect the developmental expression of *lin-4 in vivo* (Turner *et al.* 2014).

Similarly, a region downstream of the *let-7* hairpin, within sequences expressed as part of the *let-7* primary transcript, contains *let-7* complementary sites that were shown to bind to miRISC (ALG-1) *in vivo* (Zisoulis *et al.* 2012). Moreover, functional tests using *let-7* transgenes have shown that the presence of the downstream sequences containing the *let-7* sites could positively impact *let-7* microRNA expression, suggesting that mature *let-7* microRNA could act in the nucleus to promote its own biogenesis (Zisoulis *et al.* 2012).

Identification and Validation of MicroRNA Targets

Studies using *C. elegans* have contributed substantially to our understanding of the underlying principles of target recognition by microRNAs. The primacy of the 5′ part of the microRNA (eventually termed the seed) in target binding was evident from the predicted base pairing between *lin-4* and *let-7* microRNAs and their first genetically identified targets (Lee *et al.* 1993; Wightman *et al*. 1993; Reinhart *et al.* 2000). Some of the first *in vivo* structure–function analyses of microRNA–target interactions were conducted in *C. elegans* (Ha *et al.* 1996), and computational target prediction algorithms were developed using data from *C. elegans* (Hammell *et al.* 2008).

Genetic epistasis of predicted microRNA–target mRNA pairs

As base pairing of a microRNA to a target mRNA causes a decrease in either the translation of the target mRNA and/or the abundance of that mRNA, the phenotypes caused by a microRNA mutation are expected to be due to an increase in the expression of the target's protein product. Tests of epistasis—whether knockdown of a putative mRNA target can suppress the phenotype of microRNA loss-of-function—is a powerful approach for validating that a gene containing predicted microRNA target sites can function downstream of the microRNA. In some cases, major microRNA targets have been identified directly in screens for suppressors of microRNA mutants, as was the case for *lin-14* (Ambros and Horvitz 1984; Wightman *et al.* 1993) for the *lin-4* microRNA and *hbl-1*(Abrahante *et al.* 2003; Lin *et al.* 2003) for the *let-7* microRNA. Similarly, *lin-41*(*lf*) mutations were identified by epistasis to *let-7*(*lf*) in screens for suppressors of *let-7*(*lf*) (Slack *et al.* 2000), and targeting of *nhl-2* and *sup-26* by the *mir-35* family was discovered in an RNAi screen for suppressors of the subliminal masculinization of the *mir-35-family*(*lf*) animals (McJunkin and Ambros 2017). Genome-wide RNAi screens can also, in principle, identify targets of a microRNA from knockdowns that suppress the microRNA mutant phenotypes. For example, in RNAi screens for suppressors

and enhancers of *let-7* phenotypes (Hunter *et al.* 2013), suppressors can include targets of *let-7* as well as negative modulators of *let-7* activity (see *Regulators of miRISC activity*, above).

Epistasis, in itself, does not unequivocally establish a gene as a direct downstream target of a microRNA, and so the phylogenetic conservation of orthologous complementary target sites (in one or more *Caenorhabditis* species) is an additional criterion for directness. Another shortcoming of epistasis is that for redundant targets, of the sort where the overexpression of any *one* target can cause the phenotype, simultaneous knockdown of multiple targets would be required. Conversely, it can be possible to suppress a microRNA loss-of-function phenotype by knockdown of any one of a set of predicted targets (Grosshans *et al.* 2005), indicating that the phenotype of a microRNA loss-of-function could depend on the simultaneous hyperactivity of multiple genes of a coherent downstream gene network, where the levels of each gene are coupled to the levels of others in the network. Even in cases where such a set of hypothetically coupled genes all contain microRNA complementary sites (Grosshans *et al.* 2005), it is possible that only a subset of the network may be directly regulated by the microRNA.

In summary, while epistasis is a powerful means to support the supposition of a direct microRNA–target interaction (by indicating that the putative target functions downstream of the microRNA), and phylogenetic conservation of targeting can also endorse target validity (by indicating selection on the putative target sequence), further supporting evidence can come from the mutation of the microRNA complementary sites using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and from assaying for upregulation of the putative target protein, along with associated phenotypes.

Computational prediction of microRNA complementary target sites

The primary involvement of "seed pairing" (base pairing between target nucleotides and positions 2–8 of the microRNA) was apparent from the initial identification of targets for *lin-4* (Lee *et al.* 1993; Wightman *et al.* 1993) and *let-7* (Slack *et al.* 2000). When additional conserved microRNAs were identified (Lau *et al.* 2001; Lee and Ambros 2001), the primacy of the seed in target recognition was confirmed by the almost universal conservation of nucleotides 2–8 among evolutionarily related microRNAs. Therefore, target prediction algorithms rely heavily on the base pairing of nucleotides 2–8 or 2–7 of the microRNA, with additional provisions for filtering out false positive predictions by employing evolutionary conservation of UTR sequence alignment (Lall *et al.* 2006), and the conservation of targeting and/or other parameters derived from *in vivo* confirmatory data (Hammell *et al.* 2008; Agarwal *et al.* 2015).

A number of different microRNA target prediction tools are available, and generally all of them are convenient and powerful. The chief differences among them are how underlying assumptions are weighted, and different tools can yield nonidentical sets of putative targets. So, in general, it is advisable to employ the combined predictions of multiple computational tools. Another consideration is that some tools may be found to be more stringent than others, being tuned to yield fewer false positives (at the expense of perhaps missing many *bona fide* targets). Less-stringent prediction tools can be more comprehensive and sweep up most *bona fide* targets, but at the expense of more false positive predictions. Predicted targets must be validated by *in vivo* experiments, and so the choice of target prediction tool is in part governed by the logistics of target validation in a given situation. A stringent tool may be advisable when high-throughput validation tests are not available, while a more comprehensive tool, such as RNAhybrid (Rehmsmeier *et al.* 2004), could be the choice in situations where avoiding false negatives is a priority and where false positives can easily be screened out.

Direct identification of in vivo microRNA–target complexes

The caveats associated with the computational identification of microRNA targets are derived from two issues. First, even the more stringent prediction tools can yield a list of scores, or even hundreds of predicted targets for a single microRNA, yet in cases where genetic epistasis has been applied to identify functional targets of a microRNA, it is generally found that very few or only a single target is actually involved in a given context. Therefore, it appears that we do not yet understand what contextual factors govern which specific microRNA–target interactions, among all the computationally predicted potential interactions, are efficacious. Second, it is not clear that we have a comprehensive understanding of the various configurations of microRNA–target interactions (other than seed pairing) that can be functional *in vivo*. Therefore, significant advances in identifying *bona fide* microRNA target complexes *in vivo* will not only enable focused attention on functional targets for genetic evaluation, but will also permit the continued refinement of computational target prediction algorithms.

IP of miRISC using antisera against miRISC components, followed by the identification of bound mRNAs using microarray or RNA sequencing (RNAseq), has provided data sets of mRNAs stably associated with miRISC (Zhang *et al.* 2007). These data sets have been used to shape target prediction algorithms based on experimental evidence (Hammell *et al.* 2008). The shortcoming of miRISC IP followed by RNAseq is that the precise location of miRISC binding is not known, so the specific microRNAs responsible for miRISC binding to the mRNA sequences obtained by co-IP must be inferred from sequence complementarity.

Strategies such as cross-linking immunoprecipitation with high-throughput sequencing (CLIP-seq) (Zisoulis *et al.* 2010), individual nucleotide-resolution cross-linking and immunoprecipitation (iCLIP) (Broughton and Pasquinelli 2013), and chimera PCR (ChimP) (Broughton *et al.* 2016), which employ UV-cross-linking of protein–RNA complexes *in vivo*, nuclease digestion of unprotected RNA, followed by IP of ALG-1 with

its bound RNA and cDNA sequencing of the ALG-1-linked mRNA sequences, have provided genome-scale data sets of microRNA-binding sites. As these methods become more widely used, particularly for specific cell types and for specific microRNA mutants, it will be possible to more definitively match microRNA to specific targets in particular contexts. The iCLIP and CLIP-seq methods are powerful strategies for identifying miRISC-binding sites, but unambiguous assignment of the microRNA recognizing those sites is not always possible, especially for microRNAs of the same seed family. Such ambiguities in assigning specific microRNAs to specific mRNA sites are overcome by analyzing the rare sequence reads that result from the ligation of a microRNA to a fragment of mRNA target that is cocross-linked to miRISC, so that microRNA–target tandem sequences are obtained from single-cDNA sequencing reads. Such microRNA–target chimeric sequences have been found in *C. elegans* iCLIP data sets (Broughton *et al.* 2016), as well as in data sets from protocols designed to enrich for the intermolecular ligation events (Helwak *et al.* 2013; Grosswendt *et al.* 2014).

So far, microRNA-target chimera sequencing has been applied in a limited fashion for *C. elegans*, but going forward, these approaches that identify chimeric microRNA–target sequences, especially if applied with improved efficiency compared to current applications and in a tissue-specific fashion, should permit high-confidence analysis of microRNA–target regulatory networks.

While approaches that identify microRNA–target site chimeras can confirm whether or not a particular microRNA actually binds to particular targets *in vivo*, measurements of the ribosome occupancy of target mRNAs and quantitation of the levels of proteins by mass spectrometry can provide additional evidence for the efficacy of the microRNA interaction with specific mRNAs. For example, the combined application of ribosome profiling and targeted quantitative proteomics, combined with 3′-UTR reporter assays, has enabled the discovery and validation of numerous functionally relevant *let-7* and *miR-58* targets (Jovanovic *et al.* 2010).

It should be noted that nothing is perfect and there are caveats attached to every method: ribosome occupancy does not necessarily reflect translation rate (see below) and indirect effects on protein turnover can confound interpreting protein levels. Nevertheless, the experimental arsenal available to *C. elegans* researchers—including genetic epistasis, mRNA sequencing, proteomics, ribosome profiling, CLASH, and CRISPR/Cas9 for the tagging of target genes *in loco* and for surgical mutagenesis of mRNA and microRNA complementary sequences (see below)—offers a gold standard for microRNA target discovery and validation.

Mechanisms of MicroRNA Repression of Target mRNAs

How do microRNAs repress the production of proteins from target mRNAs? In addition to microRNA and Argonaute, the miRISC complex contains other effector proteins, including notably GW182 (AIN-1/2), which are understood to mediate the repression of translation and/or accelerate mRNA turnover. Studies using *C. elegans* have contributed fundamentally to our understanding of the range of mRNA regulatory mechanisms that can be elicited by microRNA and have highlighted areas for future study, namely, how it is that miRISC can be programmed for different outcomes, depending on the microRNA, the particular mRNA sequence that it recognizes, and interactions with miRISC cofactors and RNA-binding proteins (*Regulators of miRISC activity*).

mRNA translational repression and/or mRNA turnover

The current understanding of microRNA repression, from invertebrate and vertebrate experimental systems, is that the chief mode of microRNA action is via interactions *in cis*, between the miRISC complex and the polyadenylation/deadenylation machinery, resulting in shortening of the poly(A) tail, a decrease in translation, 5′ end decapping, and degradation of the mRNA.

Numerous investigators, using *C. elegans* and other systems, have sought to determine whether the primary activity of miRISC is to trigger mRNA turnover, and hence to indirectly inhibit protein output, or whether miRISC can inhibit translation independently of mRNA turnover. Many studies have focused on the effects of microRNAs on target mRNA levels, perhaps because mRNAs are so much more easily quantified than proteins, especially at the genomic scale, but also because in assays for protein production (*e.g.*, measurement of luciferase activity produced from 3′-UTR reporters), microRNA regulation of translation activity and mRNA levels have often correlated.

Measuring the impact of microRNAs on the proteome, simultaneously with the quantitation of mRNA levels, can, in principle, resolve effects of microRNAs on mRNA abundance from translational repression and can be applied in high-throughput in *C. elegans*. For example, candidate targets of *mir-58*/bantam in *C. elegans* were identified by differential co-IP of mRNAs in wild-type *vs. mir-58* family mutants and validated by targeted proteomics. In this study, the targets that were confidently validated displayed behaviors consistent with regulation primarily at the level of protein abundance rather than mRNA stability (Jovanovic *et al.* 2012). Interestingly, mRNA and proteomic quantitation of *mir-58*/bantam targets in response to a progressive depletion of *mir-58* family members uncovered additional complexity, where translational inhibition was most evident in single *mir-58* family mutants; however, with depletion of the whole family, mRNA degradation was predominant.

Because the heterochronic microRNAs are deployed in a timed sequence, the heterochronic gene pathway is well suited for exploring the dynamics of target gene protein and mRNA levels after the initiation of microRNA-mediated repression. By monitoring *lin-14* protein and mRNA in finely staged larvae, a complex dynamic of mRNA and protein decline after developmental the induction of *lin-4* microRNA could be resolved, revealing the repression of mRNA levels and a decline of protein that proceeds with distinct kinetics

(Shi *et al.* 2013). For *lin-14*, a modest (two- to threefold decrease) in mRNA level was observed between the L1 (when *lin-4* is absent) and the L2 (when lin-4 is present) stages, while at the same time, the level of *lin-14* protein decreased much more (Shi *et al.* 2013). Similarly, the developmental dynamics of *let-7* target levels support an acute regulation of protein synthesis, followed by subsequent changes in mRNA levels (Stadler *et al.* 2012). This dynamic—a relatively rapid translational repression of targets after appearance of the microRNA, accompanied by a longer time course of target mRNA decay—is similar to that observed for certain cases of microRNA repression in zebrafish embryos (Bazzini *et al.* 2012) and *Drosophila* S2 cells (Djuranovic *et al.* 2012).

Interestingly, the apparent impact of microRNA repression on target protein *vs.* mRNA can vary, depending on the experimental context. For example, studies comparing wild-type to *lin-4* or *let-7* microRNA mutants at corresponding developmental stages indicated a more potent contribution of mRNA turnover than did other studies that followed the mRNA and protein dynamics of those targets during wild-type development (Bagga *et al.* 2005; Ding and Grosshans 2009; Holtz and Pasquinelli 2009; Stadler *et al.* 2012; Shi *et al.* 2013). These contrasting findings from the same system, using different experimental approaches, highlight how examining the kinetics of stage-specific microRNA-mediated repression in wild-type worms can resolve time-dependent components of the mechanism (translational repression followed by mRNA turnover), whereas comparing mutant to wild-type worms can emphasize the final state of the system (the eventual loss of the mRNA).

There is also evidence that the physiological context can modulate the relative contribution of translational repression and mRNA destabilization. For example, in larvae developing under conditions of limited nutrients, repression of *lin-14* by *lin-4* primarily involves repression of LIN-14 protein levels, and less so repression of *lin-14* mRNA levels (Holtz and Pasquinelli 2009), in contrast to nonstarved conditions, where mRNA degradation is more evident (Bagga *et al.* 2005).

What sorts of translational repression mechanisms can be invoked by miRISC? There are indications that microRNAs in *C. elegans* can impact translation at the initiation step (Ding *et al.* 2008; Ding and Grosshans 2009), and it is reasonable to suppose that the inhibition of translational initiation would be a predominant mechanism in *C. elegans*, as in other animals. However, there is also evidence that translational repression by *C. elegans* microRNAs, at least in some contexts, could occur postinitiation. *lin-14* mRNA remains associated with polyribosomes, even at stages where *lin-4* microRNA is abundant, suggesting a mode of translational repression in this case that occurs after translation initiation (Olsen and Ambros 1999). Polyribosome fractionation analysis of *lin-28* mRNA supported a similar mechanism for *lin-28* repression by *lin-4* (Seggerson *et al.* 2002). Genome-wide ribosomal profiling across multiple developmental time points also indicates that many of the heterochronic gene microRNA targets are subject to postinitiation translational repression (Stadler *et al.* 2012).

This apparent polyribosome-associated mode of translational repression by microRNAs may not be specific to nematodes, as a similar phenomenon was observed for *let-7* in mammalian cells (Nottrott *et al.* 2006). It seems relevant here to also mention that, at least in some systems, microRNA-mediated target mRNA destabilization can occur in association with ribosomes (Antic *et al.* 2015; Tat *et al.* 2016), which could provide one explanation for how postinitiation mechanisms for microRNA activity may not be incompatible with target degradation.

The association of a subset of microRNAs with RDE-1 (Steiner *et al.* 2007; Corrêa *et al.* 2010) suggests that RDE-1 may have roles in the activity of at least certain microRNAs, in addition to its major role as an siRNA effector in RNAi. Among the microRNAs found to complex with RDE-1 *in vivo*, *mir-243* is particularly noteworthy, in that *mir-243* is preferentially loaded into RDE-1 (compared to ALG-1/2) and is completely complementary to its primary mRNA target, Y47H10AA.5. Therefore, *mir-243* is essentially an endogenous siRNA. As expected for an siRNA carried by RDE-1, *mir-243* elicits a bloom of secondary endo-siRNAs that efficiently silence Y47H10AA.5 by RNAi (Corrêa *et al.* 2010). *mir-243* is highly unusual among *C. elegans* microRNAs (and among animal microRNAs in general) in having perfect complementarity to a target; animals seem to generally stick to the partial-complementarity mode of microRNA–target base pairing, likely to avoid obligatory target destruction. The function of the silencing of Y47H10AA.5 by *mir-243* is unknown, as *mir-243* mutant animals appear superficially normal (Miska *et al.* 2007). Another microRNA that seems to cause the cleavage of a target is *mir-249*; degradome sequencing of RNA from wild-type *vs.* *mir-249*(*lf*) animals has revealed evidence of *mir-249*-dependent cleavage of the transcript ZK637.6 (Park *et al.* 2013). The functions of *mir-249* and ZK637.6 are presently unknown.

MicroRNA–target base pairing

It is possible that distinct repressive outcomes could be mediated by structural properties of the base pairing between microRNAs and their targets. Whether the microRNA–Argonaute complex engages in seed-only base pairing or 3′ compensatory base pairing could in principle affect miRISC composition and therefore outcome. Distinct outcomes could be governed by accessory factors that associate with certain miRISC-containing microRNAs (for example by sequence-specific recognition of microRNAs) and/or the effects of miRISC–target conformation on the binding of specific cofactors. Conformational modeling of the *C. elegans* *let-7* miRISC bound to its target (Gan and Gunsalus 2015) supports the concept that its pairing configuration could affect the conformation ALG-2, and perhaps thereby effect miRISC assembly and function. An influence of the configuration of 3′-UTR elements on microRNA repression has also been noted (Flamand *et al.* 2016). Structural analyses of microRNA–target complexes, exemplified by the NMR analysis of *let-7* bound to synthetic *lin-41* 3′-UTR (Cevec *et al.* 2008, 2010)

and modeling of *let-7*::*lin-41* (Gan and Gunsalus 2013), promise to reveal principles of miRISC assembly and function that can be efficiently tested using *C. elegans in vivo* genetics.

Whether and how the members of microRNA seed families, which can differ in their nonseed nucleotides, might be deployed to regulate distinct target sets has been explored in *C. elegans* using the *let-7*::*lin-41* interaction. The evidence suggests that the configuration of base pairing between the microRNA and its target site, particularly in the 3′ part of the microRNA, can govern the specificity with which a particular microRNA family member associates with the site (Broughton *et al.* 2016).

Although it is likely that the majority of microRNA–target interactions involve primarily perfect seed pairing, there is evidence that noncanonical base pairing interactions are not uncommon. Sites for *lin-4* in the 3′-UTR of *lin-14* are predicted to contain bulged seed nucleotides, the presence of which impacts target repression, suggesting that microRNA–target structure is important in certain contexts (Ha *et al.* 1996) and that perfect seed pairing is not a hard rule. Structure–function experiments on the *lsy-6*::*cog-1* regulatory interaction support the idea that perfect seed pairing is not required (Didiano and Hobert 2006). Further, in the configuration of the two sites for *let-7* in the *lin-41* 3′-UTR, extensive predicted 3′ pairing and imperfect seed pairing (one site has a bulge, while the other has a G:U base pair) indicate that perfect seed pairing is not required as long as there is sufficient 3′ compensatory pairing (Vella *et al.* 2004).

Structure–function studies of the interactions of *lin-4* and *let-7* with their chief targets, in the context of their *in vivo* developmental phenotypes, have revealed interesting differences in the constraints on these two microRNAs, suggesting that some microRNAs depend on seed sequences more than others. For *lin-4*, a strong requirement for seed sequences was apparent, while for *let-7* there was a surprising tolerance for seed mutations, suggesting that *let-7* has a greater capacity for engaging in functional noncanonical interactions than does *lin-4* (Zhang *et al.* 2015).

In vitro analysis of microRNA mechanisms

MicroRNAs are stubbornly *in vivo* entities, with many connections, partners, and modulators that determine how they function in cells and animals. For this reason, *C. elegans* is a particularly appropriate system for the exploitation of *in vivo* genetics and cell biology to probe how microRNAs function in the context of an intact developing and behaving animal. However, biochemical approaches are ultimately required to fully understand how microRNAs work. An extraordinarily promising system was been devised using *C. elegans* embryo extracts programmed with synthetic mRNAs whose translation, stability, and poly(A) status can be monitored quantitatively (Wu *et al.* 2010). In this system, reporters containing 3′-UTR sequences from putative microRNA targets were regulated by endogenous embryonic microRNAs, enabling the characterization of microRNA mechanisms. The reporters containing natural 3′-UTRs exhibited rapid microRNA-dependent deadenylation and were translationally repressed. Interestingly, single sites did not work well, suggesting that cooperativity exists between miRISC complexes colocated in a UTR.

Surprisingly, many of the targets that were rapidly deadenylated in response to microRNA activity *in vitro* were stable, suggesting a novel mechanism of microRNA repression where targets appear to be converted (perhaps reversibly) to a translationally quiescent status. It is possible that the *C. elegans* embryo extract system is an example of a context where deadenylation can be uncoupled from decapping and degradation (Wu *et al.* 2010). It is also possible that microRNA-mediated gene regulation could differ substantially between the embryo and other stages. For example, targeted mutation of ALG-1 to eliminate the tryptophan-binding sites for AIN-1 and AIN-2 has been found to have little effect on embryonic development, but disrupts microRNA function in larval stages (Jannot *et al.* 2016).

The *C. elegans* embryo cell-free system (Wu *et al.* 2010) can be leveraged for the biochemical characterization of miRISC complexes and genetic tests of candidate microRNA cofactors and effectors (by employing extracts treated with RNAi or from mutants). In this fashion, roles have been uncovered for cytoplasmic poly(A)-binding proteins in microRNA-mediated deadenylation and in poly(A)-dependent and poly(A)-independent translational repression (Flamand *et al.* 2016).

Conclusions

C. elegans has remained in the thick of microRNA research and should continue to do so. The experimental toolkit continues to grow, and the use of CRISPR/Cas9 genome editing permits essentially any defined modification being made to microRNAs and target sequences in their natural genomic context. It should be possible to explore and uncover the principles of microRNA recognition *in vivo* using *C. elegans* genetics with greater efficiency than in any other organism.

Regulators of microRNA biogenesis, stability, and activity should continue to emerge from genetic screens, miRISC proteomics, and RNAi modifier screens. Whether and how specific miRISC complexes may be customized for specific outcomes remains an open question, and much remains to be learned from *C. elegans* about how specific microRNAs can be deployed to regulate particular targets, depending on the cellular or physiological context.

Further questions remain about the potential for cell nonautonomous activity of microRNAs in multicellular animals. In *C. elegans*, cell autonomy of microRNA function has been tested for only a handful of microRNAs: *lin-4* (Zhang and Fire 2010), *let-7* (Zhi *et al.* 2017), and *mir-34* and *mir-83* (Burke *et al.* 2015). The fact that RNAi can spread among cells in *C. elegans* indicates that the worm possesses mechanisms for the intracellular transport of RNA, and so it is conceivable that microRNAs could be similarly deployed extracellularly in some contexts.

Mechanisms of how microRNAs are regulated post-transcriptionally in response to developmental and physiological

signals are ripe for investigation. For example, certain *C. elegans* microRNAs undergo rapid developmental downregulation, while others remain constant or increase. It is essentially unknown in any system how the rates of biogenesis and turnover of different microRNAs are programmed and regulated and, as usual, *C. elegans* is the ideal system to investigate such questions *in vivo*.

Acknowledgments

Research in VA's laboratory is supported by NIH GM34028. Research in GR's laboratory is supported by NIH GM44619. The authors thank Gloria Ha for help with Figure 1. The authors also thank John Kim and the WormBook editors and anonymous reviewers for advice and criticism.

Literature Cited

Abbott, A. L., E. Alvarez-Saavedra, E. A. Miska, N. C. Lau, D. P. Bartel *et al.*, 2005 The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev. Cell 9: 403–414. https://doi.org/10.1016/j.devcel.2005.07.009

Abrahante, J. E., A. L. Daul, M. Li, M. L. Volk, J. M. Tennessen *et al.*, 2003 The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. *Dev. Cell* 4: 625–637.

Agarwal, V., G. W. Bell, J. W. Nam, and D. P. Bartel, 2015 Predicting effective microRNA target sites in mammalian mRNAs. Elife 4: e05005. https://doi.org/10.7554/eLife.05005

Alessi, A. F., V. Khivansara, T. Han, M. A. Freeberg, J. J. Moresco *et al.*, 2015 Casein kinase II promotes target silencing by miRISC through direct phosphorylation of the DEAD-box RNA helicase CGH-1. *Proc. Natl. Acad. Sci. USA* 112: E7213–E7222. https://doi.org/10.1073/pnas.1509499112

Alvarez-Saavedra, E., and H. R. Horvitz, 2010 Many families of C. elegans microRNAs are not essential for development or viability. *Curr. Biol.* 20: 367–373. https://doi.org/10.1016/j.cub.2009.12.051

Ambros, V., 1989 A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell 57: 49–57. https://doi.org/10.1016/0092-8674(89)90171-2

Ambros, V., and H. R. Horvitz, 1984 Heterochronic mutants of the nematode Caenorhabditis elegans. *Science* 226: 409–416

Ambros, V., and H. R. Horvitz, 1987 The lin-14 locus of Caenorhabditis elegans controls the time of expression of specific postembryonic developmental events. Genes Dev. 1: 398–414. https://doi.org/10.1101/gad.1.4.398

Antic, S., M. T. Wolfinger, A. Skucha, S. Hosiner, and S. Dorner, 2015 General and microRNA-mediated mRNA degradation occurs on ribosome complexes in Drosophila cells. Mol. Cell. Biol. 35: 2309–2320. https://doi.org/10.1128/MCB.01346-14

Bagga, S., J. Bracht, S. Hunter, K. Massirer, J. Holtz *et al.*, 2005 Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122: 553–563.

Bazzini, A. A., M. T. Lee, and A. J. Giraldez, 2012 Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science 336: 233–237. https://doi.org/10.1126/science.1215704

Bethke, A., N. Fielenbach, Z. Wang, D. J. Mangelsdorf, and A. Antebi, 2009 Nuclear hormone receptor regulation of microRNAs controls developmental progression. *Science* 324: 95–98. https://doi.org/10.1126/science.1164899

Boehm, M., and F. Slack, 2005 A developmental timing microRNA and its target regulate life span in C. elegans. *Science* 310: 1954–1957. https://doi.org/10.1126/science.1115596

Bossé, G. D., S. Rüegger, M. C. Ow, A. Vasquez-Rifo, E. L. Rondeau *et al.*, 2013 The decapping scavenger enzyme DCS-1 controls microRNA levels in Caenorhabditis elegans. Mol. Cell 50: 281–287. https://doi.org/10.1016/j.molcel.2013.02.023

Boulias, K., and H. R. Horvitz, 2012 The C. elegans microRNA mir-71 acts in neurons to promote germline-mediated longevity through regulation of DAF-16/FOXO. *Cell Metab.* 15: 439–450. https://doi.org/10.1016/j.cmet.2012.02.014

Brenner, J. L., K. L. Jasiewicz, A. F. Fahley, B. J. Kemp, and A. L. Abbott, 2010 Loss of individual microRNAs causes mutant phenotypes in sensitized genetic backgrounds in C. elegans. *Curr. Biol.* 20: 1321–1325. https://doi.org/10.1016/j.cub.2010.05.062

Brenner, J. L., B. J. Kemp, and A. L. Abbott, 2012 The mir-51 family of microRNAs functions in diverse regulatory pathways in Caenorhabditis elegans. PLoS One 7: e37185. https://doi.org/10.1371/journal.pone.0037185

Broughton, J. P., and A. E. Pasquinelli, 2013 Identifying Argonaute binding sites in Caenorhabditis elegans using iCLIP. Methods 63: 119–125. https://doi.org/10.1016/j.ymeth.2013.03.033

Broughton, J. P., M. T. Lovci, J. L. Huang, G. W. Yeo, and A. E. Pasquinelli, 2016 Pairing beyond the seed supports microRNA targeting specificity. Mol. Cell 64: 320–333. https://doi.org/10.1016/j.molcel.2016.09.004

Brown, K. C., J. M. Svendsen, R. M. Tucci, B. E. Montgomery, and T. A. Montgomery, 2017 ALG-5 is a miRNA-associated Argonaute required for proper developmental timing in the Caenorhabditis elegans germline. Nucleic Acids Res. 45: 9093–9107. https://doi.org/10.1093/nar/gkx536

Bukhari, S. I., A. Vasquez-Rifo, D. Gagne, E. R. Paquet, M. Zetka *et al.*, 2012 The microRNA pathway controls germ cell proliferation and differentiation in C. elegans. Cell Res. 22: 1034–1045. https://doi.org/10.1038/cr.2012.31

Burke, S. L., M. Hammell, and V. Ambros, 2015 Robust distal tip cell pathfinding in the face of temperature stress is ensured by two conserved microRNAs in Caenorhabditis elegans. Genetics 200: 1201–1218. https://doi.org/10.1534/genetics.115.179184

Büssing, I., J. S. Yang, E. C. Lai, and H. Grosshans, 2010 The nuclear export receptor XPO-1 supports primary miRNA processing in C. elegans and Drosophila. EMBO J. 29: 1830–1839. https://doi.org/10.1038/emboj.2010.82

Cevec, M., C. Thibaudeau, and J. Plavec, 2008 Solution structure of a let-7 miRNA:lin-41 mRNA complex from C. elegans. *Nucleic Acids Res.* 36: 2330–2337. https://doi.org/10.1093/nar/gkn088

Cevec, M., C. Thibaudeau, and J. Plavec, 2010 NMR structure of the let-7 miRNA interacting with the site LCS1 of lin-41 mRNA from Caenorhabditis elegans. *Nucleic Acids Res.* 38: 7814–7821. https://doi.org/10.1093/nar/gkq640

Chalfie, M., H. R. Horvitz, and J. E. Sulston, 1981 Mutations that lead to reiterations in the cell lineages of C. elegans. *Cell* 24: 59–69.

Chan, S. P., and F. J. Slack, 2009 Ribosomal protein RPS-14 modulates let-7 microRNA function in Caenorhabditis elegans. Dev. Biol. 334: 152–160. https://doi.org/10.1016/j.ydbio.2009.07.011

Chang, S., R. J. Johnston, Jr., C. Frokjaer-Jensen, S. Lockery, and O. Hobert, 2004 MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. Nature 430: 785–789. https://doi.org/10.1038/nature02752

Chatterjee, S., and H. Grosshans, 2009 Active turnover modulates mature microRNA activity in Caenorhabditis elegans. *Nature* 461: 546–549. https://doi.org/10.1038/nature08349

Chatterjee, S., M. Fasler, I. Bussing, and H. Grosshans, 2011 Target-mediated protection of endogenous microRNAs in C. elegans. Dev. Cell 20: 388–396. https://doi.org/10.1016/j.devcel.2011.02.008

Chung, W. J., P. Agius, J. O. Westholm, M. Chen, K. Okamura *et al.*, 2011 Computational and experimental identification of mirtrons

in Drosophila melanogaster and Caenorhabditis elegans. Genome Res. 21: 286–300. https://doi.org/10.1101/gr.113050.110

Cochella, L., and O. Hobert, 2012 Embryonic priming of a miRNA locus predetermines postmitotic neuronal left/right asymmetry in C. elegans. Cell 151: 1229–1242. https://doi.org/10.1016/j.cell.2012.10.049

Corrêa, R. L., F. A. Steiner, E. Berezikov, and R. F. Ketting, 2010 MicroRNA-directed siRNA biogenesis in Caenorhabditis elegans. PLoS Genet. 6: e1000903. https://doi.org/10.1371/journal.pgen.1000903

Dai, L. L., J. X. Gao, C. G. Zou, Y. C. Ma, and K. Q. Zhang, 2015 mir-233 modulates the unfolded protein response in C. elegans during Pseudomonas aeruginosa infection. PLoS Pathog. 11: e1004606. https://doi.org/10.1371/journal.ppat.1004606

de Lencastre, A., Z. Pincus, K. Zhou, M. Kato, S. S. Lee *et al.*, 2010 MicroRNAs both promote and antagonize longevity in C. elegans. Curr. Biol. 20: 2159–2168. https://doi.org/10.1016/j.cub.2010.11.015

de Lucas, M. P., A. G. Saez, and E. Lozano, 2015 miR-58 family and TGF-beta pathways regulate each other in Caenorhabditis elegans. Nucleic Acids Res. 43: 9978–9993. https://doi.org/10.1093/nar/gkv923

Denli, A. M., B. B. Tops, R. H. Plasterk, R. F. Ketting, and G. J. Hannon, 2004 Processing of primary microRNAs by the microprocessor complex. Nature 432: 231–235. https://doi.org/10.1038/nature03049

Didiano, D., and O. Hobert, 2006 Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. Nat. Struct. Mol. Biol. 13: 849–851. https://doi.org/10.1038/nsmb1138

Ding, L., A. Spencer, K. Morita, and M. Han, 2005 The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. *Mol. Cell* 19: 437–447. https://doi.org/10.1016/j.molcel.2005.07.013

Ding, X. C., and H. Grosshans, 2009 Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. *EMBO J.* 28: 213–222. https://doi.org/10.1038/emboj.2008.275

Ding, X. C., F. J. Slack, and H. Grosshans, 2008 The let-7 microRNA interfaces extensively with the translation machinery to regulate cell differentiation. *Cell Cycle* 7: 3083–3090. https://doi.org/10.4161/cc.7.19.6778

Djuranovic, S., A. Nahvi, and R. Green, 2012 miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science* 336: 237–240. https://doi.org/10.1126/science.1215691

Dowen, R. H., P. C. Breen, T. Tullius, A. L. Conery, and G. Ruvkun, 2016 A microRNA program in the C. elegans hypodermis couples to intestinal mTORC2/PQM-1 signaling to modulate fat transport. Genes Dev. 30: 1515–1528. https://doi.org/10.1101/gad.283895.116

Drake, M., T. Furuta, K. M. Suen, G. Gonzalez, B. Liu *et al.*, 2014 A requirement for ERK-dependent Dicer phosphorylation in coordinating oocyte-to-embryo transition in C. elegans. Dev. Cell 31: 614–628. https://doi.org/10.1016/j.devcel.2014.11.004

Drexel, T., K. Mahofsky, R. Latham, M. Zimmer, and L. Cochella, 2016 Neuron type-specific miRNA represses two broadly expressed genes to modulate an avoidance behavior in C. elegans. Genes Dev. 30: 2042–2047. https://doi.org/10.1101/gad.287904.116

Ecsedi, M., M. Rausch, and H. Großhans, 2015 The let-7 microRNA directs vulval development through a single target. *Dev. Cell* 32: 335–344. https://doi.org/10.1016/j.devcel.2014.12.018

Euling, S., and V. Ambros, 1996 Heterochronic genes control cell cycle progress and developmental competence of C. elegans vulva precursor cells. Cell 84: 667–676. https://doi.org/10.1016/S0092-8674(00)81045-4

Ferguson, E. L., and H. R. Horvitz, 1985 Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode Caenorhabditis elegans. *Genetics* 110: 17–72.

Flamand, M. N., E. Wu, A. Vashisht, G. Jannot, B. D. Keiper *et al.*, 2016 Poly(A)-binding proteins are required for microRNA-mediated silencing and to promote target deadenylation in C. elegans. Nucleic Acids Res. 44: 5924–5935. https://doi.org/10.1093/nar/gkw276

Gan, H. H., and K. C. Gunsalus, 2013 Tertiary structure-based analysis of microRNA-target interactions. *RNA* 19: 539–551. https://doi.org/10.1261/rna.035691.112

Gan, H. H., and K. C. Gunsalus, 2015 Assembly and analysis of eukaryotic Argonaute-RNA complexes in microRNA-target recognition. *Nucleic Acids Res.* 43: 9613–9625. https://doi.org/10.1093/nar/gkv990

Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish *et al.*, 2001 Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106: 23–34. https://doi.org/10.1016/S0092-8674(01)00431-7

Grosshans, H., T. Johnson, K. L. Reinert, M. Gerstein, and F. J. Slack, 2005 The temporal patterning microRNA let-7 regulates several transcription factors at the larval to adult transition in C. elegans. Dev. Cell 8: 321–330. https://doi.org/10.1016/j.devcel.2004.12.019

Grosswendt, S., A. Filipchyk, M. Manzano, F. Klironomos, M. Schilling *et al.*, 2014 Unambiguous identification of miRNA:target site interactions by different types of ligation reactions. Mol. Cell 54: 1042–1054. https://doi.org/10.1016/j.molcel.2014.03.049

Ha, I., B. Wightman, and G. Ruvkun, 1996 A bulged lin-4/lin-14 RNA duplex is sufficient for Caenorhabditis elegans lin-14 temporal gradient formation. Genes Dev. 10: 3041–3050. https://doi.org/10.1101/gad.10.23.3041

Hallam, S. J., and Y. Jin, 1998 lin-14 regulates the timing of synaptic remodelling in Caenorhabditis elegans. Nature 395: 78–82. https://doi.org/10.1038/25757

Hammell, C. M., X. Karp, and V. Ambros, 2009a A feedback circuit involving let-7-family miRNAs and DAF-12 integrates environmental signals and developmental timing in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 106: 18668–18673. https://doi.org/10.1073/pnas.0908131106

Hammell, C. M., I. Lubin, P. R. Boag, T. K. Blackwell, and V. Ambros, 2009b nhl-2 modulates microRNA activity in Caenorhabditis elegans. Cell 136: 926–938. https://doi.org/10.1016/j.cell.2009.01.053

Hammell, M., D. Long, L. Zhang, A. Lee, C. S. Carmack *et al.*, 2008 mirWIP: microRNA target prediction based on microRNA-containing ribonucleoprotein-enriched transcripts. Nat. Methods 5: 813–819. https://doi.org/10.1038/nmeth.1247

Hayes, G. D., A. R. Frand, and G. Ruvkun, 2006 The mir-84 and let-7 paralogous microRNA genes of Caenorhabditis elegans direct the cessation of molting via the conserved nuclear hormone receptors NHR-23 and NHR-25. *Development* 133: 4631–4641. https://doi.org/10.1242/dev.02655

Helwak, A., G. Kudla, T. Dudnakova, and D. Tollervey, 2013 Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell* 153: 654–665. https://doi.org/10.1016/j.cell.2013.03.043

Holtz, J., and A. E. Pasquinelli, 2009 Uncoupling of lin-14 mRNA and protein repression by nutrient deprivation in Caenorhabditis elegans. RNA 15: 400–405. https://doi.org/10.1261/rna.1258309

Hsieh, Y.-W., C. Chang, and C.-F. Chuang, 2012 The microRNA mir-71 inhibits calcium signaling by targeting the TIR-1/Sarm1 adaptor protein to control stochastic L/R neuronal asymmetry in C. elegans. *PLoS Genet.* 8: e1002864. https://doi.org/10.1371/journal.pgen.1002864

Hunter, S. E., E. F. Finnegan, D. G. Zisoulis, M. T. Lovci, K. V. Melnik-Martinez *et al.*, 2013 Functional genomic analysis of the let-7 regulatory network in Caenorhabditis elegans. *PLoS Genet.* 9: e1003353. https://doi.org/10.1371/journal.pgen.1003353

Hurschler, B. A., D. T. Harris, and H. Grosshans, 2011 The type II poly(A)-binding protein PABP-2 genetically interacts with the let-7 miRNA and elicits heterochronic phenotypes in Caenorhabditis elegans. Nucleic Acids Res. 39: 5647–5657. https://doi.org/10.1093/nar/gkr145

Iatsenko, I., A. Sinha, C. Rodelsperger, and R. J. Sommer, 2013 New role for DCR-1/dicer in Caenorhabditis elegans innate immunity against the highly virulent bacterium Bacillus thuringiensis DB27. Infect. Immun. 81: 3942–3957. https://doi.org/10.1128/IAI.00700-13

Ibáñez-Ventoso, C., M. Yang, S. Guo, H. Robins, R. W. Padgett *et al.*, 2006 Modulated microRNA expression during adult lifespan in Caenorhabditis elegans. *Aging Cell* 5: 235–246. https://doi.org/10.1111/j.1474-9726.2006.00210.x

Isik, M., T. K. Blackwell, and E. Berezikov, 2016 MicroRNA mir-34 provides robustness to environmental stress response via the DAF-16 network in C. elegans. Sci. Rep. 6: 36766. https://doi.org/10.1038/srep36766

Jannot, G., S. Bajan, N. J. Giguère, S. Bouasker, I. H. Banville *et al.*, 2011 The ribosomal protein RACK1 is required for microRNA function in both C. elegans and humans. *EMBO Rep.* 12: 581–586. https://doi.org/10.1038/embor.2011.66

Jannot, G., P. Michaud, M. Quevillon Huberdeau, L. Morel-Berryman, J. A. Brackbill *et al.*, 2016 GW182-free microRNA silencing complex controls post-transcriptional gene expression during Caenorhabditis elegans embryogenesis. PLoS Genet. 12: e1006484 (erratum: PLoS Genet. 12: e1006534). https://doi.org/10.1371/journal.pgen.1006484

Jeon, M., H. F. Gardner, E. A. Miller, J. Deshler, and A. E. Rougvie, 1999 Similarity of the C. elegans developmental timing protein LIN-42 to circadian rhythm proteins. Science 286: 1141–1146. https://doi.org/10.1126/science.286.5442.1141

Johnson, S. M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis *et al.*, 2005 RAS is regulated by the let-7 microRNA family. Cell 120: 635–647. https://doi.org/10.1016/j.cell.2005.01.014

Johnston, R. J., and O. Hobert, 2003 A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature 426: 845–849. https://doi.org/10.1038/nature02255

Johnston, R. J., Jr., and O. Hobert, 2005 A novel C. elegans zinc finger transcription factor, lsy-2, required for the cell type-specific expression of the lsy-6 microRNA. Development 132: 5451–5460. https://doi.org/10.1242/dev.02163

Jovanovic, M., L. Reiter, P. Picotti, V. Lange, E. Bogan *et al.*, 2010 A quantitative targeted proteomics approach to validate predicted microRNA targets in C. elegans. Nat. Methods 7: 837–842. https://doi.org/10.1038/nmeth.1504

Jovanovic, M., L. Reiter, A. Clark, M. Weiss, P. Picotti *et al.*, 2012 RIP-chip-SRM–a new combinatorial large-scale approach identifies a set of translationally regulated bantam/miR-58 targets in C. elegans. Genome Res. 22: 1360–1371. https://doi.org/10.1101/gr.133330.111

Kagias, K., and R. Pocock, 2015 microRNA regulation of the embryonic hypoxic response in Caenorhabditis elegans. *Sci. Rep.* 5: 11284. https://doi.org/10.1038/srep11284

Kai, Z. S., E. F. Finnegan, S. Huang, and A. E. Pasquinelli, 2013 Multiple cis-elements and trans-acting factors regulate dynamic spatio-temporal transcription of let-7 in Caenorhabditis elegans. *Dev. Biol.* 374: 223–233. https://doi.org/10.1016/j.ydbio.2012.11.021

Karp, X., and V. Ambros, 2011 The developmental timing regulator HBL-1 modulates the dauer formation decision in Caenorhabditis elegans. Genetics 187: 345–353. https://doi.org/10.1534/genetics.110.123992

Karp, X., and V. Ambros, 2012 Dauer larva quiescence alters the circuitry of microRNA pathways regulating cell fate progression in C. elegans. Development 139: 2177–2186. https://doi.org/10.1242/dev.075986

Karp, X., M. Hammell, M. C. Ow, and V. Ambros, 2011 Effect of life history on microRNA expression during C. elegans development. RNA 17: 639–651. https://doi.org/10.1261/rna.2310111

Kasuga, H., M. Fukuyama, A. Kitazawa, K. Kontani, and T. Katada, 2013 The microRNA miR-235 couples blast-cell quiescence to the nutritional state. Nature 497: 503–506. https://doi.org/10.1038/nature12117

Kato, M., T. Paranjape, R. U. Müller, S. Nallur, E. Gillespie *et al.*, 2009 The mir-34 microRNA is required for the DNA damage response in vivo in C. elegans and in vitro in human breast cancer cells. Oncogene 28: 2419–2424 (erratum: Oncogene 28: 3008). https://doi.org/10.1038/onc.2009.106

Kato, M., X. Chen, S. Inukai, H. Zhao, and F. J. Slack, 2011 Age-associated changes in expression of small, noncoding RNAs, including microRNAs, in C. elegans. RNA 17: 1804–1820. https://doi.org/10.1261/rna.2714411

Kato, M., M. A. Kashem, and C. Cheng, 2016 An intestinal microRNA modulates the homeostatic adaptation to chronic oxidative stress in C. elegans. Aging (Albany N.Y.) 8: 1979–2005. https://doi.org/10.18632/aging.101029

Kemp, B. J., E. Allman, L. Immerman, M. Mohnen, M. A. Peters *et al.*, 2012 miR-786 regulation of a fatty-acid elongase contributes to rhythmic calcium-wave initiation in C. elegans. *Curr. Biol.* 22: 2213–2220. https://doi.org/10.1016/j.cub.2012.09.047

Kim, J. K., H. W. Gabel, R. S. Kamath, M. Tewari, A. Pasquinelli *et al.*, 2005 Functional genomic analysis of RNA interference in C. elegans. *Science* 308: 1164–1167. https://doi.org/10.1126/science.1109267

Kudlow, B. A., L. Zhang, and M. Han, 2012 Systematic analysis of tissue-restricted miRISCs reveals a broad role for microRNAs in suppressing basal activity of the C. elegans pathogen response. Mol. Cell 46: 530–541. https://doi.org/10.1016/j.molcel.2012.03.011

Lagos-Quintana, M., R. Rauhut, W. Lendeckel, and T. Tuschl, 2001 Identification of novel genes coding for small expressed RNAs. *Science* 294: 853–858. https://doi.org/10.1126/science.1064921

Lall, S., D. Grun, A. Krek, K. Chen, Y. L. Wang *et al.*, 2006 A genome-wide map of conserved microRNA targets in C. elegans. Curr. Biol. 16: 460–471. https://doi.org/10.1016/j.cub.2006.01.050

Lau, N. C., L. P. Lim, E. G. Weinstein, and D. P. Bartel, 2001 An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* 294: 858–862. https://doi.org/10.1126/science.1065062

Lee, R. C., and V. Ambros, 2001 An extensive class of small RNAs in Caenorhabditis elegans. Science 294: 862–864. https://doi.org/10.1126/science.1065329

Lee, R. C., R. L. Feinbaum, and V. Ambros, 1993 The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843–854. https://doi.org/10.1016/0092-8674(93)90529-Y

LeGendre, J. B., Z. T. Campbell, P. Kroll-Conner, P. Anderson, J. Kimble *et al.*, 2013 RNA targets and specificity of Staufen, a double-stranded RNA-binding protein in Caenorhabditis elegans. J. Biol. Chem. 288: 2532–2545. https://doi.org/10.1074/jbc.M112.397349

Lehrbach, N. J., J. Armisen, H. L. Lightfoot, K. J. Murfitt, A. Bugaut *et al.*, 2009 LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in Caenorhabditis elegans. Nat. Struct. Mol. Biol. 16: 1016–1020. https://doi.org/10.1038/nsmb.1675

Lehrbach, N. J., C. Castro, K. J. Murfitt, C. Abreu-Goodger, J. L. Griffin *et al.*, 2012 Post-developmental microRNA expression is required for normal physiology, and regulates aging in parallel to insulin/IGF-1 signaling in C. elegans. RNA 18: 2220–2235. https://doi.org/10.1261/rna.035402.112

Lewis, B. P., C. B. Burge, and D. P. Bartel, 2005 Conserved seed pairing, often flanked by adenosines, indicates that thousands of

human genes are microRNA targets. *Cell* 120: 15–20. https://doi.org/10.1016/j.cell.2004.12.035

Li, J., and I. Greenwald, 2010 LIN-14 inhibition of LIN-12 contributes to precision and timing of C. elegans vulval fate patterning. Curr. Biol. 20: 1875–1879. https://doi.org/10.1016/j.cub.2010.09.055

Li, S., B. Le, X. Ma, S. Li, C. You *et al.*, 2016 Biogenesis of phased siRNAs on membrane-bound polysomes in Arabidopsis. Elife 5: e22750. https://doi.org/10.7554/eLife.22750

Lin, S. Y., S. M. Johnson, M. Abraham, M. C. Vella, A. Pasquinelli *et al.*, 2003 The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Dev. Cell 4: 639–650. https://doi.org/10.1016/S1534-5807(03)00124-2

Liu, F., C.-X. He, L.-J. Luo, Q.-L. Zou, Y.-X. Zhao *et al.*, 2013 Nuclear hormone receptor regulation of microRNAs controls innate immune responses in *C. elegans*. PLoS Pathog. 9: e1003545. https://doi.org/10.1371/journal.ppat.1003545

Liu, Z., S. Kirch, and V. Ambros, 1995 The Caenorhabditis elegans heterochronic gene pathway controls stage-specific transcription of collagen genes. Development 121: 2471–2478.

Liu, Z. C., and V. Ambros, 1989 Heterochronic genes control the stage-specific initiation and expression of the dauer larva developmental program in Caenorhabditis elegans. Genes Dev. 3: 2039–2049. https://doi.org/10.1101/gad.3.12b.2039

Lozano, E., M. P. de Lucas, and A. G. Saez, 2016 sta-1 is repressed by mir-58 family in Caenorhabditis elegans. Worm 5: e1238560. https://doi.org/10.1080/21624054.2016.1238560

Ma, Y. C., L. Zhang, L. L. Dai, R. U. Khan, and C. G. Zou, 2017 mir-67 regulates P. aeruginosa avoidance behavior in C. elegans. Biochem. Biophys. Res. Commun. 494: 120–125. https://doi.org/10.1016/j.bbrc.2017.10.069

Martinez, N. J., M. C. Ow, M. I. Barrasa, M. Hammell, R. Sequerra *et al.*, 2008a A C. elegans genome-scale microRNA network contains composite feedback motifs with high flux capacity. Genes Dev. 22: 2535–2549. https://doi.org/10.1101/gad.1678608

Martinez, N. J., M. C. Ow, J. S. Reece-Hoyes, M. I. Barrasa, V. R. Ambros *et al.*, 2008b Genome-scale spatiotemporal analysis of Caenorhabditis elegans microRNA promoter activity. Genome Res. 18: 2005–2015. https://doi.org/10.1101/gr.083055.108

McCulloch, K. A., and A. E. Rougvie, 2014 Caenorhabditis elegans period homolog lin-42 regulates the timing of heterochronic miRNA expression. *Proc. Natl. Acad. Sci. USA* 111: 15450–15455. https://doi.org/10.1073/pnas.1414856111

McEwen, T. J., Q. Yao, S. Yun, C. Y. Lee, and K. L. Bennett, 2016 Small RNA in situ hybridization in Caenorhabditis elegans, combined with RNA-seq, identifies germline-enriched microRNAs. Dev. Biol. 418: 248–257. https://doi.org/10.1016/j.ydbio.2016.08.003

McJunkin, K., and V. Ambros, 2014 The embryonic mir-35 family of microRNAs promotes multiple aspects of fecundity in Caenorhabditis elegans. G3 (Bethesda) 4: 1747–1754. https://doi.org/10.1534/g3.114.011973

McJunkin, K., and V. Ambros, 2017 A microRNA family exerts maternal control on sex determination in C. elegans. Genes Dev. 31: 422–437. https://doi.org/10.1101/gad.290155.116

Metheetrairut, C., B. D. Adams, S. Nallur, J. B. Weidhaas, and F. J. Slack, 2017 cel-mir-237 and its homologue, hsa-miR-125b, modulate the cellular response to ionizing radiation. Oncogene 36: 512–524. https://doi.org/10.1038/onc.2016.222

Micklem, D. R., J. Adams, S. Grunert, and D. St Johnston, 2000 Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. EMBO J. 19: 1366–1377. https://doi.org/10.1093/emboj/19.6.1366

Miki, T. S., S. Ruegger, D. Gaidatzis, M. B. Stadler, and H. Grosshans, 2014 Engineering of a conditional allele reveals multiple roles of XRN2 in Caenorhabditis elegans development and substrate specificity in microRNA turnover. Nucleic Acids Res. 42: 4056–4067. https://doi.org/10.1093/nar/gkt1418

Miska, E. A., E. Alvarez-Saavedra, A. L. Abbott, N. C. Lau, A. B. Hellman *et al.*, 2007 Most Caenorhabditis elegans microRNAs are individually not essential for development or viability. PLoS Genet. 3: e215. https://doi.org/10.1371/journal.pgen.0030215

Mondol, V., B. C. Ahn, and A. E. Pasquinelli, 2015 Splicing remodels the let-7 primary microRNA to facilitate Drosha processing in Caenorhabditis elegans. *RNA* 21: 1396–1403. https://doi.org/10.1261/rna.052118.115

Moss, E. G., R. C. Lee, and V. Ambros, 1997 The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell 88: 637–646. https://doi.org/10.1016/S0092-8674(00)81906-6

Nehammer, C., A. Podolska, S. D. Mackowiak, K. Kagias, and R. Pocock, 2015 Specific microRNAs regulate heat stress responses in Caenorhabditis elegans. Sci. Rep. 5: 8866. https://doi.org/10.1038/srep08866

Nolde, M. J., N. Saka, K. L. Reinert, and F. J. Slack, 2007 The Caenorhabditis elegans pumilio homolog, puf-9, is required for the 3′UTR-mediated repression of the let-7 microRNA target gene, hbl-1. Dev. Biol. 305: 551–563. https://doi.org/10.1016/j.ydbio.2007.02.040

Nottrott, S., M. J. Simard, and J. D. Richter, 2006 Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat. Struct. Mol. Biol.* 13: 1108–1114. https://doi.org/10.1038/nsmb1173

Olsen, P. H., and V. Ambros, 1999 The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. 216: 671–680. https://doi.org/10.1006/dbio.1999.9523

Olsson-Carter, K., and F. J. Slack, 2010 A developmental timing switch promotes axon outgrowth independent of known guidance receptors. *PLoS Genet.* 6: e1001054. https://doi.org/10.1371/journal.pgen.1001054

Pagano, D. J., E. R. Kingston, and D. H. Kim, 2015 Tissue expression pattern of PMK-2 p38 MAPK is established by the miR-58 family in C. elegans. PLoS Genet. 11: e1004997 (erratum: PLoS Genet. 11: e1005317). https://doi.org/10.1371/journal.pgen.1004997

Pandit, A., V. Jain, N. Kumar, and A. Mukhopadhyay, 2014 PHA-4/FOXA-regulated microRNA feed forward loops during Caenorhabditis elegans dietary restriction. *Aging (Albany N.Y.)* 6: 835–855. https://doi.org/10.18632/aging.100697

Park, J. H., S. Ahn, S. Kim, J. Lee, J.-W. Nam *et al.*, 2013 Degradome sequencing reveals an endogenous microRNA target in C. elegans. *FEBS Lett.* 587: 964–969. https://doi.org/10.1016/j.febslet.2013.02.029

Parry, D. H., J. Xu, and G. Ruvkun, 2007 A whole-genome RNAi Screen for C. elegans miRNA pathway genes. Curr. Biol. 17: 2013–2022. https://doi.org/10.1016/j.cub.2007.10.058

Pasquinelli, A. E., B. J. Reinhart, F. Slack, M. Q. Martindale, M. I. Kuroda *et al.*, 2000 Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408: 86–89. https://doi.org/10.1038/35040556

Pedersen, M. E., G. Snieckute, K. Kagias, C. Nehammer, H. A. B. Multhaupt *et al.*, 2013 An epidermal microRNA regulates neuronal migration through control of the cellular glycosylation state. *Science* 341: 1404–1408. https://doi.org/10.1126/science.1242528

Perales, R., D. M. King, C. Aguirre-Chen, and C. M. Hammell, 2014 LIN-42, the Caenorhabditis elegans PERIOD homolog, negatively regulates microRNA transcription. PLoS Genet. 10: e1004486. https://doi.org/10.1371/journal.pgen.1004486

Rausch, M., M. Ecsedi, H. Bartake, A. Mullner, and H. Grosshans, 2015 A genetic interactome of the let-7 microRNA in C. elegans. Dev. Biol. 401: 276–286. https://doi.org/10.1016/j.ydbio.2015.02.013

Rauthan, M., J. Gong, J. Liu, Z. Li, S. A. Wescott *et al.*, 2017 MicroRNA regulation of nAChR expression and nicotine-dependent

behavior in C. elegans. Cell Rep. 21: 1434–1441. https://doi.org/10.1016/j.celrep.2017.10.043

Rehmsmeier, M., P. Steffen, M. Hochsmann, and R. Giegerich, 2004 Fast and effective prediction of microRNA/target duplexes. RNA 10: 1507–1517. https://doi.org/10.1261/rna.5248604

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger *et al.*, 2000 The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 403: 901–906. https://doi.org/10.1038/35002607

Ren, Z., and V. R. Ambros, 2015 Caenorhabditis elegans microRNAs of the let-7 family act in innate immune response circuits and confer robust developmental timing against pathogen stress. Proc. Natl. Acad. Sci. USA 112: E2366–E2375. https://doi.org/10.1073/pnas.1422858112

Ren, Z., I. Veksler-Lublinsky, D. Morrissey, and V. Ambros, 2016 Staufen negatively modulates microRNA activity in Caenorhabditis elegans. G3 (Bethesda) 6: 1227–1237. https://doi.org/10.1534/g3.116.027300

Rhoades, M. W., B. J. Reinhart, L. P. Lim, C. B. Burge, B. Bartel *et al.*, 2002 Prediction of plant microRNA targets. *Cell* 110: 513–520.

Rokavec, M., H. Li, L. Jiang, and H. Hermeking, 2014 The p53/miR-34 axis in development and disease. J. Mol. Cell Biol. 6: 214–230. https://doi.org/10.1093/jmcb/mju003

Roush, S. F., and F. J. Slack, 2009 Transcription of the C. elegans let-7 microRNA is temporally regulated by one of its targets, hbl-1. *Dev. Biol.* 334: 523–534. https://doi.org/10.1016/j.ydbio.2009.07.012

Ruby, J. G., C. H. Jan, and D. P. Bartel, 2007 Intronic microRNA precursors that bypass Drosha processing. Nature 448: 83–86. https://doi.org/10.1038/nature05983

Seggerson, K., L. Tang, and E. G. Moss, 2002 Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev. Biol. 243: 215–225. https://doi.org/10.1006/dbio.2001.0563

Shaw, W. R., J. Armisen, N. J. Lehrbach, and E. A. Miska, 2010 The conserved miR-51 microRNA family is redundantly required for embryonic development and pharynx attachment in Caenorhabditis elegans. *Genetics* 185: 897–905. https://doi.org/10.1534/genetics.110.117515

Sherrard, R., S. Luehr, H. Holzkamp, K. McJunkin, N. Memar *et al.*, 2017 miRNAs cooperate in apoptosis regulation during C. elegans development. Genes Dev. 31: 209–222. https://doi.org/10.1101/gad.288555.116

Shi, Z., and G. Ruvkun, 2012 The mevalonate pathway regulates microRNA activity in Caenorhabditis elegans. *Proc. Natl. Acad. Sci USA* 109: 4568–4573. https://doi.org/10.1073/pnas.1202421109

Shi, Z., G. Hayes, and G. Ruvkun, 2013 Dual regulation of the lin-14 target mRNA by the lin-4 miRNA. PLoS One 8: e75475. https://doi.org/10.1371/journal.pone.0075475

Simon, D. J., J. M. Madison, A. L. Conery, K. L. Thompson-Peer, M. Soskis *et al.*, 2008 The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions. *Cell* 133: 903–915. https://doi.org/10.1016/j.cell.2008.04.035

Slack, F. J., M. Basson, Z. Liu, V. Ambros, H. R. Horvitz *et al.*, 2000 The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol. Cell 5: 659–669. https://doi.org/10.1016/S1097-2765(00)80245-2

Stadler, M., K. Artiles, J. Pak, and A. Fire, 2012 Contributions of mRNA abundance, ribosome loading, and post- or peri-translational effects to temporal repression of C. elegans heterochronic miRNA targets. Genome Res. 22: 2418–2426. https://doi.org/10.1101/gr.136515.111

Stefani, G., X. Chen, H. Zhao, and F. J. Slack, 2015 A novel mechanism of LIN-28 regulation of let-7 microRNA expression revealed by in vivo HITS-CLIP in C. elegans. *RNA* 21: 985–996. https://doi.org/10.1261/rna.045542.114

Steiner, F. A., S. W. Hoogstrate, K. L. Okihara, K. L. Thijssen, R. F. Ketting *et al.*, 2007 Structural features of small RNA precursors determine Argonaute loading in Caenorhabditis elegans. Nat. Struct. Mol. Biol. 14: 927–933. https://doi.org/10.1038/nsmb1308

Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok *et al.*, 1999 The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* 99: 123–132.

Tat, T. T., P. A. Maroney, S. Chamnongpol, J. Coller, and T. W. Nilsen, 2016 Cotranslational microRNA mediated messenger RNA destabilization. Elife 5: e12880. https://doi.org/10.7554/eLife.12880

Than, M. T., B. A. Kudlow, and M. Han, 2013 Functional analysis of neuronal microRNAs in Caenorhabditis elegans dauer formation by combinational genetics and Neuronal miRISC immunoprecipitation. PLoS Genet. 9: e1003592. https://doi.org/10.1371/journal.pgen.1003592

Thompson-Peer, K. L., J. Bai, Z. Hu, and J. M. Kaplan, 2012 HBL-1 patterns synaptic remodeling in C. elegans. *Neuron* 73: 453–465. https://doi.org/10.1016/j.neuron.2011.11.025

Tsialikas, J., M. A. Romens, A. Abbott, and E. G. Moss, 2017 Stage-specific timing of the microRNA regulation of lin-28 by the heterochronic gene lin-14 in Caenorhabditis elegans. Genetics 205: 251–262. https://doi.org/10.1534/genetics.116.195040

Turner, M. J., A. L. Jiao, and F. J. Slack, 2014 Autoregulation of lin-4 microRNA transcription by RNA activation (RNAa) in C. elegans. *Cell Cycle* 13: 772–781. https://doi.org/10.4161/cc.27679

Van Wynsberghe, P. M., Z. S. Kai, K. B. Massirer, V. H. Burton, G. W. Yeo *et al.*, 2011 LIN-28 co-transcriptionally binds primary let-7 to regulate miRNA maturation in Caenorhabditis elegans. Nat. Struct. Mol. Biol. 18: 302–308. https://doi.org/10.1038/nsmb.1986

Van Wynsberghe, P. M., E. F. Finnegan, T. Stark, E. P. Angelus, K. E. Homan *et al.*, 2014 The Period protein homolog LIN-42 negatively regulates microRNA biogenesis in C. elegans. Dev. Biol. 390: 126–135. https://doi.org/10.1016/j.ydbio.2014.03.017

Vasquez-Rifo, A., G. Jannot, J. Armisen, M. Labouesse, S. I. Bukhari *et al.*, 2012 Developmental characterization of the microRNA-specific C. elegans Argonautes alg-1 and alg-2. PLoS One 7: e33750. https://doi.org/10.1371/journal.pone.0033750

Vasquez-Rifo, A., G. D. Bosse, E. L. Rondeau, G. Jannot, A. Dallaire *et al.*, 2013 A new role for the GARP complex in microRNA-mediated gene regulation. PLoS Genet. 9: e1003961. https://doi.org/10.1371/journal.pgen.1003961

Vella, M. C., E. Y. Choi, S. Y. Lin, K. Reinert, and F. J. Slack, 2004 The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3′UTR. Genes Dev. 18: 132–137. https://doi.org/10.1101/gad.1165404

Vora, M., M. Shah, S. Ostafi, B. Onken, J. Xue *et al.*, 2013 Deletion of microRNA-80 activates dietary restriction to extend C. elegans healthspan and lifespan. PLoS Genet. 9: e1003737. https://doi.org/10.1371/journal.pgen.1003737

Wang, C., P. Gupta, L. Fressigne, G. D. Bossé, X. Wang *et al.*, 2017 TEG-1 CD2BP2 controls miRNA levels by regulating miRISC stability in C. elegans and human cells. Nucleic Acids Res. 45: 1488–1500. https://doi.org/10.1093/nar/gkw836

Wang, D., L. Hou, S. Nakamura, M. Su, F. Li *et al.*, 2017 LIN-28 balances longevity and germline stem cell number in Caenorhabditis elegans through let-7/AKT/DAF-16 axis. Aging Cell 16: 113–124. https://doi.org/10.1111/acel.12539

Wightman, B., I. Ha, and G. Ruvkun, 1993 Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* 75: 855–862.

Wu, E., C. Thivierge, M. Flamand, G. Mathonnet, A. A. Vashisht *et al.*, 2010 Pervasive and cooperative deadenylation of 3′UTRs by embryonic microRNA families. Mol. Cell 40: 558–570. https://doi.org/10.1016/j.molcel.2010.11.003

Wu, H., C. Huang, F. A. Taki, Y. Zhang, D. L. Dobbins *et al.*, 2015 Benzo-alpha-pyrene induced oxidative stress in Caenorhabditis elegans and the potential involvements of microRNA. Chemosphere 139: 496–503. https://doi.org/10.1016/j.chemosphere.2015.08.031

Wu, Q., Y. Zhao, G. Zhao, and D. Wang, 2014 microRNAs control of in vivo toxicity from graphene oxide in Caenorhabditis elegans. *Nanomedicine* 10: 1401–1410. https://doi.org/10.1016/j.nano.2014.04.005

Yang, J., D. Chen, Y. He, A. Melendez, Z. Feng *et al.*, 2013 MiR-34 modulates Caenorhabditis elegans lifespan via repressing the autophagy gene atg9. Age (Dordr.) 35: 11–22. https://doi.org/10.1007/s11357-011-9324-3

Yi, Y. H., T. H. Ma, L. W. Lee, P. T. Chiou, P. H. Chen *et al.*, 2015 A genetic cascade of let-7-ncl-1-fib-1 modulates nucleolar size and rRNA pool in Caenorhabditis elegans. PLoS Genet. 11: e1005580. https://doi.org/10.1371/journal.pgen.1005580

Yoo, A. S., and I. Greenwald, 2005 LIN-12/Notch activation leads to microRNA-mediated down-regulation of Vav in C. elegans. Science 310: 1330–1333. https://doi.org/10.1126/science.1119481

Zhang, H., and S. W. Emmons, 2009 Regulation of the Caenorhabditis elegans posterior Hox gene egl-5 by microRNA and the polycomb-like gene sop-2. Dev. Dyn. 238: 595–603. https://doi.org/10.1002/dvdy.21876

Zhang, H., and A. Z. Fire, 2010 Cell autonomous specification of temporal identity by Caenorhabditis elegans microRNA lin-4. *Dev. Biol.* 344: 603–610. https://doi.org/10.1016/j.ydbio.2010.05.018

Zhang, H., K. L. Artiles, and A. Z. Fire, 2015 Functional relevance of "seed" and "non-seed" sequences in microRNA-mediated promotion of C. elegans developmental progression. *RNA* 21: 1980–1992. https://doi.org/10.1261/rna.053793.115

Zhang, L., L. Ding, T. H. Cheung, M.-Q. Dong, J. Chen *et al.*, 2007 Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. *Mol. Cell* 28: 598–613. https://doi.org/10.1016/j.molcel.2007.09.014

Zhang, L., M. Hammell, B. A. Kudlow, V. Ambros, and M. Han, 2009 Systematic analysis of dynamic miRNA-target interactions during C. elegans development. Development 136: 3043–3055. https://doi.org/10.1242/dev.039008

Zhang, P., and H. Zhang, 2013 Autophagy modulates miRNA-mediated gene silencing and selectively degrades AIN-1/GW182 in C. elegans. *EMBO Rep.* 14: 568–576. https://doi.org/10.1038/embor.2013.53

Zhang, X., R. Zabinsky, Y. Teng, M. Cui, and M. Han, 2011 microRNAs play critical roles in the survival and recovery of Caenorhabditis elegans from starvation-induced L1 diapause. Proc. Natl. Acad. Sci. USA 108: 17997–18002. https://doi.org/10.1073/pnas.1105982108

Zhao, Z., T. J. Boyle, Z. Liu, J. I. Murray, W. B. Wood *et al.*, 2010 A negative regulatory loop between microRNA and Hox gene controls posterior identities in Caenorhabditis elegans. PLoS Genet. 6: e1001089. https://doi.org/10.1371/journal.pgen.1001089

Zhi, L., Y. Yu, X. Li, D. Wang, and D. Wang, 2017 Molecular control of innate immune response to Pseudomonas aeruginosa infection by intestinal let-7 in Caenorhabditis elegans. PLoS Pathog. 13: e1006152. https://doi.org/10.1371/journal.ppat.1006152

Zinovyeva, A. Y., S. Bouasker, M. J. Simard, C. M. Hammell, and V. Ambros, 2014 Mutations in conserved residues of the C. elegans microRNA Argonaute ALG-1 identify separable functions in ALG-1 miRISC loading and target repression. PLoS Genet. 10: e1004286. https://doi.org/10.1371/journal.pgen.1004286

Zinovyeva, A. Y., I. Veksler-Lublinsky, A. A. Vashisht, J. A. Wohlschlegel, and V. R. Ambros, 2015 Caenorhabditis elegans ALG-1 antimorphic mutations uncover functions for Argonaute in microRNA guide strand selection and passenger strand disposal. Proc. Natl. Acad. Sci. USA 112: E5271–E5280. https://doi.org/10.1073/pnas.1506576112

Zisoulis, D. G., M. T. Lovci, M. L. Wilbert, K. R. Hutt, T. Y. Liang *et al.*, 2010 Comprehensive discovery of endogenous Argonaute binding sites in Caenorhabditis elegans. Nat. Struct. Mol. Biol. 17: 173–179. https://doi.org/10.1038/nsmb.1745

Zisoulis, D. G., Z. S. Kai, R. K. Chang, and A. E. Pasquinelli, 2012 Autoregulation of microRNA biogenesis by let-7 and Argonaute. *Nature* 486: 541–544. https://doi.org/10.1038/nature11134

Zou, Y., H. Chiu, D. Domenger, C.-F. Chuang, and C. Chang, 2012 The lin-4 microRNA targets the LIN-14 transcription factor to inhibit netrin-mediated axon attraction. *Sci. Signal.* 5: ra43. https://doi.org/10.1126/scisignal.2002437

Zou, Y., H. Chiu, A. Zinovyeva, V. Ambros, C.-F. Chuang *et al.*, 2013 Developmental decline in neuronal regeneration by the progressive change of two intrinsic timers. *Science* 340: 372–376. https://doi.org/10.1126/science.1231321

Communicating editor: J. Kim