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Uncovering New Functions for microRNAs in *Caenorhabditis elegans*

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Abstract: In the nematode *Caenorhabditis elegans*, microRNA (miRNA) regulation of development was first observed in the striking abnormalities of *lin-4* and *let-7* loss of function mutants. However, after these first two miRNA mutant phenotypes were described, progress on the identification of miRNA functions in worms slowed considerably. Recent advances reveal new functions for miRNAs in embryonic and larval development as well as in the regulation of lifespan and stress response. Results from a combination of computational, biochemical, and genetic approaches have deepened our understanding of miRNA regulation of target mRNAs and support the hypothesis that miRNAs have an important role in ensuring the robustness of developmental and physiological pathways.

Introduction

In the race to understand microRNA (miRNA) functions in development and physiology, *Caenorhabditis elegans* investigators were the first out of the gate with the cloning and analysis of the *lin-4* and *let-7* miRNAs [1,2]. The starting point of strong, penetrant loss of function phenotypes facilitated these advancements. However, subsequent functional analysis of miRNAs in *C. elegans* was hampered

by the lack of easily observable, loss of function phenotypes [3]. There are several possible models to account for this observation. First, redundancy between related miRNAs can account for the absence of phenotypes in mutants missing individual miRNA genes [4,5]. Second, miRNAs may also function redundantly with unrelated miRNAs or other regulatory mechanisms. Third, identification of miRNA functions may require the analysis of specific cells during development, assays typically not included in initial broad phenotypic analyses. For example, the *lisy-6* miRNA is an essential regulator of a chemosensory neuron cell fate in *C. elegans* [6]. Such a specialized function would not have been identified in broad phenotypic analyses. Finally, miRNAs may act to 'fine-tune' gene expression, to maintain protein levels of targets in an optimal range. Loss of this relatively minor regulatory input by miRNAs would not be expected to result in penetrant, observable defects under normal conditions. Recent work has analyzed the functions of individual miRNAs under conditions of environmental or physiological stress. With these approaches, functions for individual miRNAs, which remain elusive under normal growth conditions, have been uncovered. These stresses can be introduced through genetic mutations, environmental perturbations, or through the normal aging process. These results are consistent with the hypothesis that miRNAs act to ensure the robustness of developmental or physiological pathways [7].

Searching for miRNA Targets

Identification of direct miRNA targets is central to the characterization of miRNA functions. miRNAs largely act to down-regulate protein levels of target mRNAs by acting as guide molecules for the miRISC (miRNA induced silencing complex). miRISC activity promotes translational repression and in many cases destabilization of the target mRNA [8]. In nearly all cases, miRNAs in animals bind imperfectly to their target mRNAs. Typical miRNA-binding sites are located in 3' untranslated regions (UTRs) and have a perfect match to the miRNA seed region, which comprises nucleotides 2–8 of the mature miRNA sequence [9]. miRNAs that have identical seed sequences are grouped into miRNA families (Table 1) and are predicted to regulate a set of shared target mRNAs. A subset of targets display atypical miRNA binding sites, which can be located in

the 5' UTR or in the coding region and also can bind imperfectly to the miRNA seed region, often with stronger compensatory binding at the 3' end of the miRNA sequence [9]. Algorithms to predict miRNA targets, such as Targetscan, PicTar, and mirWIP [10-12] have been designed to emphasize specific characteristics of miRNA-binding sites including perfect 7–8 nucleotide seed pairing, the free energy of the miRNA:mRNA duplex, evolutionary conservation, or target site accessibility. Because of these different emphases, there is only moderate concordance between algorithms and it is likely that all algorithms result in many false positive predictions. In addition, most target prediction algorithms would fail to identify atypical binding sites that may be nevertheless biologically relevant. The variation observed in the small number of miRNA targets that have been shown to be biologically relevant precludes making a defined set of rules whereby miRNAs can recognize target mRNAs [13]. Heikkinen *et al.* [14] recently reported a machine learning-based method to create a 'self-organizing map' using *C. elegans* experimentally validated target 3' UTRs. While this approach successfully identifies verified targets and fails to identify verified false targets, it is unclear whether it is able to accurately identify novel, biologically relevant target mRNAs.

Table 1. Summary of functions identified for miRNAs in *C. elegans*.

miRNA gene/family	Seed sequence	Observed function	Target	References
<i>lin-4</i>	CCCUGAG	Developmental timing, lifespan, HSN axon growth	<i>lin-14</i> , <i>lin-28</i>	[24,31–34]
<i>let-7</i> , <i>mir-48</i> , <i>-84</i> , <i>-241</i>	GAGGUAG	Developmental timing, vulval cell fate specification	<i>def-12</i> , <i>hbl-1</i> , <i>let-60</i> , <i>lin-41</i>	[2,4,35–38]
<i>hsy-6</i>	UUUGUUAU	ASEL/R neuron fate specification	<i>cog-1</i>	[8]
<i>mir-1</i>	GGAAUGU	Synaptic transmission	<i>unc-29</i> , <i>unc-63</i> , <i>mef-2</i>	[39]
<i>mir-34</i>	GGCAGUG	DNA damage response		[28]
<i>mir-35</i> , <i>-36</i> , <i>-37</i> , <i>-38</i> , <i>-39</i> , <i>-40</i> , <i>-41</i> , <i>-42</i>	CACCGGG	Embryogenesis		[5]
<i>mir-51</i> , <i>-52</i> , <i>-53</i> , <i>-54</i> , <i>-55</i> , <i>-56</i>	ACCCGUA	Embryogenesis, pharynx attachment, developmental timing	<i>cdh-3</i>	[5,21,23]
<i>mir-57</i>	ACCCUGU	Posterior cell fate specification	<i>nab-1</i>	[30]
<i>mir-58</i>	GAGAUCC	Locomotion, body size, egg laying, dauer entry		[5]
<i>mir-59</i>	CGAAUCC	Gonad migration, embryogenesis, adult viability		[23]
<i>mir-80</i> , <i>-81</i> , <i>-82</i>	GAGAUCA	Locomotion, body size, egg laying, dauer entry		[5]
<i>mir-71</i>	GAAAGAC	Lifespan		[26]
<i>mir-77</i>	UCAUCAG	Embryogenesis		[23]
<i>mir-83</i>	AGCACCA	Gonad migration		[23]
<i>mir-124</i>	AAGGCAC	Gonad migration		[23]
<i>mir-228</i>	AUGGCAC	Developmental timing, embryogenesis		[23]
<i>mir-235</i>	AUUGCAC	Adult viability		[23]
<i>mir-238</i> , <i>-239a/b</i>	UUUUAU	Developmental timing, lifespan		[23,26]
<i>mir-240</i>	ACUGGCC	Defecation cycling, fertility		[23]
<i>mir-244</i>	CUUUGGU	Developmental timing		[23]
<i>mir-246</i>	UACAUGU	Embryogenesis, lifespan		[23,26]
<i>mir-247</i>	GACUAGA	Gonad migration		[23]
<i>mir-259</i>	AAUCUCA	Developmental timing, gonad migration		[23]
<i>mir-786</i>	AAUGCCC	Defecation cycling, fertility		[3,23]
<i>mir-797</i>	AUCACAG	Gonad migration		[23]

Recently, several groups have employed biochemical approaches to identify target mRNAs in *C. elegans*. Immunoprecipitation of miRISC protein components along with their associated RNAs, including a *C. elegans* GW182 ortholog, AIN-1, and a miRNA pathway Argonaute protein, ALG-1, has generated lists of mRNAs that are likely enriched in miRNA targets [15-17]. In addition, a targeted proteomics method (selected reaction monitoring) to measure protein levels of individual candidate miRNA targets has identified novel targets for *let-7* and *mir-58* [18]. Data from biochemical approaches will lead to further optimization of algorithms to predict miRNA targets and will facilitate identification and validation of biologically relevant miRNA targets.

Despite the central importance of the 3' UTRs for post-transcriptional regulation by miRNAs as well as other regulatory RNAs or proteins, until recently most genes in *C. elegans* lacked annotated 3' UTRs. This gap has now been corrected by using biochemical cloning and high throughput sequencing to define the 3' UTRs of *C. elegans* mRNAs [19,20]. Interestingly, in both 3' UTR datasets from Jan *et al.* and Mangone *et al.* [19,20] many genes (31–43%) were found to have alternative, shorter isoforms from proximal cleavage sites or from alternative 3' exons. This indicates that the ability of a miRNA to bind to a particular target mRNA can be developmentally regulated through splicing or cleavage and polyadenylation, thus adding an additional layer of complexity to miRNA regulation of mRNA targets.

Digging Deeper to Find Elusive miRNA Functions in Worms

Whereas most loss of function mutants for individual miRNAs do not present easily detectable abnormalities in worms [3], compound mutants that are missing multiple miRNA family members can display penetrant developmental phenotypes [4,5]. A summary of functions identified in worms through analysis of loss of function miRNA mutants is presented in Table 1. Loss of most or all of the *mir-35* or *mir-51* family members results in embryonic or larval lethality, while loss of *mir-58* miRNA family members results in a pleiotropic phenotype with observed defects in locomotion, body size, egg laying, and dauer larva

formation [5]. For the compound mutants generated for the remaining twelve miRNA families, no defects in development or viability were observed. In further analysis of the compound mutants missing the *mir-51* family member miRNAs, Shaw *et al.* [21] found that the observed failure of pharynx attachment was likely due, in part, to the regulation of the cadherin ortholog, *cdh-3*. In many cases, family members likely function redundantly to regulate overlapping sets of targets, since expression of any miRNA family member can rescue compound mutant phenotypes. While this rescue is observed for the *let-7* [22], *mir-35* [5] and the *mir-51* [5,21] families of miRNAs, it is likely that the *let-7* family members have both overlapping and non-overlapping targets since *mir-48*, *mir-84*, and *mir-241* do not repress the activity of the *let-7* target, *lin-41* [4]. Redundancy between miRNA family members accounts for some, but not all, of the scarcity of loss of function phenotypes for single gene miRNA mutants. To address whether miRNAs function with unrelated miRNAs or with other gene regulatory mechanisms, loss of miRNA-encoding genes were examined in a sensitized genetic background in which all miRNAs are lower or in which chromatin modification or transcriptional regulation is perturbed. Using this approach, mutant phenotypes were detected for 25 out of 31 miRNAs studied [23]. Thus, in many cases the phenotypic consequences of loss of a single miRNA gene may only be evident in conditions where other regulatory mechanisms are compromised.

miRNAs Function to Regulate Lifespan and Stress Response

Cellular and genetic regulatory mechanisms can be compromised by conditions of environmental or physiological stress. Recent functional analysis indicates that certain miRNAs regulate lifespan in *C. elegans*. A major pathway that functions to regulate lifespan in worms is the insulin/insulin-like growth factor-1 (IGF-1) pathway, which has been shown to be regulated by the *lin-4* miRNA [24]. *lin-4* mutants display a shortened lifespan with accelerated aging and increased sensitivity to stresses such as heat shock. In contrast, loss of the *lin-4* target, *lin-14*, is associated with lengthened lifespan and delayed aging [24]. Interestingly, *lin-4* is not the only miRNA that regulates aging. Using microarrays, 50 out of 114 miRNAs analyzed were found to change in expression level during *C. elegans* aging [25].

The majority of these miRNAs decrease in expression, suggesting that loss of miRNA regulation may promote the observed cell and tissue declines, such as the loss of muscle integrity. Recently, it has been demonstrated that loss of function mutants in four individual miRNAs that show significant changes in expression during aging also display lifespan defects [26]. In this study, deep sequencing was used to profile small RNAs during worm aging, allowing the authors to analyze all miRNAs and to discover novel miRNAs: 7 miRNAs were found to be strongly upregulated (2.2 to 5.9 fold change) during aging while 23 were found to be strongly downregulated. (2.1 to 7.4 fold change). Of those miRNAs that showed strong upregulation, loss of function mutations in these genes demonstrated that four displayed lifespan defects (*mir-71*, *mir-238*, *mir-239*, and *mir-246*). It is noteworthy that while *mir-71*, *mir-238*, and *mir-246* mutants displayed decreased lifespans, *mir-239* mutant worms showed a significant extension in lifespan. In addition, *mir-71*, *mir-238*, and *mir-246* mutants showed reduced stress resistance while *mir-239* mutants showed enhanced resistance. Thus, regulation of gene expression by miRNAs during aging is complex, with miRNAs showing opposing functions.

Starting with a similar approach of profiling miRNA expression levels, Karp *et al.* [27] identified 14 individual miRNAs that showed differences in expression between worms that developed continuously, under optimal conditions and worms that went through the dauer developmental program. Worms enter the dauer stage after the L2 stage in response to unfavorable conditions, such as crowding or starvation. When conditions again become favorable, worms exit the dauer stage, complete the L3 and L4 stages of larval development, and become adults. Interestingly, two miRNA genes, *mir-34* and *mir-71*, shown to be significantly upregulated during aging [26] are also upregulated in response to development through the dauer pathway [27], indicating a more general role for these miRNAs in the worm's response to physiological and environmental stress. In addition, *mir-34* mutants display sensitivity to the stress of exposure to radiation [28]. The critical next step will be to further characterize the pathways and direct mRNA targets regulated by miRNAs in aging and stress response in worms.

miRNAs Function to Regulate Embryonic and Larval Development

Although the initial characterization of a set of worms carrying deletion alleles covering 87 miRNA genes did not identify mutant phenotypes for the majority of individual miRNA genes [3], it is important to note that in many cases identification of mutant phenotypes will depend upon more specific, focused analysis. For example, miR-124 has been shown to fine-tune gene expression in ciliated neurons [29]. Using FACS sorting of *mir-124* expressing cells and transcriptome analysis of wild-type and *mir-124* mutant worms, Clark *et al.* [29] described a set of genes that are likely directly regulated by miR-124. Further analysis of this set of miR-124 regulated genes will elucidate how miR-124 functions to regulate the cell fate specification or the physiology of ciliated neurons. Also, Zhao *et al.* [30] recently described a function for miR-57 by first determining that this miRNA is specifically expressed in the posterior of the worm during embryogenesis and early larval development. Because *mir-57* shows a posterior-specific expression pattern, the authors then focused their analysis on *mir-57* loss of function mutants and were able to identify a weakly penetrant developmental defect: upon growth at an elevated temperature (26°C) about 12% of the mutants die during embryonic or larval development and 32% are sterile. Most significantly, a small percentage of worms (2.7%) display tail abnormalities that are consistent with defects in posterior cell fate specification. In an effort to induce a more penetrant phenotype, the authors overexpressed *mir-57* and indeed found more penetrant posterior cell fate defects. About 30% of *mir-57* overexpressing worms display a Nob or Vab phenotype with abnormal bulges observed near the tail. Using a candidate approach the authors identified the posterior Hox gene *nob-1* as both an upstream regulator of *mir-57* expression as well as a downstream target for miR-57 repression. Thus, *mir-57* and *nob-1* are in a negative feedback loop that likely functions in the robust execution of posterior cell fate specification. In addition, this experimental approach of using a combination of expression analysis, the sensitized conditions of elevated temperature, overexpression of miRNA genes, and focused analysis on specific cell fates is a model that is sure to be effective in the future characterization of miRNA functions in worms.

Summary

Defects associated with the loss of 44 miRNAs have now been described in *C. elegans*. This represents 28% of the 159 confidently identified miRNAs encoded in the *C. elegans* genome [19]. While this represents a significant leap forward in our understanding of miRNA biology, many challenges remain. One such challenge is to identify the pathways and direct targets that are misregulated in these miRNA mutants. In addition, we have little to no information about the function of newly identified miRNAs. For the many miRNAs for which functions remain unknown, exposure to genetic or environmental stresses will facilitate the identification of new miRNA-dependent pathways. Genetic analysis of miRNAs in *C. elegans* and other model organisms will continue to uncover new functions for miRNAs in the regulation of development and physiology.

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