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Loss of Individual MicroRNAs Causes Mutant Phenotypes in Sensitized Genetic Backgrounds in *C. elegans*

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Summary: MicroRNAs (miRNAs) are small, non-coding RNAs that regulate the translation and/or the stability of their mRNA targets. Previous work

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showed that for most miRNA genes of *C. elegans*, single gene knockouts did not result in detectable mutant phenotypes [1]. This may be due, in part, to functional redundancy between miRNAs. However, in most cases, worms carrying deletions of all members of a miRNA family do not display strong mutant phenotypes [2]. They may function together with unrelated miRNAs or with non-miRNA genes in regulatory networks, possibly to ensure the robustness of developmental mechanisms. To test this, we examined worms lacking individual miRNAs in genetically sensitized backgrounds. These include genetic backgrounds with reduced processing and activity of all miRNAs or with reduced activity of a wide array of regulatory pathways [3]. Using these two approaches, mutant phenotypes were identified for 25 out of 31 miRNAs included in this analysis. Our findings describe biological roles for individual miRNAs and suggest that use of sensitized genetic backgrounds provides an efficient approach for miRNA functional analysis.

Results

Loss of individual miRNAs can enhance or suppress alg-1 developmental defects

Genetic analysis demonstrates that development of worms, flies, fish and mice requires miRNAs [4-9]. While it is clear that normal development of the worm requires miRNA biogenesis, functions have been described for only a few individual miRNAs. In *C. elegans*, the majority of individual miRNAs are not required for viability or for development, as most loss of function miRNA mutants display no obvious developmental abnormalities [1]. To test the hypothesis that the absence of phenotypes in miRNA mutant worms is due to functional redundancy with other miRNAs, we determined if loss of specific miRNAs in a sensitized genetic background resulted in observable mutant phenotypes. For a sensitized background, we used a loss of function allele of *alg-1* (*argonaute like 1)*, which is one of two Argonaute-encoding genes that function in the miRNA pathway in worms. Compared to wild-type worms, *alg-1* mutants have lower levels of mature miRNAs [6, 10]. Whereas worms that lack *alg-1* and *alg-2* activities die during embryogenesis [6], *alg-1* single mutant worms are viable, displaying developmental timing defects, molting defects and early adult-stage lethality [11-14].

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A subset of 25 miRNA deletion alleles covering 31 miRNA-encoding genes were chosen for analysis (Table 1). As a subset of miRNAs are clustered in the genome, some alleles affect multiple miRNAs. For example, *nDf58* is a deletion of three miRNAs: *mir-54, mir-55*, and *mir-56* (*mir-54-56)*. We selected miRNAs that show evolutionary conservation of miRNA family members based on their seed sequence or a developmentally-regulated expression pattern [15-19]. First, all of the strains carrying deletion alleles were outcrossed with wild-type N2 worms (see Table S1 available online). A set of multiply mutant strains was then constructed that carried a single miRNA deletion allele and the *gk214* allele of *alg-1*. We performed phenotypic analysis of our collection of 25 *alg-1;mir* multiply mutant strains. This analysis focused on readily-observable, quantifiable, developmental phenotypes: embryonic, larval, and adult lethality, gross morphology and motility, dauer formation, gonad migration, and alae formation. A summary of the phenotypic analysis is shown in Table 1, which includes the identification of novel enhanced or synthetic phenotypes for 19 of the 25 strains analyzed.

Table 1. Phenotypic Characterization of miRNA Mutants in *alg-1*-Sensitized Genetic Background

		Developmental Timing	Gonad Migration	Embryonic Lethality	Adult Lethality
Strain	Genotype	% Incomplete Alae Formation[a]	% Abnormal[b]	% Unhatched[c]	% Dead at 72 hr[d]
N2	wild type	0%	0%	0%	0%
RF54	*alg-1(gk214)*	81%	8%	3%	63%
RF70	*mir-1(n4102); alg-1(gk214)*	57%	25%**	3%	53%
RF129	*mir-34(n4276) alg-1(gk214)*	59%	13%	5%	72%
RF420	*mir-51(n4473); alg-1(gk214)*	31%**	7%	8%*	51%
RF411	*mir-52(n4114); alg-1(gk214)*	3%**	0%	6%	16%**
RF398	*mir-53(n4113); alg-1(gk214)*	60%	17%	1%	57%
RF410	*mir-54-55(nDf45) alg-1(gk214)*	4%**	11%	2%	12%**
RF89	*mir-54-56(nDf58) alg-1(gk214)*	23%**	4%	3%	5%**
RF133	*mir-57(gk175); alg-1(gk214)*	51%	5%	8%**	73%
RF137	*mir-59(n4604); alg-1(gk214)*	69%	23%**	10%**	83%**
RF153	*mir-72(n4130); alg-1(gk214)*	56%	2%	5%	48%
RF81	*mir-73-74(nDf47) alg-1(gk214)*	75%	7%	3%	40%**
RF178	*mir-77(n4285); alg-1(gk214)*	54%	8%	12%**	59%
RF65	*mir-83(n4636); alg-1(gk214)*	51%	25%**	3%	77%
RF141	*mir-85(n4117); alg-1(gk214)*	48%	4%	3%	54%
RF77	*mir-124(n4255); alg-1(gk214)*	69%	18%*	2%	69%
RF145	*mir-228(n4382); alg-1(gk214)*	39%**	5%	13%**	53%
RF93	*mir-234(n4520); alg-1(gk214)*	53%	5%	3%	57%
RF182	*mir-235(n4504); alg-1(gk214)*	56%	7%	0%	80%**
RF85	*mir-237(n4296) alg-1(gk214)*	47%	12%	3%	75%
RF183	*mir-238(n4112); mir-239a-b(nDf62) alg-1(gk214)*	22%**	3%	1%	11%**
RF80	*mir-240 mir-786(n4541) alg-1(gk214)*	50%	13%	7%**	45%*
RF186	*mir-244(n4367); alg-1(gk214)*	13%**	9%	5%	53%
RF149	*mir-246(n4636); alg-1(gk214)*	44%	7%	8%**	71%
RF368	*mir-247 mir-797(n4505) alg-1(gk214)*	41%	25%**	4%	57%
RF343	*mir-259(n4106); alg-1(gk214)*	34%**	28%**	4%	59%

*p < 0.05; **p < 0.01 by the chi-square test, as compared to *alg-1* single mutants.
[a] Alae were scored at the L4m by DIC microscopy. n > 39 (range: 39–204) worms scored for each strain.
[b] Gonad morphology was scored in young adult worms by DIC microscopy. n > 41 (range: 41–262) worms scored for each strain.
[c] Embryos were transferred to a new plate and scored after 16–24 hr for the presence of unhatched embryos. n > 78 (range: 78–548) embryos scored for each strain.
[d] Synchronized L1-stage worms were transferred to plates to initiate development. Lethality was scored 72 hr after plating at 20°C; n > 76 (range: 76–172) worms scored for each strain.

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First, we observed that loss of mir-51, mir-57, mir-59, mir-77, mir-228, mir-240 mir 786, or *mir-246* in the *alg-1* sensitized background resulted in a significant increase in the percentage of embryonic lethality, from 3% in *alg-1* single mutants to 7-13% in the *alg-1;mir* multiple mutants (Table 1). Consistent with a role in embryogenesis, embryonic expression has been reported for *mir-51, mir-57, mir-59*, and *mir-228* [17, 20]. However, no embryonic expression has been reported for *mir-77, mir-240 mir-786*, or *mir-246*. The point at which these embryos arrested was not determined and because the increase in embryonic lethality was relatively modest, transgenic rescue was not performed. Recently, it has been shown that worms lacking all six members of the *mir-51* miRNA family display a penetrant embryonic lethal phenotype [2], demonstrating an essential role for this miRNA family in embryogenesis.

Second, we observed that loss of *mir-1, mir-59, mir-83, mir-124, mir-247 mir-797*, or *mir-259* in the *alg-1* sensitized background resulted in a significant increase in the percentage of worms that had defective distal tip cell migration as determined by the gonad morphology in young adult worms (Table 1). In contrast to wild-type animals that execute a single, developmentally-regulated reflex of each gonad arm, 8% of *alg-1* single mutants displayed abnormal distal tip cell migration, with the distal tip cell executing an extra turn during development. The distal tip cell of the gonad arms migrated normally to the anterior or posterior, made a dorsal turn, initiated migration back to the midline normally but then executed an extra turn back away from the midline. The extra turn was observed to occur predominantly, but not exclusively, in the posterior gonad arm. In all six *alg-1; mir* strains, this enhanced gonad migration phenotype was rescued by the extra-chromosomal expression of a wild-type genomic fragment containing the miRNA stem-loop sequence along with flanking sequence (Table S1). The expression patterns of most of these miRNAs are consistent with a possible role in the regulation of secreted or membrane-bound guidance molecules: *mir-1* is expressed in the body wall muscles [21] *mir-59* and *mir-83* are expressed in the intestine [20] and *mir-247 mir-797* is expressed in the distal tip cell [20]. Proteins from the body wall muscle and intestinal cells are required for guidance cues in the body wall basement membrane over which the distal tip cells migrate [22, 23].

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Third, we observed that loss of *mir-59* and *mir-235* resulted in an increased level of adult lethality compared to *alg-1* single mutants (Table 1). *alg-1* single mutants appeared healthy at the L4-to-adult transition but 63% die as young adults (Table 1). About 62% of *alg-1* worms enter a supernumerary molt in the adult stage (Table S2). Nearly all of the adult worms that enter the molt subsequently die with a "bag of worms" phenotype (Table S2), likely due to a failure to properly complete the molting cycle that compromises the worm's ability to lay embryos. Execution of a supernumerary molt is a heterochronic phenotype also observed in worms missing *let-7* family members [24, 25]. This adult lethality was enhanced in *mir-59;alg-1* and *mir-235;alg-1* worms, with 83% and 80% adult lethality, respectively (Table 1). The increase in adult lethality observed in these strains is primarily due to an increase in the percentage of worms that enter a supernumary lethargus (Table S2).

In contrast to this enhancement of the *alg-1* adult lethality phenotype, we found that loss of other miRNAs suppressed *alg-1* phenotypes. In particular, loss of *mir-73-74, mir-238/mir-239a-b, mir-240 mir-786*, or certain *mir-51* family members (*mir-52, mir-54-55*, and *mir-54-56*) in the *alg-1* background resulted in a reduced level of adult lethality when compared to *alg-1* single mutants (Table 1). This decrease in adult lethality is primarily due to a decrease in the percentage of worms that enter a supernumary lethargus (Table S2). Similarly, we observed that loss of *mir-228, mir-238/mir-239a-b, mir-244, mir-259* or *mir-51* family members (*mir-51, mir-52, mir-54-55*, and *mir-54-56*), resulted in a reduced penetrance of developmental timing defects, as assayed by the formation of an adult-specific cuticle structure called alae (Table 1). About 60% of *alg-1* single mutants showed incomplete alae formation owing to a partial reiteration of larval stage programs, which is likely due to reduced levels of *lin-4* and *let-7* family miRNAs [6]. Incomplete alae formation indicates an inappropriate larval cell fate for some of the cells in the hypodermis at the L4-to-adult transition. Introduction of a transgene with the *mir-54-56*, *mir-238, mir-244*, or *mir-259* genomic loci into the respective *alg-1;mir* strain, restored the level of incomplete alae formation to that observed in *alg-1* single mutants (Table S1). Four strains, *mir-52; alg-1, mir-54-55 alg-1, mir-54-56 alg-1* and *mir-238; mir-239a-b alg-1* displayed both reduced adult lethality as well as reduced alae formation defects (Table 1).

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Loss of individual miRNAs confers enhanced or synthetic sterility with knockdown of a set of chromatin regulator "hub" genes

We next tested whether miRNA genes functionally interact with broadly-acting pathways of gene regulation. A small set of genes, termed "hub" genes, have been identified that show a high level of connectivity to many developmental processes [3]. These hub genes encode chromatin regulatory proteins and interact with a wide range of signaling pathways that are critical for normal development, including the Wnt, EGF, and Notch pathways [3]. Therefore, we hypothesized that the reduced activity of these hub genes could provide a sensitized genetic background in which to reveal additional relationships between individual miRNA mutants and other developmental processes. To address this question directly, we determined whether individual miRNA deletion alleles displayed enhanced or synthetic interactions upon knockdown of one of five hub genes by RNAi: *egl-27, din-1, hmg-1.2, dpy-22*, and *trr-1* (Table 2). We performed phenotypic assays on eleven strains carrying miRNA deletion alleles to identify embryonic lethal or sterile phenotypes upon knockdown of hub genes.

Table 2. Summary of hub Genes and Synthetic Interactions with Developmental Pathways

Gene	Description[a]	Enhancement with Developmental Regulatory Components[b]
din-1	Ortholog of human transcriptional corepressor SHARP/SPEN, encodes large RNA-binding protein of RRM superfamily	EGF, Wnt, Notch, ephrin receptor
dpy-22	Ortholog of the human transcriptional Mediator protein TRAP230	EGF, Wnt, Notch, E2F/synMuvB, ephrin receptor, RAC1/GTPase
egl-27	Ortholog of human MTA1, part of nucleosome remodeling and histone deacetylation (NURD) complex	EGF, Notch, E2F/synMuvB, ephrin receptor
hmg-1.2	DNA binding protein with HMG box	EGF, Wnt, Notch, ephrin receptor
trr-1	Ortholog of component of the NuA4/Tip60 histone acetyltransferase complexes, atypical protein kinase of TRAAP subfamily	EGF, Wnt, Notch, E2F/synMuvB, GTPase

[a] Information from http://www.wormbase.org.
[b] See [3] for details.

We found that RNAi knockdown of *egl-27, din-1, hmg-1.2, dpy-22*, and *trr-1* in wild-type worms did not result in a significant number of worms that were sterile as compared to the negative control RNAi (Table 3). However, four miRNA mutant strains displayed a synthetic sterile phenotype following knockdown of individual hub genes. When *egl-27* or *hmg-1.2* activity were knocked down in *mir-1* mutant worms, 24% and 29% of worms were sterile, respectively (Table 3). No sterility was observed when *mir-1* worms were fed bacteria containing an empty vector construct. Like *mir-1* worms, *mir-59* worms showed a synthetic sterile phenotype with knockdown of *egl-27*

or *hmg-1.2* (Table 3). When either *egl-27* or *trr-1* activity was knocked down in *mir-247 mir-797* mutant worms 29% and 40% of worms were sterile, respectively, compared to 3% in the negative control group (Table 3). The strongest synthetic phenotype was observed in *mir-240 mir-786* worms following knockdown of *hmg-1.2* or *trr-1*, with 76% and 83% sterility (Table 3). These worms exhibited germline defects (Figure S1). For all strains that displayed a sterile phenotype upon hub gene knockdown, there was no significant embryonic lethality (Table S3). Few, if any, embryos were found on plates with *mir-240 mir-786* worms following knockdown of *hmg-1.2* or *trr-1* activity (Table S3). These data suggest that *mir-1, mir-59, mir-240 mir-786*, and *mir-247 mir-797* function in regulatory pathways that are essential for germline development. Of these, only *mir-240 mir-786* has been shown to be expressed in the gonad using miRNA promoter reporter transgenes, with expression observed in the uterus, spermatheca, and gonadal sheath [20]. It is possible that *mir-1, mir-59*, and *mir-247 mir-797* are expressed in the germline but this expression is undetectable using reporter transgenes or that these miRNAs function to control developmental signals required for fertility from outside of the germline.

Table 3. Phenotypic Characterization of miRNA Mutants after Knockdown of hub Gene Activity by RNAi

		% Sterile Worms					
Strain	Genotype	Empty-Vector RNAi	*din-1* RNAi	*dpy-22* RNAi	*egl-27* RNAi	*hmg1.2* RNAi	*trr-1* RNAi
N2	wild-type	0%	11%	4%	3%	7%	14%
RF71	*mir-1(n4102)*	0%	18%	4%	24%*	29%*	16%
RF124	*mir-34(n4276)*	3%	2%	0%	7%	3%	0%
RF90	*mir-54-56(nDf58)*	0%	4%	0%	0%	5%	21%
RF15	*mir-59(n4604)*	7%	24%	8%	21%*	26%*	27%
RF66	*mir-83(n4638)*	0%	5%	5%	0%	11%	7%
RF78	*mir-124(n4255)*	0%	4%	0%	0%	14%	24%
RF94	*mir-234(n4520)*	0%	7%	0%	9%	11%	9%
RF86	*mir-237(n4296)*	2%	0%	0%	0%	0%	12%
RF61	*mir-240 mir-786(n4541)*	0%	17%	13%	4%	76%**	83%**
RF126	*mir-246(n4636)*	0%	5%	3%	15%	16%	33%
RF24	*mir-247 mir-797 (n4505)*	3%	11%	9%	29%**	13%	40%*

*p < 0.05; **p < 0.01 by the chi-square test as compared to wild-type worms with the corresponding hub gene RNAi.

Discussion

The goal of this work was to test the hypothesis that miRNAs have overlapping or redundant functions and to identify phenotypes associated with the loss of individual miRNAs. We found that loss of individual miRNAs resulted in developmental abnormalities in an *alg-1* genetic background, which has lower total miRNA activity, and in backgrounds with reduced hub gene activity, in which multiple

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regulatory pathways are compromised. Our analysis described mutant phenotypes associated with loss of 25 out of the 31 miRNAs included in our study.

These results identified only a limited number of phenotypes in the *alg-1;mir* strains. This indicates that only a subset of miRNA-regulated pathways are sufficiently sensitized in *alg-1* worms, while others may require a further reduction in the activity of miRNAs to become sensitized. Additional knockdown of *alg-2* activity, which is the other Argonaute-encoding gene that acts in the miRNA pathway, could be used to enhance the sensitivity of this assay. However, complete loss of *alg-1* and *alg-2* results in embryonic lethality [6]. The synthetic sterility observed with individual miRNAs and hub genes indicates that pathways that are essential for germline development are sensitized in these backgrounds. Additional work is needed to identify the specific regulatory pathways and targets that are controlled by individual miRNAs.

Our assays identified multiple miRNAs that interact with either *alg-1* or individual hub genes to give similar phenotypes. One parsimonious model for this observation is that these sets of miRNAs may regulate shared mRNA targets. For example, *mir-1, mir-59*, and *mir-240 mir-786* all interact with *hmg-1.2 (RNAi)* to give a synthetic sterile phenotype. To examine this hypothesis, we identified predicted mRNA targets using Targetscan [19] or mirWIP [26]. Both prediction algorithms identified possible shared targets for pairs of these miRNAs (Table S4), with three targets that are possible shared targets for three miRNAs (miR-1, miR-59, and miR-786): *daf-12, hbl-1*, and *cfim-2*. Interestingly, *cfim-2* encodes an mRNA cleavage and polyadenylation factor that is required for fertility [27]. This analysis of target predictions provides a platform to identify the biologically-relevant targets and to test the hypothesis that multiple miRNAs regulate shared targets. Alternatively, the phenotypes we observe may reflect miRNA regulation of distinct targets in convergent developmental pathways.

Interestingly, loss of a subset of miRNAs, *mir-228, mir-238;mir-239a/b, mir-244*, *mir-259* or certain *mir-51* family members (*mir-51, mir-52*, and *mir-54-56)* resulted in a suppression of *alg-1* alae formation defects. This could indicate regulation of specific targets in

the developmental timing pathway. In this model, some miRNAs could function antagonistically to the *lin-4* or *let-7* family miRNAs, which are largely responsible for the *alg-1* developmental timing defects [6]. An alternative explanation for the observed suppression of *alg-1* phenotypes by certain miRNA gene mutations could be that these miRNAs regulate specific targets that control miRISC activity, such that in their absence, miRISC activity is enhanced. For example, the miRISC cofactor, *nhl-2* [13], is predicted to be a target of miR-244 and miR-228 using TargetScan [19] and mirWIP [26] miRNA target prediction algorithms, suggesting a possible mechanism for suppression. It is also possible that the observed suppression may be due to a more general effect on miRISC activity. If individual miRNAs compete for loading into miRISC, then loss of a highly abundant miRNA, such as miR-52 [17, 19], may allow for greater activity of remaining miRNAs, including the *let-7* family. However, this can not account for the observed suppression in all *alg-1;mir* strains as loss of abundant miRNAs does not suppress *alg-1* defects in all cases. Additionally, strong suppression of *alg-1* defects is observed with loss of weakly expressed miRNAs, such as *mir-238* or *mir-259* [19]. Interestingly, Targetscan identifies *alg-2* as a candidate target for miR-259 [19].

Few phenotypes have been described for individual miRNA mutants [see 28]. In addition, few phenotypes are observed in strains that lack most or all miRNA family members, suggesting that the lack of detectable phenotypes for individual miRNA mutants is not due primarily to overlapping function with related miRNA family members [2]. Our results indicate that miRNAs may function together with unrelated miRNAs or non-miRNA genes, perhaps functioning together to ensure the robustness of developmental mechanisms. The use of sensitized genetic backgrounds is a fruitful approach to identify phenotypes associated with the loss of specific miRNAs. Genetic backgrounds can be selected that have compromised activity of specific regulatory pathways, which may render them sensitive to the loss of individual miRNAs. It is possible that functions of some miRNAs may be revealed only when sufficient genetic or environmental variation is introduced.

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Experimental Procedures

General Methods and strains

C. elegans strains were maintained under standard conditions as previously described [29]. Worms were kept on NGM plates seeded with *E. coli* strain AMA1004 [30]. The wild type strain used was var. Bristol N2 [31]. RF54 was used as the *alg-1(gk214)X* control in phenotypic analysis of *mir; alg-1* strains. All strains were kept at 20°C unless otherwise indicated. miRNA mutant strains were first outcrossed to wild-type N2 (Table S1). For building multiply mutant strains, presence of the miRNA deletion alleles and the *alg-1* allele in F2s were identified by performing PCR with primers that amplified the genomic region flanking the deletion mutation. Sequences for primers used for genotyping can be found in Table S1. Fluorescence and DIC microscopy was performed using a Nikon Eclipse 80i equipped with a Photometrics CoolSNAP HQ2 monochrome digital camera and RS Image software (Roper Scientific).

RNAi experiments

Four L4-stage worms were placed on RNAi plates (NGM with 0.2% (w/v) lactose and 100 µg/ml ampicillin) seeded with bacteria to knockdown *egl-27, din-1, hmg-1.2, dpy-22*, or *trr-1*. Empty vector (L4440) was used as the negative control. Bacteria for RNAi experiments were isolated from the Ahringer RNAi library [32]. Worms were placed at 20°C overnight. 48 F1 embryos were transferred to a new RNAi plate, seeded with the same bacteria. The next day live worms and unhatched embryos were scored. The surviving larval worms were cloned into 96 well culture plates, with each well containing RNAi liquid media (M9 containing 0.2% Lactose and 100 µg/ml ampicillin) plus bacteria. The bacteria were from frozen bacterial pellets taken from 5 mL stationary phase overnight cultures of appropriate bacteria strain in LB containing 0.2% lactose and 100 µg/ml ampicillin. 96-well plates were placed at 20°C for 96 hours and then wells were scored for the presence of F2 progeny. Wells in which the F1 worm died were not scored.

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Transgene Rescue experiments

To create transgenic animals, germline transformation was performed as described [33]. Injection mixes contained 5-25 ng/μl of the rescue plasmid, 25-100 ng/μl of a co-injection marker (*myo-2::gfp* or *myo-2::dsRed)* containing plasmid, and pRS413 plasmid for a final DNA concentration of 150 ng/μl (Table S1). Transgenic animals expressing GFP or dsRed in the pharynx were assayed for alae formation or for gonad migration defects.

Go to:

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Footnotes

Supplemental Information Supplemental Information, which includes five tables and one figure, can be found with this article online.

Mutant phenotypes identified for 25 out of 31 miRNAs in sensitized backgrounds. Loss of subset of miRNAs can suppress developmental phenotypes of *alg-1* mutants.

Use of sensitized backgrounds is an effective approach to study miRNA functions.

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Supplementary Material

01 Supplemental Information

Identification of mutant phenotypes associated with loss of individual microRNAs in sensitized genetic backgrounds in *Caenorhabditis elegans.*

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Inventory of Supplemental Information

Table S1, related to Table 1. Shows microRNA deletion allele information and primers for identification of deletion allele and transgenic rescue of mutant phenotypes in *alg-1;mir* strains.
Table S2, related to Table 1. Analysis of enhancement or suppression of *alg-1* lethality phenotype.
Table S3, related to Table 3. Knockdown of hub gene activity does not result in embryonic lethality.

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Table S4, related to Table 3. Predictions of candidate shared targets for pairs of miRNAs that interact with *hmg-1.2(RNAi)*

Figure S1, related to Table 1. Representative pictures of phenotypes described in Table 1 and Table 3.

Figure legends

Figure S1. Representative DIC images of developmental timing, gonad migration, and germline morphology phenotypes. Nomarski DIC image of (A) wild-type adult-stage worm with complete alae formation. (B) alg-1 adult-stage worm with incomplete alae formation. Black arrows point to breaks in the alae structure. (C) wild-type adult-stage worm with normal gonad morphology. The gonad arm is a U shaped structure with the distal end (marked with asterisk) in close proximity to the vulva. The worm is oriented with the posterior is to the right. (D) alg-1 adult-stage worm with the posterior gonad arm displaying a supernumerary turn. The gonad arm shows migration to the midline but then an extra turn such that the distal end (marked with asterisk) is in close proximity to the tail. The worm is oriented with the posterior is to the right. (E) wild-type adult-stage worm following knockdown of hmg-1.2 by RNAi with essentially normal gonad morphology. Black arrow points to two cell embryo at proximal end of gonad arm. The distal end is marked with an asterisk. (F) mir-240 mir-786 adult-stage worm following knockdown of hmg-1.2 by RNAi with abnormal germline morphology with defects observed in the proximal end of the gonad arm (Black arrow). The distal end is marked with an asterisk.

Table S2. Analysis of enhancement or suppression of *alg-1* lethality phenotype

Strain	Genotype	% Total adult lethality	% Adults that enter lethargus[a]	% Lethality of worms that enter lethargus[b]	% Adult bursting at vulva	% Non-lethargic Bag of worms[c]
N2	wild type	3%	0%	--	3%	0%
RF54	*alg-1(gk214)*	67%	68%	90%	3%	3%
RF411	*mir-52(n4114); alg-1(gk214)*	**17%****	**0%****	--	5%	12%
RF410	*mir-54-55(nDf45) alg-1(gk214)*	**33%****	**21%****	100%	3%	8%
RF89	*mir-54-56(nDf58) alg-1(gk214)*	**18%****	**4%****	100%	3%	12%
RF137	*mir-59(n4604); alg-1(gk214)*	**87%****	**88%****	92%	3%	4%
RF81	*mir-73-74(nDf47) alg-1(gk214)*	55%	**53%***	96%	2%	4%
RF182	*mir-235(n4504); alg-1(gk214)*	70%	72%	90%	2%	3%
RF163	*mir-238(n4112), mir-239a-b(nDf62) alg-1(gk214)*	**27%****	**17%****	100%	1%	9%
RF60	*mir-240 mir-786(n4541);alg-1(gk214)*	**27%****	**14%****	100%	7%	5%

* $p < 0.05$, ** $p < 0.01$ by the chi-square test, as compared to *alg-1* single mutants.
[a] plates were examined every hour from 12 to 20 hours after the L4m for worms that entered lethargus as defined by cessation of pharyngeal pumping and reduced locomotion.
[b] Worms that entered lethargus were transferred to a new plate and scored after 16 hours for lethality. Worms died 28-36 hours after the L4m with embryos that hatched within the adult worm. Represented as # of worms that entered lethargus and died / total # of worms that entered lethargus.
[c] Worms that did not enter a supernumerary lethargus but died (36 hours after the L4m) with embryos that hatched within the adult worm ("bag of worms")

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Table S3. Knockdown of hub gene activity does not result in embryonic lethality

Strain	Genotype	% Embryonic Lethality[a]			
		empty vector RNAi	*egl-27* RNAi	*hmg1.2* RNAi	*trr-1* RNAi
N2	wild-type	0	1.6	2.1	1.3
RF71	*mir-1(n4102)*	2.0	4.9	1.0	–[b]
RF15	*mir-59(n4604)*	0	7.0	3.8	–
RF61	*mir-240 mir-786(n4541)*	4.2	–	**[c]	**
RF24	*mir-247 mir-797 (n4505)*	1.0	2.9	–	5.6

[a] 10-20 L4-stage F1 animals were transferred to a new RNAi plate. 61-122 F2 embryos were collected and transferred to a plate without bacteria. The number of unhatched embryos were scored after 16-24 hours.

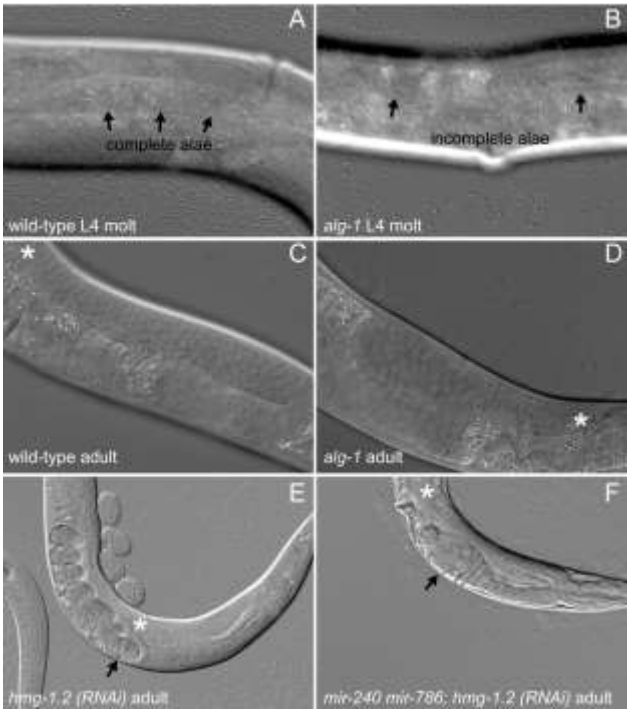
[b] – indicates that the assay was not performed

[c] ** indicates that insufficient (< 10) embryos were found on the F1 plates such that the assay could not be performed.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2946380/bin/NIHMS218551-supplement-01.pdf (72K, pdf)

02 Supplemental Information

Figure S1, Brenner et al.



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http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2946380/bin/NIHMS218551-supplement-02.pdf (1.6M, pdf)

03 Supplemental Information

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2946380/bin/NIHMS218551-supplement-03.xls (25K, xls)

04 Supplemental Information

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2946380/bin/NIHMS218551-supplement-04.xls (29K, xls)