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Many families of *Caenorhabditis elegans* microRNAs are not essential for development or viability

Ezequiel Alvarez-Saavedra and **H. Robert Horvitz**
Howard Hughes Medical Institute, Department of Biology, MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Summary

MicroRNAs (miRNAs) are approximately 23 nt regulatory RNAs that posttranscriptionally inhibit the functions of protein-coding mRNAs. We previously found that most *C. elegans* miRNAs are individually not essential for development or viability and proposed that paralogous miRNAs might often function redundantly 1. To test this hypothesis, we generated mutant *C. elegans* strains that each lack multiple or all members of one of 15 miRNA families. Mutants for 12 of these families did not display strong synthetic abnormalities, suggesting that these miRNA families have subtle roles during development. By contrast, mutants deleted for all members of the *mir-35* or *mir-51* families died as embryos or early larvae, and mutants deleted for four members of the *mir-58* family showed defects in locomotion, body size and egg laying and an inability to form dauer larvae. Our findings indicate that the regulatory functions of most individual miRNAs and most individual families of miRNAs related in sequence are not critical for development or viability. Conversely, because in some cases miRNA family members act redundantly, our findings emphasize the importance of determining miRNA function in the absence of miRNAs related in sequence.

Results and Discussion

Genetic, computational and genome-wide expression analyses indicate that miRNAs function primarily by targeting sites in the 3' untranslated regions (UTRs) of their target genes [2]. miRNA regulation results in translational inhibition and/or mRNA destabilization and, more rarely, in mRNA cleavage. The “seed” nucleotides 2-7 of the miRNA are the most important determinant for target regulation, and miRNAs with identical seed sequences can be grouped into families [2]. In *C. elegans,* approximately half of the miRNAs (76 of 136) define 23 families with two to eight members each (Table S1); eight of these 23 are conserved with humans [3, 4]. Whether miRNAs within a family regulate the same set of target genes has not been studied in detail; differences in regulation of targets among family members might result from differences in pairing at the 3′ end of a miRNA, sequences surrounding the target site, and the timing, space and levels of miRNA gene expression [5-12]. Although functional redundancy among animal miRNA family members has been reported for the *C. eleganslet-7*[13] and mouse *mir-133*[14] families, it is unclear whether such redundancy is widespread.

Corresponding author. horvitz@mit.edu; FAX (617) 253-8126.

To investigate functional redundancy among miRNA family members, we constructed double, triple, or quadruple multiply mutant strains that each carried mutations in members of a given family for 15 of the 23 known *C. elegans* miRNA families (Table 1; Tables S1 and S2). We performed a broad phenotypic analysis and examined morphology, growth, development and behavior. We find that even when several or all members of most of these miRNA families are deleted, *C. elegans* appears to be at least superficially wild-type in phenotype. Animals lacking these miRNA families might have subtler synthetic abnormalities not assayed in out studies. By contrast, we discovered that mutations affecting multiple members of the *mir-35, mir-51* or *mir-58* families caused striking abnormalities (Table 1). [1, 15]

The *mir-35* family comprises eight miRNAs, *mir-35* through *mir-42*, located in two genomic miRNA clusters separated by 350 kb on LGII. One cluster contains *mir-35* through *mir-41* (*mir-35-41*), and the other contains *mir-42*, *mir-43* and *mir-44* (*mir-42-44*) (Figure 1A; Figure S1A) [16]. *mir-43* and *mir-44* are not related to the *mir-35* family by sequence, and *mir-43* and *mir-44* belong to two different miRNA families (Table S1). A *mir-35::GFP* reporter was expressed strongly in most if not all cells of the embryo beginning at the onset of gastrulation and peaking at the onset of elongation (Figure S1B). A *mir-42::GFP* reporter was expressed in cells of the hypodermis, including the seam cells, vulval cells and the rectal epithelial cells during embryonic and postembryonic development (Figure S1C; ref. 11).[16]

We obtained three deletions that affect *mir-35* family members in the *mir-35-41* or *mir-42-44* clusters and constructed strains lacking most or all *mir-35* family members (Figures S1A and S1D). While deletion of *mir-36-41* (*nDf48* mutants), *mir-42-44* (*nDf49* mutants, referred to as *mir-42*) or *mir-36-42* (*nDf48 nDf49* double mutants) did not result in obvious abnormalities, deletion of *mir-35-41* (*nDf50* mutants) resulted in temperature-sensitive embryonic or L1 (first larval stage) larval lethality: almost half of *mir-35-41* animals died during the embryonic or L1 stages at 15°C, and more than 99% died at 26°C (Figures 1B and 1C). By contrast, deletion of all eight *mir-35* family members (*nDf50 nDf49* double mutants) resulted in fully penetrant embryonic or L1 lethality that was not temperature-sensitive (Figure 1B and data not shown). Thus, miRNAs of the *mir-35* family act redundantly and expression of only *mir-35* (in *nDf48 nDf49* mutants) or *mir-42* (in *nDf50* mutants) is sufficient to sustain embryonic development.

The *mir-35* family contains members that are tightly clustered in the genome, and the deletions we isolated affected all or multiple members of each cluster (Figure S1A and S1D). To examine the functions of individual *mir-35* family miRNAs we expressed individual miRNAs from transgenes and found that each *mir-35* family miRNA but not the unrelated *mir-43* and *mir-44* miRNAs was sufficient to rescue the defects caused by deletion of all family members (Figure 1D). In short, all members of the *mir-35* family can act redundantly.

We examined the *mir-35* family mutant phenotype in more detail. Animals deleted for all *mir-35* family members underwent developmental arrest at the two-fold or three-fold stage of embryonic development (Figure 1C; Figure S1E). In some embryos morphogenesis appeared to fail, resulting in the formation of an amorphous mass of cells (Figure S1E). By the 2-fold stage, almost all embryonic cell divisions have occurred and several organs and tissues, such as the hypodermis, the pharynx, and the intestine, can be identified readily [17]. The morphologies of these organs and tissues appeared normal in *mir-35-41 mir-42* mutant embryos, except that in most cases the pharynx failed to elongate and to attach to the hypodermis that links the digestive tract to the hypodermis surrounding the embryo (Figure 1C). Using time-lapse microscopy we found that the development of *mir-35-41 mir-42*

mutants proceeds more slowly than that of wild-type animals (Figure 1C; Movie S1). For example, ventral enclosure started at approximately 260 min after the first cleavage in the wild type but at approximately 340 min in *mir-35-41 mir-42* mutants, and wild-type embryos reached the two-fold stage at approximately 340 min while *mir-35-41 mir-42* mutants did so at approximately 470 min. The elongation of mutant embryos was blocked but animals were not paralyzed, indicating that muscles were functional [18]. Initial cell divisions, gastrulation, dorsal intercalation, and ventral enclosure appeared normal as viewed with Nomarski optics (data not shown).

To seek direct and indirect targets of the *mir-35* family miRNAs, we performed large-scale genetic screens for suppressors of the embryonic lethality of *mir-35-41* and *mir-35-41 mir-42* mutants (Supplemental Experimental Procedures). We were unable to identify any such suppressors, suggesting either that the *mir-35* family controls regulation of several genes that act in parallel in development or that loss-of-function mutations in *mir-35* family target genes result in embryonic lethality.

Using miRNA microarrays and northern blots we found that the *mir-35-41* cluster genes were expressed to much greater extent in oogenesis than in spermatogenesis; furthermore, these miRNAs were the only *C. elegans* miRNAs that showed such differential germline expression. Specifically, we compared animals that produce oocytes but no sperm (*fem-2(b245ts)*[19]) to animals that produce sperm but no oocytes (*fem-3*(*q20ts*)[20]) (Figure 1E; Figure S1F). Most *mir-35-41* miRNA expression originates from the gonad, because gonadless animals do not express these miRNAs at levels detectable by northern blots (Figure S1F; ref. 16). Consistent with the presence of *mir-35-41* miRNAs in oocytes, the embryonic lethality of *mir-35-41 mir-42* mutants was rescued by maternal expression of *mir-35-41* (Table S3). Expression of *mir-35-41* or *mir-35* alone but not *mir-42* exclusively from the embryo (without maternal expression of the miRNAs) was also sufficient for normal development (Table S4), indicating that the essential function of *mir-35* family members is required after embryonic transcription has initiated. The striking germline specificity, maternal contribution and early zygotic expression of *mir-35-41* miRNAs together with the embryonic lethality caused by deletion of *mir-35* family members demonstrate an essential role for this miRNA family during embryonic development.

The *mir-51* family comprises the six miRNAs *mir-51* through *mir-56* [16] and has a human counterpart with three family members (Figure 2A; Figure S2A). The *C. elegans mir-51* family members are highly abundant and are expressed throughout development [4, 21, 22]. Mutation of *mir-100,* the only orthologous *Drosophila melanogaster* miRNA, causes no obviously abnormal phenotype [23]. *C. elegans* mutants deleted for single *mir-51* family members do not have obvious abnormalities [1]. We constructed a strain lacking all *mir-51* family members and found that these mutant animals died as late embryos or shortly after hatching (Figures 2B and 2C; Figure S2B), indicating that family members act redundantly. Frequently, the pharynx was not attached to the hypodermis in arrested embryos and the animals that hatched did so without having fully elongated and displayed gross deformations (Figure 2C). Unlike the embryonic lethality of *mir-35* family mutants the embryonic lethality of *mir-51* family mutants was not rescued by a maternal contribution of miRNAs, since we were unable to recover viable homozygote mutant progeny from a heterozygote. The embryonic lethality caused by deletion of *mir-51* family members could be rescued by transgene expression of any single *mir-51* family miRNA (Figure 2D), demonstrating that all family members act redundantly.

To assess the relative importance of different *mir-51* family members we generated strains lacking all possible combinations of *mir-51* family mutations (Figure 2B; Figure S2C). Animals expressing only *mir-53* died as young larvae, but no other strain displayed

significant embryonic or larval lethality (Figure S2C). Several mutant combinations developed at slower rates than did the wild type (Figure 2B). From these observations, we deduced the contribution of a lack of each of these miRNAs to the mutant phenotype as *mir-52* ≅ *mir-54-6* > *mir-51* > *mir-53*, which correlates with the level of endogenous expression of these miRNAs (*mir-52* > *mir-54-6* > *mir-51* ≅ *mir-53*) [4, 21]. The difference in phenotype might also be explained by differences in miRNA sequence or site or time of expression. The range of mutant phenotypes, from slow growth to larval lethality to embryonic lethality, suggests that the *mir-51* family has roles during both embryonic and larval development and is consistent with expression at all developmental stages [21, 22].

The *mir-58* family comprises four highly abundant members, *mir-58, mir-80, mir-81* and *mir-82* and the recently discovered *mir-1834* (Figure 3A; Figure S3A) [16, 24]. *mir-58* family members are orthologs of *bantam* (Figure 3A), a *Drosophila* miRNA that controls cell proliferation and apoptosis [25]. Homologs of these miRNAs have been found in other nematodes and insects but not mammals [26]. In *C. elegans*, *mir-58, mir-80, mir-81* and *mir-82* are expressed at all developmental stages[21, 22].

Single mutants of the *mir-58* family do not display obvious abnormalities [1]. By contrast, we found that mutant adults lacking all *mir-58* family members for which a deletion is available, referred to as *mir-58,80,81,82,* were sluggish (Table 1) and smaller than the wild type, comparable in size to mutants defective in the regulation of body size such as *sma-1, sma-2* and *dbl-1* mutants (Figure 3B; Figure S3B)[27]. In addition, one-day old *mir-58,80,81,82* hermaphrodites but not single *mir-58* family mutants laid eggs at an abnormally wide range of stages, with over 25% retained *in utero* until at least the three-fold stage, reflecting both a longer retention of embryos *in utero* and slower rates of egg-laying than the wild type (Figure 3C; Figure S3C). The locomotion, body size and egg-laying defects could be rescued by expression of *mir-80* from a transgene (Figures 3B and 3C; Figures S3B, S3C and S3D). We found that *mir-58,80,81,82* mutants but not any other combination of *mir-58* family mutants were unable to form dauer larvae (Table 1; Figure 3D; Figure S3E), an arrested developmental stage that forms in response to environmental stress [28]. The dauer formation defect could be rescued by expression of *mir-58, mir-80,* or *mir-82* but not of *mir-81* from transgenes (Figure 3D and Supplemental Data).

Our phenotypic analysis of 15 miRNA families identified two miRNA families with essential roles in embryogenesis and one miRNA family that affects both development and behavior. We found that expression of individual miRNAs from transgenes was sufficient to rescue the defects caused by deletion of all corresponding family members for 17 of the 18 miRNAs we tested, indicating that members of each miRNA family act redundantly with other family members and suggesting that complementarity to miRNA 5′ sequences that include the seed is sufficient for efficient regulation of target genes. That miRNAs within families act redundantly with each other reveals the importance of eliminating sequence-related miRNAs to uncover the full effect of regulation by miRNAs. Surprisingly, we found that 12 of the 15 families analyzed did not show strong synthetic abnormalities, indicating that the regulatory roles of many miRNAs are not critical for development and suggesting that either most miRNA families act redundantly with other miRNAs, miRNA families or non-miRNA genes or that most miRNAs play relatively minor biological roles.

Experimental Procedures

Nomenclature

We named each family according to the miRNA with the lowest gene number (e.g., *mir-58* for the *mir-58,80,81,82,1834* family).

Strains and genetics

C. elegans was cultured as described previously [29]. N2 was the wild-type strain. The mutations used are described in Supplemental Experimental Procedures.

Rescue experiments

For rescue experiments we amplified fragments from wild-type genomic DNA using PCR and cloned them into either the pGEM-T Easy (Promega), pCR2.1 or pCRII-TOPO (Invitrogen) vectors. We used site-directed ligase-independent mutagenesis [30] to generate control plasmids in which the mature miRNA sequence was deleted and to generate plasmids with single miRNAs when miRNAs were clustered in the genome. Germline transformation experiments were performed as described [31]. Injection mixes contained plasmids at 50 ng/μl (for *mir-80* and a *mir-80* control plasmid with the mature *mir-80* sequence deleted), 40 ng/μl (for *mir-51, mir-53* and *mir-54*), 20 ng/μl (for *mir-35* through *mir-44*, *mir-58* and *mir-82*), 10 ng/μl (for *mir-52* and *mir-56*), or 1 ng/μl (for *mir-55*). All mixes contained 20 ng/μl of pTG96 (*sur-5::gfp*) [32]as a co-transformation marker and 80 ng/μl of 1 kb+ DNA ladder (Invitrogen) as carrier DNA. We unsuccessfully attempted to rescue the dauer-defective phenotype of *mir-80(nDf53); mir-58(n4640); mir-81-82(nDf54)* using *mir-81* constructs injected at 50 ng/μl and 100 ng/μl (Supplemental Data). The *T07D1.2::mir-82* transgene is described in Supplemental Data.

Microarray analysis

Total RNA was isolated from staged one-day-old adult populations of *C. elegans* by freeze-cracking [33] and extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Synchronized animals were grown at 25°C and harvested 24 h after the late L4 stage. A total of 20 μg of total RNA was used for each microarray experiment. The results shown are the averages of data obtained from two biological replicates. Short RNAs were isolated using denaturing polyacrylamide gels [34], and RNA was labeled by ligation to pCU-Cy3 [11]. Our microarrays represented 108 miRNAs and miRNA candidates that corresponded to miRBase release 3.0. Microarray probes were oligonucleotides with sequences complementary to miRNAs printed on custom microarrays as described [11]. Each oligonucleotide was printed four times. Data were scaled based on total array intensities (sum of the normalized spot intensities), and data for each sample and each gene were averaged. Microarray data is deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE---------.

Phenotypic analyses

Locomotion—The number of body bends during a 20 s period was counted after transferring one-day-old adults to Petri plates without bacteria (food). A total of 20 animals from four Petri plates were scored per genotype. The average for the four plates is reported.

Egg retention—Single one-day-old adults were placed in a drop of a 1M NaOH, 5% hypochlorite solution to dissolve the animal, allowing the eggs to be counted. At least 10 animals per genotype were scored. Assays for defecation, pharyngeal pumping, dye filling, dauer entry and dauer exit were done as described [1].

Isolation of dauer larvae—Dauer larvae were isolated by washing animals from plates with a 1% SDS solution five days after food was exhausted and incubating for 1 h.

Growth rate—We determined time it takes embryos that had been laid within an interval of four to six h to reach the L4 stage as a measure of growth rate. The late L4 stage was identified by the presence of a crescent-shaped developing vulva.

Time-lapse microscopy—Wild-type and *mir-35-41(nDf50) mir-42(nDf49)* one-day-old adults were dissected to obtain early-stage embryos. Photographs of the embryos were taken every min for a total of 800-1000 min in a temperature-controlled room set to 22.5°C. The pictures were converted to a time-lapse movie with 16.7 frames per s.

Stage of freshly laid embryos—One-day-old adults were allowed to lay embryos on a plate for 1.5 h and removed. The stages of the embryos were scored immediately after the adults were removed[35].

Rate of egg laying—Six one-day-old adults were placed on Petri plates seeded with bacteria (food) and removed after 12 h. The number of embryos and larva on the plate was counted.

Highlights

- Most miRNA families are not critical for *C. elegans* development or viability
- The *mir-35* and *mir-51* families are required for normal embryonic development
- The *mir-58* family is required for normal development and behavior

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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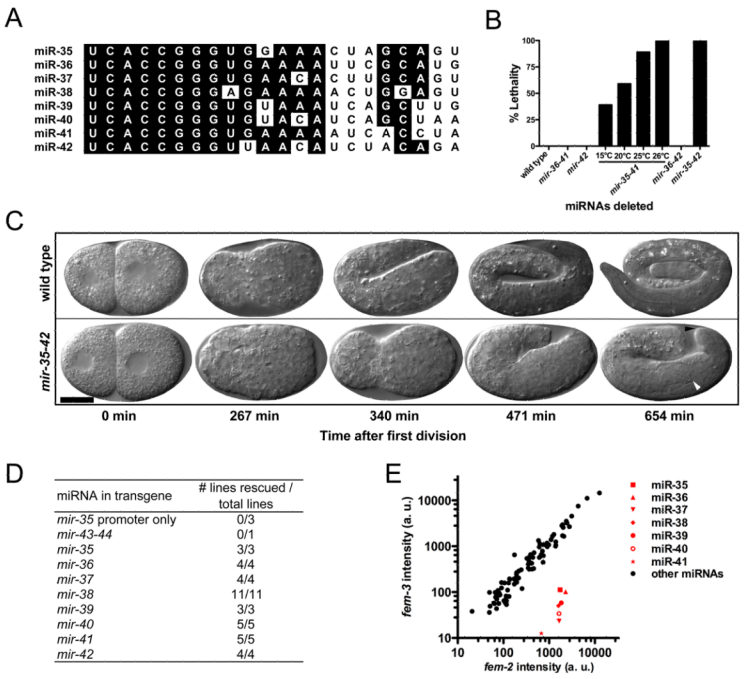
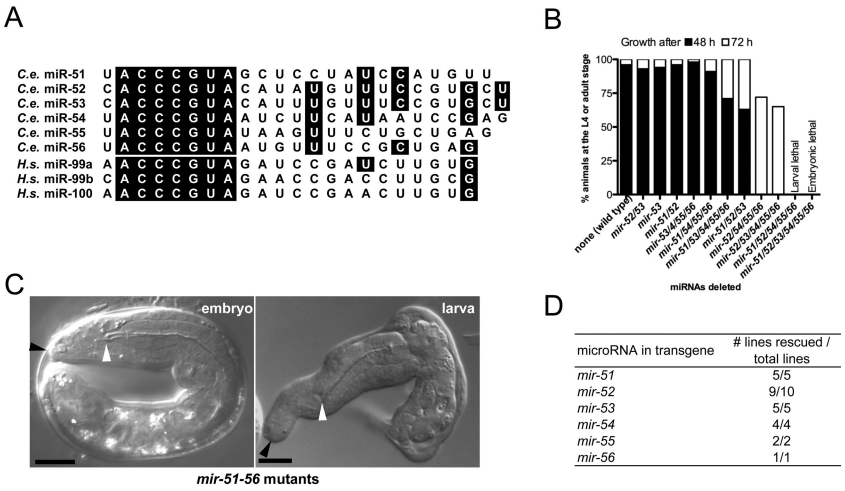


Figure 1. *mir-35* family sequences and mutant phenotypes

(A) Sequences of the eight *mir-35* family members. Nucleotides are shown in white letters on black background if more than half of the family members are identical. (B) Mutations in the *mir-35* family result in embryonic or L1 lethality. n>200 for each genotype. All experiments were conducted at 20°C except where noted. (C) *mir-35-41(nDf50) mir-42(nDf49)* mutant animals develop abnormally slowly and arrest during embryogenesis. Timepoints are from time-lapse recordings of individual embryos. Time = 0 min corresponds to the first cell division. In the *mir-35-41(nDf50) mir-42(nDf49)* embryo the anterior pharynx (white arrowhead) is not attached to the mouth hypodermal cells that normally link the digestive tract to the hypodermis surrounding the embryo (black arrowhead). The wild-type embryo hatched at 654 min. Scale bar, 10 μm. (D) Transgenic expression of any *mir-35* family member rescues the embryonic lethality of *mir-35-41(nDf50) mir-42(nDf49)* animals. We injected mir-35-41(nDf50) mir-42(nDf49)/ mIn1[mIs14 dpy-10(e128)] animals with constructs encoding single miRNAs and established transgenic lines. We concluded that a transgene had rescued the lethality if we could maintain a homozygous *mir-35-41(nDf50) mir-42(nDf49)* strain carrying the transgene. (E) Scatter plot of the normalized intensities from a miRNA microarray comparing *fem-3(q20ts)* to *fem-2(b245ts)* expression. The germline of *fem-3(q20ts)* animals produces only sperm, while the germline of *fem-2(b245ts)* animals produces only oocytes at the restrictive temperature. See also Figure S1.



microRNA in transgene	# lines rescued / total lines
mir-51	5/5
mir-52	9/10
mir-53	5/5
mir-54	4/4
mir-55	2/2
mir-56	1/1

Figure 2. *mir-51* family sequences and mutant phenotypes

(A) Sequences of the *C. elegans* (*C.e.*) *mir-51* family members and their human (*H.s.*) homologs. Nucleotides are shown in white letters on black background if more than half of the *C. elegans* family members are identical. (B) Some *mir-51* family mutant combinations result in slow growth and embryonic or early larval lethality. The percent of animals that had progressed beyond the late fourth larval (L4) stage 48 h or 72 h after they were laid as embryos is indicated; n>100 for each genotype and timepoint. Table S9 lists the genotypes of the strains scored. (C) Animals lacking all *mir-51-56* family members (mir-51 mir-53(nDf67); mir-52(n4114); mir-54-56(nDf58)) died as embryos or early larvae. Micrographs were taken 16-18 h after the first cell division. Scale bars, 10 μm. In the mir-51 mir-53(nDf67); mir-52(n4114); mir-54-56(nDf58) embryo the anterior pharynx (white arrowhead) is not attached to the hypodermal cells of the mouth that normally link the digestive tract to the hypodermis surrounding the embryo (black arrowhead). The newly hatched larva shows gross posterior body abnormalities. (D) Expression of single *mir-51* family members rescues the embryonic lethality caused by lack of all members. We injected heterozygote mir-51 mir-53(nDf67) mir-52(n4114)/nT1 [qIs51]; mir-54-56(nDf58) animals with constructs containing single miRNAs and established transgenic lines. We concluded that a transgene had rescued the lethality if we could maintain a homozygous mir-51 mir-53(nDf67) mir-52(n4114); mir-54-56(nDf58) strain carrying the transgene. See also Figure S2.

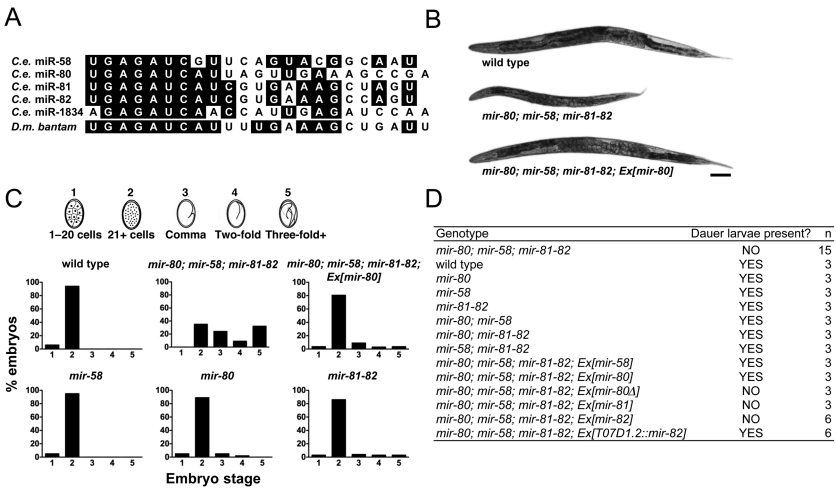


Figure 3. *mir-58* family sequences and mutant phenotypes

(A) Sequences of the *C. elegans* (*C.e.*) *mir-58* family and the *Drosophila melanogaster* (*D.m.*) homolog *bantam*. Nucleotides are shown in white letters on black background if more than half of the *C. elegans* family members are identical. (B) *mir-80(nDf53); mir-58(n4640); mir-81-82(nDf54)* mutants are smaller than the wild type. A transgene carrying *mir-80* (*Ex[mir-80]*) rescued the small body size defect of *mir-80(nDf53); mir-58(n4640); mir-81-82(nDf54)* mutants. Micrographs were taken 48 h after the late L4 (fourth larval) stage. Scale bars, 100 μm. (C) *mir-58* family mutants lay late-stage embryos. Distributions of the developmental stages of eggs laid by wild-type animals, single and combinations of *mir-58* family mutants, and *mir-58,80,81,82* animals carrying a *mir-80* transgene (*Ex[mir-80]*). At least 100 embryos were scored for each genotype. (D) *mir-58* family mutants are defective in the formation of dauer larvae. “YES”, dauers were isolated from every plate assayed; “NO”, no dauers were isolated from any of the plates assayed. n, number of plates. Two independently isolated transgenic lines were tested for the *mir-82* and *T07D1.2::mir-82* transgenes (three plates for each line), and one line was tested for each of the other transgenes. *mir*-82 is contained within an intron of *T07D1.2*. The transgene designated *[mir-80Δ]* had the sequence corresponding to *mir-80* deleted. See also Figure S3.

Table 1

Phenotypic characterization of miRNA family mutants

Family	Locomotion (body bends/ 20s)	Pumping (pumps/ 20s)	Defecation (defecation interval, s)	Egg retention (no. eggs *in utero)*	DiO fill	Dauer entry	Dauer exit	Synthetic abnormalities
wild-type	16.0 ± 0.9	75.4 ± 7.8	55.6 ± 6.2	17.1 ± 4.3	+	+	+	
Defective mutants (controls)	9.9 *** ± 1.8	17.0 *** ± 6.9	326.8 *** ± 198.0	33.2 *** ± 6.5	−	−	−	
lin-4,mir-237	0 *** ± 0.0	66.0 ± 17.6	63.1 ± 16.2	10.3 ** ± 4.6	+	−	NA	#
let-7,***mir-48,84,241***,*793,794,795*	5.9 *** ± 3.1	67.0 ± 12.1	56.9 ± 8.9	21.5 ± 3.1	+	+	+	# Developmental timing
mir-2,43,250,797	15.0 ± 1.1	65.6 ± 4.8	68.0 ± 12.6	8.0 *** ± 5.3	+	+	+	#
mir-35,36,37,38,39,40,41,42	NA	NA	NA	NA	NA	NA	NA	Embryonic lethality
mir-44, *45*,***61,247*** &	13.4 ± 2.8	75.0 ± 8.2	64.4 ± 12.8	8.5 *** ± 5.3	+	+	+	#
mir- *44*,***45,61,247*** &	13.3 ± 3.4	71.8 ± 4.4	67.6 ± 21.0	9.5 *** ± 5.1	+	+	+	#
mir-46,47	12.8 ± 3.1	76.2 ± 6.4	68.0 ± 11.3	16.3 ± 1.6	+	+	+	
mir-51,52,53,54,55,56	NA	NA	NA	NA	NA	NA	NA	Embryonic lethality
mir-58,80,81,82,1834	3.6 *** ± 3.3	67.0 ± 10.1	93.0 ± 35.0	11.8 ± 5.1	+	−	NA	Locomotion, dauer entry, egg-laying, body size
mir-63,64,65,66,229	12.8 ± 1.2	73.0 ± 10.2	52.9 ± 7.9	18.6 ± 5.2	+	+	+	
mir-72,73,74	12.5 ± 0.5	79.2 ± 7.5	55.9 ± 15.9	20.6 ± 5.6	+	+	+	
mir-75,79	13.6 ± 0.9	77.8 ± 9.8	54.5 ± 16.0	14.0 ± 3.8	+	+	+	
mir-87,233	12.5 ± 0.8	64.8 ± 20.5	50.0 ± 3.9	19.5 ± 6.4	+	+	+	
mir-232,357	11.7 ± 2.1	74.8 ± 11.2	72.5 ± 16.0	15.9 ± 5.3	+	+	+	
mir-238,239a,239b	11.8 ± 2.3	72.2 ± 7.2	55.3 ± 15.1	16.9 ± 6.8	+	+	+	
mir-251,252	13.1 ± 0.9	73.2 ± 10.0	57.9 ± 13.1	20.7 ± 4.4	+	+	+	

miRNAs that were deleted for each family are indicated in bold. Average and standard deviation are reported for each assay (AVG±SD). The complete genotype for strains is given in Supplementary Table 2. The mutants with established relevant phenotypic defects used as controls were *mod-5(n3314)* for locomotion, *eat-2(ad465)* for pumping, *dec-1(sa48)* for defecation, *egl-5(n945)* for egg retention, *daf-16(mgDf50)* for dauer entry and *daf-21(p673)* for dauer exit. Phenotypic assays are described in Materials and Methods. +, normal; -, defective; NA, not applicable. Strains were compared using analysis of variance followed by a Dunnett's multiple comparison test using the wild type as the reference.

let-7 family animals were scored after passage through dauer to suppress the early adult lethality 2.

*** p value<0.001,

** p value<0.01.

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[&] *mir-44* and *mir-45* are located 9 kb apart, and our efforts to identify a deletion affecting both miRNAs or to obtain a recombinant chromosome with deletions affecting both miRNAs were unsuccessful. Instead, we constructed and examined two strains, each missing three of the four members of the *mir-44* family.

[#] The defects observed for the *let-7*, *lin-4* and the *mir-2* and *mir-44* families are not synthetic (See also Tables S1 and S2; refs. 1,2,14)