OPEN ACCESS Freely available online

PLoS GENETICS

Most *Caenorhabditis elegans* microRNAs Are Individually Not Essential for Development or Viability

Eric A. Miska[1,2¤a☯], Ezequiel Alvarez-Saavedra[1,2☯], Allison L. Abbott[3☯¤b], Nelson C. Lau[4¤c], Andrew B. Hellman[1,2¤d], Shannon M. McGonagle[1,2¤e], David P. Bartel[1,2,4], Victor R. Ambros[3], H. Robert Horvitz[1,2*]

1 Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America, **2** Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America, **3** Department of Genetics, Dartmouth Medical School, Hanover, New Hampshire, United States of America, **4** Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, United States of America

MicroRNAs (miRNAs), a large class of short noncoding RNAs found in many plants and animals, often act to post-transcriptionally inhibit gene expression. We report the generation of deletion mutations in 87 miRNA genes in *Caenorhabditis elegans*, expanding the number of mutated miRNA genes to 95, or 83% of known *C. elegans* miRNAs. We find that the majority of miRNAs are not essential for the viability or development of *C. elegans*, and mutations in most miRNA genes do not result in grossly abnormal phenotypes. These observations are consistent with the hypothesis that there is significant functional redundancy among miRNAs or among gene pathways regulated by miRNAs. This study represents the first comprehensive genetic analysis of miRNA function in any organism and provides a unique, permanent resource for the systematic study of miRNAs.

Citation: Miska EA, Alvarez-Saavedra E, Abbott AL, Lau NC, Hellman AB, et al. (2007) Most *Caenorhabditis elegans* microRNAs are individually not essential for development or viability. PLoS Genet 3(12): e215. doi:10.1371/journal.pgen.0030215

Introduction

MicroRNAs (miRNAs) were discovered in *C. elegans* during studies of the control of developmental timing [1–5]. miRNAs are approximately 22-nucleotide noncoding RNAs that are thought to regulate gene expression through sequence-specific base-pairing with target mRNAs [6]. miRNAs have been identified in organisms as diverse as roundworms, flies, fish, frogs, mammals, flowering plants, mosses, and even viruses, using genetics, molecular cloning, and predictions from bioinformatics [7–16]. In *C. elegans* about 115 miRNA genes have been confidently identified [10,11,17–20].

In animals, miRNAs are transcribed as long RNA precursors (pri-miRNAs), which are processed in the nucleus by the RNase III enzyme complex Drosha-Pasha/DGCR8 to form the approximately 70-base pre-miRNAs [21–25] or are derived directly from introns [26,27]. Pre-miRNAs are exported from the nucleus by Exportin-5 [28], processed by the RNase III enzyme Dicer, and incorporated into an Argonaute-containing RNA-induced silencing complex (RISC) [29]. Within the silencing complex, metazoan miRNAs pair to the mRNAs of protein-coding genes, usually through imperfect base-pairing with the 3′-UTR, thereby specifying the posttranscriptional repression of these target mRNAs [6,30]. Binding of the silencing complex causes translational repression [31–33] and/or mRNA destabilization, which is sometimes through direct mRNA cleavage [34,35], but usually through other mechanisms [36–40]. Because many messages have been under selective pressure to preserve pairing to a 6mer in the 5′ region of the miRNA known as the miRNA seed (nucleotides 2–7), targets of metazoan miRNAs can be predicted above the background of false-positives by searching for conserved matches to the seed region [41–45]. In nematodes, at least 10% of the protein-coding messages appear to be conserved targets of miRNAs [46].

The in vivo functions of a few miRNAs have been established. In *C. elegans*, the *lin-4* miRNA and the *let-7* family of miRNAs control the timing of aspects of larval development. For example, the *lin-4* miRNA controls hypodermal cell-fate decisions during early larval development by negatively regulating the *lin-14* and *lin-28* mRNAs [1–3,5,47]. The *let-7* miRNA controls hypodermal cell-fate decisions during late-larval development by regulating the *lin-41*, *hbl-1*, *daf-12*, and *pha-4* mRNAs [48–51]. Three additional *C. elegans* *let-7*-like miRNAs, miR-48, miR-84, and miR-241, also act in

Editor: Michael T. McManus, University of California San Francisco Diabetes Center, United States of America

Received August 30, 2007; **Accepted** October 12, 2007; **Published** December 14, 2007

A previous version of this article appeared as an Early Online Release on October 15, 2007 (doi:10.1371/journal.pgen.0030215.eor).

Abbreviations: miRNA, microRNA

* To whom correspondence should be addressed. E-mail: horvitz@mit.edu

☯ These authors contributed equally to this work.

¤a Current address: Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom

¤b Current address: Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin, United States of America

¤c Current address: Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts, United States of America

¤d Current address: Department of Biological Sciences, Stanford University, Stanford, California, United States of America

¤e Current address: Molecular and Cellular Biology Program, University of Washington, Seattle, Washington, United States of America

Author Summary

MicroRNAs (miRNAs) are tiny endogenous RNAs that regulate gene expression in plants and animals. Individual miRNAs have important roles in development, immunity, and cancer. Although the investigation of miRNA function is of great importance, to date few miRNAs have been studied in the intact organism because of a lack of mutants in which specific miRNAs have been inactivated. Here we describe a collection of loss-of-function mutants representing the majority of all known miRNA genes in the nematode *Caenorhabditis elegans*. This study identifies a new role for miRNAs in *C. elegans* and also demonstrates that most miRNAs are not essential for viability or development. Our findings suggest that many miRNAs act redundantly with other miRNAs or other pathways. We expect that this collection of miRNA mutants will become a widely used resource to further our understanding of the biology of miRNAs.

the control of developmental timing and likely regulate the *hbl-1* mRNA, but act earlier in development than the *let-7* miRNA [52,53]. The *C. elegans lsy-6* miRNA acts in the asymmetric differentiation of the left and right ASE chemosensory neurons. Specifically, the *lsy-6* miRNA targets the *cog-1* mRNA, resulting in a shift of marker gene expression in the left ASE to resemble marker gene expression in the right ASE [20]. The first miRNA studied functionally in *Drosophila* is encoded by the *bantam* locus, which had previously been identified in a screen for deregulated tissue growth [54]. The *bantam* miRNA stimulates cell proliferation and reduces programmed cell death. *bantam* directly regulates the pro-apoptotic gene *hid*. A second *Drosophila* miRNA, miR-14, also reduces programmed cell death [55]. The muscle-specific *Drosophila* miRNA miR-1 is required for larval development and cardiac differentiation [56,57]. Dmir-7 regulates the transcription factor Yan [58]. Finally, *Drosophila* miR-9a is required for sensory organ precursor specification [59], and *Drosophila* miR-278 is required for energy homeostasis [60]. The first loss-of-function studies of miRNAs in the mouse have been reported demonstrating a role for miR-1 and miR-208 in cardiac growth in response to stress [61,62] and miR-155/BIC in normal immune function [63,64].

miRNA function has also been inferred from studies in which miRNAs have been misexpressed in worms, flies, frogs, mice, and cultured mammalian cells [65]. In addition, miRNA function has been explored by perturbing the functions of genes in the pathway for miRNA biogenesis and by reducing miRNA levels using antisense oligonucleotides. For example, mutants defective in Dicer, which is essential for miRNA biogenesis, have been studied for *C. elegans* [66,67], *Drosophila* [68,69], the zebrafish [70,71], and the mouse [72–75]. In all cases, Dicer was found to be essential for normal development. In addition, members of the AGO subfamily of Argonaute proteins, which act in the miRNA pathway, are essential for normal *C. elegans* and mouse development [67,76].

In *Drosophila*, 2′ O-methyl antisense oligoribonucleotides have been used in miRNA depletion studies [77]. This technique was initially described for human cells and *C. elegans* [78,79] and appears to offer sequence-specific inhibition of small RNAs for a limited time span. Injection of individual 2′ O-methyl antisense oligoribonucleotides complementary to the 46 miRNAs known to be expressed in the fly embryo resulted in a total of 25 different abnormal phenotypes, including defects in patterning, morphogenesis, and cell survival [77]. Knockdown of miRNAs using modified 2′ O-methyl antisense oligoribonucleotides also has been reported for the mouse [80]. Very recently, a study reported the use of morpholinos to knockdown miRNA function in zebrafish and identified a role for miR-375 in pancreatic islet development [81].

To gain a broader understanding of miRNA function, we generated a collection of deletion mutants of the majority of known miRNA genes in *C. elegans*. We found that mutations in most miRNA genes do not result in striking abnormalities, and therefore most miRNA genes likely have subtle or redundant roles. This permanent collection provides a resource for detailed studies of miRNA function not possible previously.

Results

The cloning of many miRNAs from *C. elegans* using molecular biological techniques prompted us to take a genetic approach to study miRNA function in vivo in *C. elegans* through the generation of loss-of-function mutants. We isolated deletion mutants using established *C. elegans* techniques [82,83]. We made extensive use of the "poison" primer method, which increases the sensitivity of detection of small deletions [84]. Most *C. elegans* miRNAs were cloned and verified in northern blot experiments [10,11,17,85]. Some miRNAs were predicted based on pre-miRNA folds and verified using northern blotting or PCR with specific primers and cloned miRNA libraries [17,18,85,86]. The public database for miRNAs, miRBase release 9.0, listed 114 *C. elegans* miRNAs [87,88]. Of these 114, 96 miRNAs are confidently identified, based on expression and the likelihood of being derived from stem-loop precursors, whereas many of the others do not appear to be authentic miRNAs [17–19]. Recently, two studies using high-throughput sequencing methods identified 21 additional miRNAs [19,26] bringing the total number of miRNAs identified with high confidence in *C. elegans* to 115 and the total number of annotated miRNA candidates to 135.

We isolated knockout mutants covering 87 miRNA genes. We previously described our studies of knockouts of three additional miRNA genes [52], and deletions in two other miRNA genes had been obtained by the *C. elegans* knockout consortium (D. Moerman, personal communication) [84]. Three miRNA genes had been mutated in genetic screens, *lin-4*, *let-7*, and *lsy-6* [2,4,20]. Thus, 95 *C. elegans* miRNAs can now be functionally analyzed using mutants (Table 1). Additional alleles for a subset of these miRNA genes were also isolated by the *C. elegans* knockout consortium (D. Moerman, personal communication) [84].

The median size of the deletions we isolated was 911 bases with a range of 181–6,288 bases (Tables 1 and S1). Some deletions likely affect neighboring genes in the case of intergenic miRNA genes or host genes in the case of miRNA genes found in introns. For example, the lethality linked to *mir-50(n4099)* (Table 2) might be a consequence of a loss-of-function of *mir-50* or of an effect on the predicted host gene Y71G12B.11a (Table 1).

We performed a broad phenotypic study of all available miRNA loss-of-function mutants, including mutants that had been reported earlier [2,4,20,52]. We focused on phenotypic

Table 1. miRNA Mutants

miRNA Gene(s)	Allele(s)	Chromosome	Deletion Size (bp)	Other Locus Information
let-7	*n2853ts*[a]	X	Point mutation	
lin-4	*e912*[b]	II	Not determined	
lsy-6	*ot71*[c]	V	1,071	
mir-1	*n4101, n4102*	I	380, 823	
mir-2	*n4108*	I	556	
mir-34	*n4276*	X	630	
mir-35–41	*nDf50*	II	1,261	Intron of Y62F5A.9
mir-42–44	*nDf49*	II	1,103	
mir-45	*n4280*	II	1,495	
mir-46	*n4475*	III	1,637	
mir-47	*gk167*[d]	X	1,110	
mir-48	*n4097* [e]	V	293	
mir-48, mir-241	*nDf51*[e]	V	5,930	
mir-50	*n4099*	I	1,015	Intron of Y71G12B.11a
mir-51	*n4473*	IV	1,504	
mir-52	*n4100, n4114, n4125*	IV	398, 148, 559	
mir-53	*n4113*	IV	805	
mir-54–56	*nDf45, nDf58*	X	150, 1,805	
mir-57	*gk175*[d]	II	474	
mir-58	*n4640*	IV	785	Intron of Y67D8A.1
mir-59	*n4604*	IV	1,483	
mir-60	*n4947*	II	787	
mir-61, mir-250	*nDf59*	V	1,142	
mir-62	*n4539*	X	993	Intron of *ugt-50*
mir-63	*n4568*	X	657	
mir-64, mir-229	*nDf52*	III	652	
mir-64–66, mir-229	*nDf63*	III	3,124	
mir-67	*n4899*	III	526	Intron of *zmp-1*
mir-70	*n4109, n4110*	V	738, 203	Intron of T10H9.5
mir-71	*n4105, n4115*	I	354, 181	
mir-72	*n4130*	II	968	
mir-73–74	*nDf47*	X	326	
mir-75	*n4472*	X	1,972	
mir-76	*n4474*	III	941	
mir-77	*n4286*	II	1,036	
mir-78	*n4637*	IV	738	
mir-79	*n4126*	I	386	
mir-80, mir-227	*nDf53*	III	728	
mir-81–82	*nDf54*	X	6,288	Also deletes T02D1.2
mir-83	*n4638*	IV	823	
mir-84	*n4037*[e]	X	791	
mir-85	*n4117*	II	563	Intron of F49E12.8, antisense
mir-86	*n4607*	III	1,062	Intron of Y56A3A.7
mir-87	*n4104, n4123, n4124*	V	514, 254, 615	
mir-124	*n4255*	IV	211	Intron of *trpa-1*
mir-228	*n4382*	IV	1,026	
mir-230	*n4535*	X	957	
mir-231	*n4571*	III	1,104	
mir-232	*nDf56*	IV	2,148	Also deletes F13H10.5
mir-233	*n4761*	X	669	Intron of W03G11.4
mir-234	*n4520*	II	1,178	
mir-235	*n4504*	I	781	
mir-237	*n4296*	X	614	
mir-238	*n4112*	III	536	
mir-239a-b	*nDf62*	X	2351	
mir-240, mir-786	*n4541*	X	1185	
mir-241	*n4315*[e], *n4316*[e]	V	506, 458	
mir-242	*n4605*	IV	949	
mir-243	*n4759*	IV	1,102	
mir-244	*n4367*	I	1,832	
mir-245	*n4798*	I	1,064	
mir-246	*n4636*	IV	518	
mir-247, mir-797	*n4505*	X	611	
mir-249	*n4983*	X	734	
mir-251	*n4606*	X	976	
mir-252	*n4570*	II	1,447	
mir-253	*nDf64*	V	1,095	Intron of F44E7.5
mir-254	*n4470*	X	484	Intron of *gcy-9*
mir-256[f]	*n4471*	V	1,027	Upstream of *mec-1*
mir-257[f]	*n4548*	V	785	
mir-258[f]	*n4797*	X	992	
mir-259	*n4106*	V	529	
mir-260[f]	*n4601*	II	911	
mir-261[f]	*n4594*	II	993	Also deletes B0034.4
mir-265[f]	*n4534*	IV	1,215	
mir-268[f]	*n4639*	V	1,010	
mir-269[f]	*n4641*	IV	496	
mir-270[f]	*n4595*	IV	954	
mir-272[f]	*nDf66*	III	1,054	
mir-273[f]	*n4438*	I	762	
mir-353	*nDf61*	I	521	Also deletes *rpl-24.1*
mir-355	*n4618*	V	1,106	
mir-357–8	*nDf60*	V	1,594	
mir-359	*n4540*	X	627	Also deletes Y41G9A.10
mir-360	*n4635*	X	1,307	

For miRNA clusters "-" indicates that all miRNAs are deleted inclusively, e.g., *mir-35–41* means that *mir-35*, *mir-36*, *mir-37*, *mir-38*, *mir-39*, *mir-40*, and *mir-41* are all deleted. Genes or predicted genes near to or overlapping with miRNA genes are as annotated in WormBase Release WS170 at http://ws170.wormbase.org/ [107].

[a] Previously described in [4].

[b] Previously described in [5].

[c] Previously described in [20].

[d] Mutant alleles were generated by the *C. elegans* knockout consortium [93].

[e] Previously described in [52].

[f] Unlikely to encode miRNAs [18,19].

doi:10.1371/journal.pgen.0030215.t001

assays that are relatively rapid and that examine *C. elegans* morphology, growth, development, and behavior. The assays we performed are shown in Table 3 and the phenotypes we observed are summarized in Table 2. Our initial phenotypic analysis revealed a single new abnormality linked to miRNA loss-of-function: deletion of the *mir-240 mir-797* miRNA cluster resulted in abnormal defecation cycle lengths. This defecation defect was rescued by the introduction of a transgene carrying the *mir-240 mir-797* genomic locus (Table S2). In addition, we observed other abnormal phenotypes. Mutation of the *mir-35–41* miRNA cluster resulted in temperature-sensitive embryonic and larval lethality; this lethality was rescued by the introduction of a transgene carrying the *mir-35–41* genomic locus (unpublished data). We were unable to generate homozygotes for alleles of *mir-50* and *mir-353*. *mir-50* and *mir-353* are in introns of genes that when inactivated by RNAi result in embryonic lethality and may explain why we could not isolate homozygotes for our new deletions. Indeed, the introduction of a transgene carrying the *mir-50* genomic locus failed to rescue the lethality associated with the *mir-50* allele (unpublished data). The number of times each of the deletion strains has been outcrossed is shown in Table 2. It is conceivable that some of the miRNA deletion strains harbor additional mutations that suppress abnormalities conferred by miRNA deletion alleles and that could be

Table 2. Phenotypic Characterization of miRNA Mutants

Controls and Alleles	Strain	Number of Outcrosses	Genotype	Locomotion (Body Bends)	Pumping	Defecation	Egg Retention	DiO filling	DAPI (L1)	Dauer Behavior			Abnormalities
				AVG ± SD	AVG ± SD	AVG ± SD	AVG ± SD			Entry	Constitutive (%)	Exit	
Controls	N2		Wild-type	13.3 ± 2.5	75.6 ± 4.9	60.9 ± 11.0	14.4 ± 4.5	+	+	+	0	+	
	GR1307		*daf-16(mgDf50)*							–	0	NA	Dauer formation
	CB1370		*daf-2(e1370ts)*							+	100	+	Dauer formation
	PR673		*daf-21(p673)*							+	78	–	Dauer exit
	JT48		*dec-1(sa48)*			150.8 ± 142.6							
	DA465		*eat-2(ad465)*		17.0 ± 6.9								Pumping
	MT1975		*egl-5(n945)*				20.4 ± 4.4						Egg-laying
	MT2426		*goa-1(n1134)*	15.5 ± 1.4									Locomotion
	MT9795		*mod-5(n3314)*	3.1 ± 3.4									Locomotion
miRNA alleles	MT7853		*let-7(n2853ts)*[d]	7.0 ± 2.0	65.6 ± 4.3	70.2 ± 7.0		+	+	+	0	+	Developmental timing, larval viability
	MT873		*lin-4(e912)*[c]		69.6 ± 13.9	68.8 ± 21.4		+	+	+	0	+	Developmental timing
	OH2535		*lsy-6(ot71)*	12.6 ± 2.0	64.5 ± 13.2	55.4 ± 4.3	14.5 ± 3.1	+	+	+	0	+	ASEL/R differentiation
	MT12955		*mir-1(n4102)*	11.2 ± 1.5		41.1 ± 11.6	13.5 ± 5.6	+	+	+	0	+	
	MT17810	6	*mir-1(n4102)*		80.2 ± 6.6								
	VT1361	2	*mir-2(n4108)*	8.0 ± 1.2	64.6 ± 5.9	62.7 ± 11.0	12.6 ± 2.6	+	+	+	0	+	
	MT13406		*mir-34(n4276)*	12.5 ± 1.6	75.8 ± 4.5	63.3 ± 10.3	13.8 ± 2.2	+	+	+	0	+	
	MT14119[b]	9	*mir-35–41(nDf50)*	10.9 ± 1.7	83.0 ± 15.2	57.2 ± 3.4							Embryonic/larval viability
	MT13372		*mir-42–44(nDf49)*	10.3 ± 1.4	76.0 ± 2.9	51.6 ± 9.9	13.8 ± 1.9	+	+	+	0	+	
	MT13433		*mir-45(n4280)*	9.7 ± 3.9	64.0 ± 6.3	65.6 ± 15.0	10.1 ± 3.2	+	+	+	0	+	
	MT14452		*mir-46(n4475)*	9.3 ± 2.4	68.2 ± 9.8	80.9 ± 23.0	14.3 ± 5.2	+	+	+	0	+	
	VC328		*mir-47(gk167)*	14.4 ± 1.7	77.0 ± 9.1	57.9 ± 10.7	13.9 ± 4.1	+	+	+	0	+	
	MT13650	8	mir-48(n4097)	11.0 ± 2.4	68.4 ± 9.4	57.1 ± 19.3	12.7 ± 4.1	+	+	+	0	+	Developmental timing
	MT12944[a]		*mir-50(n4099)*										Viability
	MT14450		*mir-51(n4473)*	10.1 ± 2.5	62.2 ± 9.6	49.2 ± 11.4	10.0 ± 2.1	+	+	+	0	+	
	MT12945		*mir-52(n4100)*				10.5 ± 2.9	+	+	+	0	+	
	MT12990		*mir-52(n4114)*	12.6 ± 2.7	68.8 ± 6.7	51.9 ± 9.8							
	MT12989		*mir-53(n4113)*	11.2 ± 2.4	73.6 ± 2.9	56.1 ± 4.1	10.6 ± 3.9	+	+	+	0	+	
	MT14767		*mir-54–56(nDf58)*	10.1 ± 2.4	58.2 ± 9.4	54.3 ± 6.7	12.1 ± 2.3	+	+	+	0	+	
	VC347		*mir-57(gk175)*	12.4 ± 1.1	82.4 ± 10.4	73.7 ± 19.5	13.7 ± 4.2	+	+	+	0	+	
	MT15024		*mir-58(n4640)*	8.1 ± 3.3	61.0 ± 17.1	58.8 ± 7.5	14.5 ± 2.9	+	+	+	0	+	
	MT14935		*mir-59(n4604)*	11.9 ± 1.8	79.8 ± 6.7	69.5 ± 26.3	14.4 ± 4.4	+	+	+	0	+	
	MT14875		*mir-61 mir-250(nDf59)*	11.1 ± 3.5	66.8 ± 9.2	74.1 ± 45.4	15.4 ± 3.8	+	+	+	0	+	
	VT1289		*mir-63(n4568)*	8.0 ± 2.3	75.4 ± 15.0	66.7 ± 14.3	10.3 ± 2.6	+	+	+	0	+	
	MT15982		*mir-67(n4899)*	10.3 ± 3.2	65.6 ± 9.3	57.5 ± 6.5	12.1 ± 3.4	+	+	+	0	+	
	MT12978		*mir-70(n4109)*	12.9 ± 2.4	63.0 ± 6.2	64.0 ± 12.5							
	VT1362	4	*mir-70(n4109)*	10.2 ± 2.7	66.4 ± 3.4		15.6 ± 2.5	+	+	+	0	+	
	MT12993		*mir-71(n4115)*	12.0 ± 2.9	74.8 ± 9.2	50.7 ± 4.3	20.3 ± 4.2	+	+	+	0	+	
	MT13015		*mir-72(n4130)*	12.3 ± 3.6	78.8 ± 5.0	51.4 ± 5.1	10.0 ± 5.5	+	+	+	0	+	
	MT13078		*mir-73–74(nDf47)*	11.4 ± 2.3	67.2 ± 8.9	66.2 ± 12.3	13.2 ± 4.1	+	+	+	0	+	
	MT18037	3	*mir-75(n4472)*	14.2 ± 1.7	74.0 ± 5.5	53.5 ± 10.7							
	SX168	4	*mir-75(n4472)*				14.3 ± 3.6	+	+	+	0	+	
	MT14451		*mir-76(n4474)*	11.2 ± 1.7	74.8 ± 11.9	71.8 ± 25.4	14.2 ± 6.1	+	+	+	0	+	
	MT16311		*mir-77(n4286)*	11.2 ± 1.2	71.2 ± 4.0	65.9 ± 15.6	15.1 ± 2.3	+	+	+	0	+	
	MT15021		*mir-78(n4637)*	12.1 ± 1.7	79.8 ± 5.3	56.3 ± 5.2	14.1 ± 2.8	+	+	+	0	+	
	MT14091	13	*mir-79(n4126)*	14.6 ± 1.5	69.6 ± 6.1	57.3 ± 5.2	12.8 ± 4.5	+	+	+	0	+	

Table 2. Continued.

Controls and Alleles	Strain	Number of Outcrosses	Genotype	Locomotion (Body Bends)	Pumping	Defecation	Egg Retention	DiO filling	DAPI (L1)	Dauer Behavior			Abnormalities
				AVG ± SD	AVG ± SD	AVG ± SD	AVG ± SD			Entry	Constitutive (%)	Exit	
	MT13949		*mir-80 mir-227(nDf53)*	11.7 ± 1.9	76.8 ± 8.5	72.9 ± 10.8	16.5 ± 3.6	+	+	+	0	+	
	MT13954		*mir-81 mir-82(nDf54)*	11.2 ± 1.9	66.0 ± 4.3	57.3 ± 15.3	17.2 ± 5.2	+	+	+	0	+	
	MT15501	2	*mir-83(n4638)*	12.5 ± 2.7		57.8 ± 17.4	10.1 ± 2.4	+	+	+	0	+	
	SX169	4	*mir-83(n4638)*		74.5 ± 6.1								
	MT13651	5	*mir-84(n4037)*	12.5 ± 3.0	71.6 ± 14.4	54.5 ± 15.3	13.2 ± 3.3	+	+	+	0	+	
	MT12999		*mir-85(n4117)*	12.1 ± 1.8	75.8 ± 6.6	50.9 ± 6.9	14.2 ± 2.3	+	+	+	0	+	
	MT14938		*mir-86(n4607)*	10.9 ± 1.7	76.4 ± 7.8	70.4 ± 4.7	17.6 ± 4.1	+	+	+	0	+	
	MT12958		*mir-87(n4104)*	12.3 ± 1.7	72.2 ± 5.4	55.3 ± 8.3	14.0 ± 3.7	+	+	+	0	+	
	MT13292	2	*mir-124(n4255)*	14.6 ± 2.2	56.9 ± 12.3	67.3 ± 35.3	9.7 ± 2.9	+	+	+	0	+	
	MT14446	2	*mir-228(n4382)*	7.7 ± 3.3	73.6 ± 15.6	48.3 ± 7.4	10.6 ± 2.9	+	+	+	0	+	
	MT15784		*mir-229 mir-64–66(nDf63)*				10.8 ± 3.2	+	+	+	0	+	
	MT16494	2	*mir-229 mir-64–66(nDf63)*	11.9 ± 1.8	78.8 ± 5.0	58.6 ± 11.9							
	MT14662		*mir-230(n4535)*	8.1 ± 2.1	68.6 ± 10.3	62.2 ± 11.0	12.2 ± 2.8	+	+	+	0	+	
	MT14768		*mir-231(n4571)*	10.1 ± 1.9	71.2 ± 8.9	72.6 ± 19.6	10.2 ± 5.2	+	+	+	0	+	
	MT14449		*mir-232(nDf56)*	9.8 ± 2.7	57.2 ± 10.8	65.4 ± 8.6	14.2 ± 4.2	+	+	+	1	+	
	MT15517		*mir-233(n4761)*	10.6 ± 3.0	62.6 ± 15.5	62.7 ± 21.4	13.9 ± 3.1	+	+	+	0	+	
	MT14588		*mir-234(n4520)*	12.8 ± 2.3	79.4 ± 8.3	65.7 ± 17.7	14.5 ± 4.9	+	+	+	0	+	
	MT14522		*mir-235(n4504)*	10.7 ± 1.8		54.7 ± 7.4	15.6 ± 5.5	+	+	+	0	+	
	MT17997	2	*mir-235(n4504)*		73.8 ± 10.2								
	MT13653		*mir-237(n4296)*	14.4 ± 1.6	79.8 ± 13.6	60.1 ± 17.1	12.4 ± 3.6	+	+	+	0	+	
	MT12983		*mir-238(n4112)*	11.2 ± 3.4	60.0 ± 9.1	52.8 ± 5.5	11.9 ± 3.4	+	+	+	0	+	
	MT15312		*mir-239a mir-239b(nDf62)*	15.1 ± 1.6	77.2 ± 8.2	53.4 ± 9.5	19.3 ± 2.3	+	+	+	0	+	
	MT15873	1	*mir-240 mir-786(n4541)*	12.4 ± 1.6	73.2 ± 9.0		16.3 ± 2.7	+	+	+	0	+	
	MT18043	3	*mir-240 mir-786(n4541)*			109.3 ± 9.7							
	MT13896		*mir-241(n4315)*	10.6 ± 2.2	78.0 ± 10.6	60.0 ± 13.1	14.3 ± 4.5	+	+	+	0	+	Developmental timing
	MT14936		*mir-242(n4605)*	10.3 ± 2.7	65.4 ± 3.5	60.4 ± 16.4	12.8 ± 4.4	+	+	+	0	+	
	MT15454		*mir-243(n4759)*	10.4 ± 2.2	68.6 ± 6.0	48.7 ± 5.1	13.8 ± 2.9	+	+	+	0	+	
	MT14064		*mir-244(n4367)*	8.3 ± 4.5		52.7 ± 8.8	11.9 ± 3.3	+	+	+	0	+	
	MT16033	2	*mir-244(n4367)*		65.6 ± 10.4								
	MT16337	2	*mir-245(n4798)*	10.1 ± 2.0	72.4 ± 7.2	60.6 ± 10.0	12.7 ± 2.1	+	+	+	0	+	
	MT15020		*mir-246(n4636)*	11.3 ± 2.5	70.4 ± 8.3	55.5 ± 5.9	11.4 ± 4.2	+	+	+	0	+	
	MT16309	2	*mir-247 mir-797(n4505)*	10.5 ± 2.1	67.2 ± 10.8	60.1 ± 9.5	9.4 ± 4.2	+	+	+	0	+	
	MT14937		*mir-251(n4606)*	10.3 ± 2.0	72.6 ± 8.8	73.0 ± 32.7	15.3 ± 2.7	+	+	+	0	+	
	MT16308	2	*mir-252(n4570)*	11.5 ± 2.1	73.0 ± 4.7	57.1 ± 8.1	13.1 ± 4.3	+	+	+	0	+	
	MT16060		*mir-253(nDf64)*	11.4 ± 2.3	62.0 ± 3.4	68.0 ± 14.3	17.1 ± 2.8	+	+	+	0	+	
	MT14525		*mir-254(n4470)*			60.3 ± 18.4	14.2 ± 3.3	+	+	+	0	+	
	MT16506	1	*mir-254(n4470)*	11.5 ± 2.2	73.4 ± 5.2								
	MT14682		*mir-257(n4548)*	12.2 ± 2.3	66.4 ± 10.5	60.5 ± 7.2	12.7 ± 3.0	+	+	+	0	+	
	MT15767		*mir-258(n4797)*	12.1 ± 2.8	67.0 ± 13.3	68.9 ± 19.8	11.1 ± 1.6	+	+	+	0	+	
	MT12969		*mir-259(n4106)*	10.4 ± 1.9	61.4 ± 8.3	59.1 ± 8.4	18.6 ± 4.1	+	+	+	0	+	
	MT14919		*mir-260(n4601)*	10.7 ± 2.5	69.6 ± 7.8	56.2 ± 17.2	12.4 ± 2.6	+	+	+	0	+	
	MT14876		*mir-261(n4594)*	13.5 ± 2.1	76.2 ± 9.3	57.6 ± 5.1	11.3 ± 3.4	+	+	+	0	+	
	MT14661		*mir-265(n4534)*	11.0 ± 2.7	59.4 ± 12.2	59.2 ± 8.9	10.1 ± 2.6	+	+	+	0	+	
	MT15023		*mir-268(n4639)*		69.6 ± 6.5	64.7 ± 19.1	13.2 ± 2.5	+	+	+	0	+	
	SX170	4	*mir-268(n4639)*	12.3 ± 1.1									
	MT16310	2	*mir-269(n4641)*	11.2 ± 1.2	74.6 ± 11.9	65.7 ± 1.2	15.1 ± 3.5	+	+	+	0	+	

Table 2. Continued.

Controls and Alleles	Strain	Number of Outcrosses	Genotype	Locomotion (Body Bends)	Pumping	Defecation	Egg Retention	DiO filling	DAPI (L1)	Dauer Behavior			Abnormalities
				AVG ± SD	AVG ± SD	AVG ± SD	AVG ± SD			Entry	Constitutive (%)	Exit	
	MT14878		*mir-270(n4595)*	10.4 ± 1.6	74.8 ± 7.3	75.9 ± 18.7	15.2 ± 2.7	+	+	+	0	+	
	MT14347		*mir-273(n4438)*	12.1 ± 1.3	72.3 ± 12.2	55.3 ± 11.9	15.8 ± 2.3	+	+	+	0	+	
	MT15026[a]		*mir-353(nDf61)*										Viability
	MT16316	2	*mir-355(n4618)*	10.7 ± 1.3	67.8 ± 11.6	53.9 ± 4.4	18.1 ± 5.2	+	+	+	0	+	
	MT15019		*mir-357–8(nDf60)*	12.3 ± 1.5	68.6 ± 6.4	76.7 ± 15.1	13.9 ± 4.1	+	+	+	0	+	
	MT14673		*mir-359(n4540)*	11.1 ± 1.5	75.2 ± 7.9	59.7 ± 7.3	14.0 ± 2.7	+	+	+	0	+	
	MT15018		*mir-360(n4635)*	10.1 ± 3.2	67.4 ± 12.7	65.1 ± 17.3	11.4 ± 2.0	+	+	+	0	+	

Phenotype assays are described in Table 3 and Materials and Methods.

[a] Heterozygous strain.

[b] Assays were carried out at 20 °C.

[c] In some cases no pBoc was observed for 4 min.

[d] For locomotion, pumping and defecation assays this strain was grown at 15 °C and assayed at 22 °C. Colored background indicates data that are at least two standard deviations higher (orange) than the average of all miRNA deletion mutants and the wild-type control. All strains carrying miRNA deletions that were generated in this study have been submitted to the *Ceanorhabditis* Genetics Center, University of Minnesota, Twin Cities, Minnesota.

AVG, average; SD, standard deviation; +, normal; –, defective; NA, not applicable; no entry, not scored.

doi:10.1371/journal.pgen.0030215.t002

Table 3. Phenotypes Examined

Phenotype	Assay
Locomotion	Body bends in 20 s
Pharyngeal pumping	Movement of grinder in 20 s
Defecation	Timing of defecation cycle
Egg laying	Eggs retained in 24-h adult
Presence of chemosensory neurons	Dye filling
Cell number and nuclear morphology	DAPI staining
Dauer larva formation	Dauer entry and exit, constitutive dauer formation

For methods concerning the phenotypic characterization of microRNA mutants, see Materials and Methods.

doi:10.1371/journal.pgen.0030215.t003

revealed by outcrossing. To uncover subtle abnormalities in the miRNA mutant strains will require more detailed analyses, as has been performed for *lin-4*, *let-7*, *lsy-6*, *mir-48*, *mir-84*, and *mir-241*. Nevertheless, we note one striking conclusion: the majority of miRNAs are not essential for *C. elegans* viability and development.

Discussion

Here we report the first large-scale collection of miRNA loss-of-function mutants for any organism. We isolated new deletion alleles for 87 miRNA genes. Together with two publicly available deletion mutants, three mutants that we described elsewhere, and three mutants generated in genetic screens, there are now mutants for 95 *C. elegans* miRNA genes [2,4,20,52]. We hope that this collection will become a widely used resource for the study of miRNA function.

Loss-of-Function versus Misexpression Studies

The overexpression of the miRNAs miR-84 and miR-61 from transgenes in *C. elegans* affects vulval development [89,90]. The overexpression of miR-61 leads to the expression in Pn.p cells that do not normally generate vulval cell fates of reporter genes indicative of vulval cell fates [89]. We examined if miR-61 and the closely related miR-247 were required for the normal induction of primary or secondary vulval cell fates by the Pn.p cells. We found that Pn.p cell induction was normal in *mir-61* mutants and in *mir-61; mir-247* double mutants (Table S3), although we did not test the effects of combining these mutants with mutants of *mir-44* and *mir-45*, which have the same seed and thus are predicted to target the same messages. Similarly, *let-60 RAS* has been suggested to be a target of miR-84, based on the observation that overexpression of miR-84 from a transgene suppresses the multivulva phenotype of *let-60 RAS* activation mutants. If *let-60 RAS* is a target of miR-84, loss of *mir-84* might result in *let-60 RAS* overexpression and possibly a multivulva phenotype [91,92]. However, as we reported previously, *mir-84* single mutants or *mir-48 mir-241; mir-84* triple mutants do not have a multivulva phenotype [52]. Thus, for both miR-84 and miR-61, we were unable to confirm a role in vulval development based on loss-of-function alleles. We conclude that these miRNAs are not required for vulval development and suggest that either they act redundantly with other miRNAs or other pathways in vulval development or they do not normally act in vulval development at all.

Redundancy of miRNAs and Their Regulatory Pathways

One difference between most protein-coding genes and most miRNA genes in *C. elegans* is the number of paralogs. Whereas fewer than 25% of protein-coding genes have a recognizable paralog in the *C. elegans* genome [93], about 60% of miRNAs are members of a family of two to eight genes [19]. A higher number of paralogs might be a consequence of smaller gene size, which could allow a greater opportunity for gene duplication. As a consequence, miRNAs might act redundantly with other miRNAs and mutation of all paralogs of a miRNA or a miRNA family might result in synthetic abnormal synthetic phenotypes. Alternatively, some nematode miRNAs might act in parallel with other regulatory pathways that can compensate gene expression when the miRNAs are lost. For example, genetic data indicate that *Drosophila mir-7* directly regulates the transcriptional repressor Yan in the fly eye, but that loss of *mir-7* does not appreciably alter eye development, probably because of redundant protein turnover mechanisms that can also downregulate Yan [58]. In such a scenario, disruptions in the other mechanisms would be needed to reveal the miRNA function.

Roles for Evolutionary Conserved miRNAs

The discovery that the *let-7* miRNA is conserved among bilateria, including such disparate organisms as *C. elegans* and humans [94], appears not to have been an exception: for 15 miRNA families, miRNAs with identical seeds have been found in *C. elegans*, flies, fish, and mammals, and several additional families are predicted to be conserved throughout these diverse lineages [19,95–97]. The conservation is not only for primary miRNA sequences, but also, at least in some cases, for patterns of expression. For example, the miRNA miR-1 is expressed in muscles of *Drosophila*, the zebrafish, and the mouse [11,56,98]. However, the predicted mRNA targets of miRNAs might not share the same degree of conservation as miRNA expression patterns—the spectrum of predicted mRNA targets varies significantly among metazoans [99]. With several miRNA loss-of-function mutants of *Drosophila* now available, we can begin to compare miRNA functions between *C. elegans* and *Drosophila*. Among the microRNAs for which mutations exist for flies and worms, Dmir-1 and *C. elegans* miR-1 are the most similar in sequence [56]. Whereas Dmir-1 loss-of-function mutant fly larvae display muscle degeneration and die [56], we found that *C. elegans* miR-1 loss-of-function mutant animals are fully viable. Despite these differences, the *mir-1* miRNA family could have a conserved role in muscle homeostasis and function. For example, the severity of the muscle defect of *C. elegans mir-1* mutants might depend on physiological conditions, as is the case for the Dmir-1 mutant phenotypes of *Drosophila* [56].

We expect that as additional miRNA mutants become available for flies and other animals there will be future comparative studies of the biological functions of miRNAs using the collection of *C. elegans* miRNA mutants we have generated. More generally, we believe that the functions of miRNA genes, like the functions of protein-coding genes, will often prove to be conserved among animals, and that the collection of miRNA mutants we have generated will help define, test, and analyze general biological roles of miRNAs.

Materials and Methods

Nematode methods. *C. elegans* was grown using standard conditions [100]. The wild-type strain was var. Bristol N2 [101]. Nematodes were grown at 25 °C, except where otherwise indicated. Details about the mutant alleles we generated are shown in Table S1. All strains generated in this study have been submitted to the *Caenorhabditis* Genetics Center. Deletion allele information can be accessed directly from WormBase (http://www.wormbase.org).

Generation of deletion mutants. Deletion mutants were isolated from a frozen library of worms mutagenized with ethyl methanesulphonate (EMS), 1,2,3,4-diepoxybutane (DEB), or a combination of UV irradiation and thymidine monophosphate (UV-TMP) [82,83]. In most instances, to enhance the detection of deletions one or two "poison" primers were included in the first round of nested PCR reactions [84]. These poison primers were designed to anneal close to the mature miRNA sequence. In the first round of PCR, the three primers in the reaction (external forward, external reverse, and poison primers) generated both a full-length (from external primers) and a shorter product (from external and poison) from the wild-type allele. The shorter product was amplified more efficiently and thereby out-competed the amplification of full-length product. A deletion allele that removed the miRNA sequence and therefore removed the poison primer-binding site generated a product only from the external primers. In the second round of PCR, two internal primers designed just inside of the external primers amplified the full-length product but not the shorter product from the wild-type allele and a single product from the deletion allele. Mutant strains were outcrossed with the wild-type strain as indicated (Table S1).

Phenotypic analysis. The minimum number of individual animals scored in each assay is given as n in parentheses below. (1) Locomotion: Number of body bends during a 20-s period were counted 4 min after transferring 1-d-old adult animals to fresh plates containing food ($n = 10$). (2) Pharyngeal pumping: Frequency of grinder displacement was counted for 20 s by eye, but otherwise as described previously [102] ($n = 5$). (3) Defecation: The time between defecation cycles marked by posterior body muscle contraction events was measured [103] ($n = 3$, 5 events per animal). (4) Egg laying: 1-d-old adult animals were lysed in bleaching solution for 10 min in the well of a round-bottom 96-well plate, and eggs were counted [100] ($n = 20$). (5) Chemosensory neurons: L2 or L3 larvae were stained with DiO dye (Invitrogen) and filling of the neurons ASI, ASJ, ASH, ASK, AWS, ADL, PHA, PHB was scored [104] ($n = 15$). (6) Cell number/nuclear morphology: L1 larvae were fixed and stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) as described previously [105]. Nuclei of the ventral cord and intestine were counted [106] ($n = 15$). (7) Dauer development: To assay dauer larva entry, three L4 animals were incubated at 25 °C until the F2/F3 progeny had been starved for at least five days. Animals were washed from plates using 1% SDS in de-ionized H_2O for 30 min. Dauer larvae were identified by observing their thrashing and re-plated onto plates containing food to assay dauer exit. Constitutive dauer entry was scored by testing animals from plates with food for the presence of dauer larvae isolated after SDS treatment as described above ($n = 50$).

Supporting Information

Table S1. Deletion Alleles Described in This Study

Found at doi:10.1371/journal.pgen.0030215.st001 (61 KB XLS).

Table S2. Rescue of Defecation Defect of *mir-240 mir-786* Mutants

Found at doi:10.1371/journal.pgen.0030215.st002 (35 KB XLS).

Table S3. Normal Induction of 1° and 2° Fates in the Pn.ps of *mir-61* and *mir-247* Mutants

Found at doi:10.1371/journal.pgen.0030215.st003 (37 KB XLS).

Accession Numbers

The miRNA sequences discussed in this paper can be found in the miRNA Registry (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml). The *C. elegans* miRNA genes, their genomic location and deletion allele information can de accessed directly from WormBase (http://www.wormbase.org) [107].

Acknowledgments

We thank Beth Castor for DNA sequence determinations and screening of the deletion library, Na An for strain management, Ines Alvarez-Garcia and Rob Shaw for help with the characterization of mutant phenotypes, and M. Lucila Scimone for data entry.

Author contributions. EAM, EAS, ALA, DPB, VRA, and HRH conceived and designed the experiments and wrote the paper. EAM, EAS, ALA, NCL, ABH, and SMM performed the experiments. EAM, EAS, and ALA analyzed the data. NCL contributed reagents/materials/analysis tools.

Funding. EAM was a Wellcome Trust Prize Traveling Research Fellow (061641). EAS was supported by a grant from the Ellison Medical Foundation (AG-SS-1319-04). ALA was supported by a Ruth L. Kirschstein NRSA postdoctoral fellowship (5F32GM065721-02). DPB and VRA were supported by grants from the National Institutes of Health (GM067031 to DPB and GM34028 to VRA). HRH is the David H. Koch Professor of Biology at MIT. HRH and DPB were supported by and are Investigators of the Howard Hughes Medical Institute.

Competing interests. The authors have declared that no competing interests exist.

References

1. Ambros V, Horvitz HR (1984) Heterochronic mutants of the nematode *Caenorhabditis elegans*. Science 226: 409–416.
2. Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 75: 843–854.
3. Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. Cell 75: 855–862.
4. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, et al. (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 403: 901–906.
5. Chalfie M, Horvitz HR, Sulston JE (1981) Mutations that lead to reiterations in the cell lineages of *C. elegans*. Cell 24: 59–69.
6. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
7. Arazi T, Talmor-Neiman M, Stav R, Riese M, Huijser P, et al. (2005) Cloning and characterization of micro-RNAs from moss. Plant J 43: 837–848.
8. Axtell MJ, Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. Plant Cell 17: 1658–1673.
9. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294: 853–858.
10. Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 294: 858–862.
11. Lee RC, Ambros V (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294: 862–864.
12. Lim LP, Glasner ME, Yekta S, Burge CB, Bartel DP (2003) Vertebrate microRNA genes. Science 299: 1540.
13. Llave C, Kasschau KD, Rector MA, Carrington JC (2002) Endogenous and silencing-associated small RNAs in plants. Plant Cell 14: 1605–1619.
14. Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16: 1616–1626.
15. Watanabe T, Takeda A, Mise K, Okuno T, Suzuki T, et al. (2005) Stage-specific expression of microRNAs during *Xenopus* development. FEBS Lett 579: 318–324.
16. Pfeffer S, Zavolan M, Grasser FA, Chien M, Russo JJ, et al. (2004) Identification of virus-encoded microRNAs. Science 304: 734–736.
17. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, et al. (2003) The microRNAs of *Caenorhabditis elegans*. Genes Dev 17: 991–1008.
18. Ohler U, Yekta S, Lim LP, Bartel DP, Burge CB (2004) Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification. RNA 10: 1309–1322.
19. Ruby JG, Jan C, Player C, Axtell MJ, Lee W, et al. (2006) Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. Cell 127: 1193–1207.
20. Johnston RJ, Hobert O (2003) A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. Nature 426: 845–849.
21. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. Nature 432: 231–235.
22. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, et al. (2004) The Microprocessor complex mediates the genesis of microRNAs. Nature 432: 235–240.
23. Han J, Lee Y, Yeom KH, Kim YK, Jin H, et al. (2004) The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18: 3016–3027.
24. Landthaler M, Yalcin A, Tuschl T (2004) The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis. Curr Biol 14: 2162–2167.
25. Lee Y, Ahn C, Han J, Choi H, Kim J, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425: 415–419.
26. Ruby JG, Jan CH, Bartel DP (2007) Intronic microRNA precursors that bypass Drosha processing. Nature 448: 83–86.
27. Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC (2007) The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*. Cell 130: 89–100.
28. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U (2003) Nuclear export of microRNA precursors. Science 303: 95–98.
29. Du T, Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. Development 132: 4645–4652.
30. Pillai RS (2005) MicroRNA function: multiple mechanisms for a tiny RNA? RNA 11: 1753–1761.
31. Olsen PH, Ambros V (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.
32. Petersen CP, Bordeleau ME, Pelletier J, Sharp PA (2006) Short RNAs repress translation after initiation in mammalian cells. Mol Cell 21: 533–542.
33. Seggerson K, Tang L, Moss EG (2002) Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene *lin-28* after translation initiation. Dev Biol 243: 215–225.
34. Yekta S, Shih IH, Bartel DP (2004) MicroRNA-directed cleavage of HOXB8 mRNA. Science 304: 594–596.
35. Mansfield JH, Harfe BD, Nissen R, Obenauer J, Srineel J, et al. (2004) MicroRNA-responsive "sensor" transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. Nat Genet 36: 1079–1083.
36. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, et al. (2005) Regulation by *let-7* and *lin-4* miRNAs results in target mRNA degradation. Cell 122: 553–563.
37. Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, et al. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell 120: 623–634.
38. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, et al. (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312: 75–79.
39. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, et al. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433: 769–773.
40. Wu L, Fan J, Belasco JG (2006) MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103: 4034–4039.
41. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.
42. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115: 787–798.
43. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, et al. (2005) Combinatorial microRNA target predictions. Nat Genet 37: 495–500.
44. Brennecke J, Stark A, Russell RB, Cohen SM (2005) Principles of microRNA-target recognition. PLoS Biol 3: e85. doi:10.1371/journal.pbio.0030085
45. Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, et al. (2005) Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. Nature 434: 338–345.
46. Lall S, Grun D, Krek A, Chen K, Wang YL, et al. (2006) A genome-wide map of conserved microRNA targets in *C. elegans*. Curr Biol 16: 460–471.
47. Moss EG, Lee RC, Ambros V (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.
48. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, 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.
49. Abrahante JE, Daul AL, Li M, Volk ML, Tennessen JM, 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.
50. Grosshans H, Johnson T, Reinert KL, Gerstein M, Slack FJ (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.
51. Lin SY, Johnson SM, Abraham M, Vella MC, Pasquinelli A, et al. (2003) The *C. elegans hunchback* homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. Dev Cell 4: 639–650.
52. Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, 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.
53. Li M, Jones-Rhoades MW, Lau NC, Bartel DP, Rougvie AE (2005) Regulatory mutations of *mir-48*, a *C. elegans let-7* family MicroRNA, cause developmental timing defects. Dev Cell 9: 415–422.
54. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. Cell 113: 25–36.
55. Xu P, Vernooy SY, Guo M, Hay BA (2003) The *Drosophila* microRNA mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol 13: 790–795.
56. Sokol NS, Ambros V (2005) Mesodermally expressed *Drosophila* micro-

RNA-1 is regulated by Twist and is required in muscles during larval growth. Genes Dev 19: 2343–2354.

57. Kwon C, Han Z, Olson EN, Srivastava D (2005) MicroRNA1 influences cardiac differentiation in *Drosophila* and regulates Notch signaling. Proc Natl Acad Sci U S A 102: 18986–18991.
58. Li X, Carthew RW (2005) A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the *Drosophila* eye. Cell 123: 1267–1277.
59. Li Y, Wang F, Lee JA, Gao FB (2006) MicroRNA-9a ensures the precise specification of sensory organ precursors in *Drosophila*. Genes Dev 20: 2793–2805.
60. Teleman AA, Maitra S, Cohen SM (2006) *Drosophila* lacking microRNA miR-278 are defective in energy homeostasis. Genes Dev 20: 417–422.
61. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, et al. (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. Science: 316: 575–579.
62. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, et al. (2007) Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1–2. Cell 129: 303–317.
63. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, et al. (2007) Requirement of bic/microRNA-155 for normal immune function. Science 316: 608–611.
64. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, et al. (2007) Regulation of the germinal center response by microRNA-155. Science 316: 604–608.
65. Miska EA (2005) How microRNAs control cell division, differentiation, and death. Curr Opin Genet Dev 15: 563–568.
66. Knight SW, Bass BL (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. Science 293: 2269–2271.
67. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, 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.
68. Lee YS, Nakahara K, Pham JW, Kim K, He Z, et al. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117: 69–81.
69. Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, et al. (2005) Stem cell division is regulated by the microRNA pathway. Nature 435: 974–978.
70. Wienholds E, Koudijs MJ, van Eeden FJ, Cuppen E, Plasterk RH (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet 35: 217–218.
71. Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, et al. (2005) MicroRNAs regulate brain morphogenesis in zebrafish. Science 308: 833–838.
72. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, et al. (2003) Dicer is essential for mouse development. Nat Genet 35: 215–217.
73. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ (2005) The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc Natl Acad Sci U S A 102: 10898–10903.
74. Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, et al. (2005) Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem 280: 9330–9335.
75. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X (2006) Dicer function is essential for lung epithelium morphogenesis. Proc Natl Acad Sci U S A 103: 2208–2213.
76. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, et al. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305: 1437–1441.
77. Leaman D, Chen PY, Fak J, Yalcin A, Pearce M, et al. (2005) Antisense-mediated depletion reveals essential and specific functions of microRNAs in *Drosophila* development. Cell 121: 1097–1108.
78. Hutvagner G, Simard MJ, Mello CC, Zamore PD (2004) Sequence-specific inhibition of small RNA function. PLoS Biol 2: e98. doi:10.1371/journal.pbio.0020098
79. Meister G, Landthaler M, Dorsett Y, Tuschl T (2004) Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA 10: 544–550.
80. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, et al. (2005) Silencing of microRNAs in vivo with "antagomirs." Nature 438: 685–689.
81. Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RHA (2007) Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol 5: e203. doi:10.1371/journal.pbio.0050203
82. Jansen G, Hazendonk E, Thijssen KL, Plasterk RH (1997) Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. Nat Genet 17: 119–121.
83. Liu LX, Spoerke JM, Mulligan EL, Chen J, Reardon B, et al. (1999) High-throughput isolation of *Caenorhabditis elegans* deletion mutants. Genome Res 9: 859–867.
84. Edgley M, D'Souza A, Moulder G, McKay S, Shen B, et al. (2002) Improved detection of small deletions in complex pools of DNA. Nucleic Acids Res 30: e52.
85. Ambros V, Lee RC, Lavanway A, Williams PT, Jewell D (2003) MicroRNAs and other tiny endogenous RNAs in *C. elegans*. Curr Biol 13: 807–818.
86. Grad Y, Aach J, Hayes GD, Reinhart BJ, Church GM, et al. (2003) Computational and experimental identification of *C. elegans* microRNAs. Mol Cell 11: 1253–1263.
87. Griffiths-Jones S (2004) The microRNA Registry. Nucleic Acids Res 32: D109–D111.
88. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets, and gene nomenclature. Nucleic Acids Res 34: D140–D144.
89. Yoo AS, Greenwald I (2005) LIN-12/Notch activation leads to microRNA-mediated downregulation of Vav in *C. elegans*. Science 310: 1330–1333.
90. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, et al. (2005) RAS is regulated by the *let-7* microRNA family. Cell 120: 635–647.
91. Beitel GJ, Clark SG, Horvitz HR (1990) *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. Nature 348: 503–509.
92. Han M, Aroian RV, Sternberg PW (1990) The *let-60* locus controls the switch between vulval and nonvulval cell fates in *Caenorhabditis elegans*. Genetics 126: 899–913.
93. The *C. elegans* Sequencing Consortium (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science 282: 2012–2018.
94. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, et al. (2000) Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. Nature 408: 86–89.
95. Ruby JG, Stark A, Johnston W, Kellis M, Bartel DP, et al. (2007) Biogenesis, expression, and target predictions for an expanded set of microRNA genes in *Drosophila*. Genome Res. In press.
96. Sempere LF, Cole CN, McPeek MA, Peterson KJ (2006) The phylogenetic distribution of metazoan microRNAs: insights into evolutionary complexity and constraint. J Exp Zoolog B Mol Dev Evol 306: 575–588.
97. Prochnik SE, Rokhsar DS, Aboobaker AA (2007) Evidence for a microRNA expansion in the bilaterian ancestor. Dev Genes Evol 217: 73–77.
98. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, et al. (2005) MicroRNA expression in zebrafish embryonic development. Science 309: 310–311.
99. Grun D, Wang YL, Langenberger D, Gunsalus KC, Rajewsky N (2005) microRNA target predictions across seven *Drosophila* species and comparison to mammalian targets. PLoS Comput Biol 1: e13. doi:10.1371/journal.pcbi.0010013
100. Wood WB and the Community of *C. elegans* Researchers (1988) The nematode *Caenorhabditis elegans*. Cold Spring Harbor, New York: Cold Spring Harbor Press.
101. Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77: 71–94.
102. Avery L, Horvitz HR (1987) A cell that dies during wild-type *C. elegans* development can function as a neuron in a *ced-3* mutant. Cell 51: 1071–1078.
103. Thomas JH (1990) Genetic analysis of defecation in *Caenorhabditis elegans*. Genetics 124: 855–872.
104. Sawin ER (1996) Genetic and cellular analysis of modulated behaviors in *Caenorhabditis elegans* [Ph.D.]. Cambridge: Massachusetts Institute of Technology.
105. Fixsen WD (1985) The genetic control of hypodermal cell lineages during nematode development [Ph.D.]. Cambridge: Massachusetts Institute of Technology.
106. Horvitz HR, Sulston JE (1980) Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. Genetics 96: 435–454.
107. Schwarz EM, Antoshechkin I, Bastiani C, Bieri T, Blasiar D, et al. (2006) WormBase: better software, richer content. Nucleic Acids Res 34: D475–D478.