Inventory of Supplemental Information

File 1 : Wu et al Supp. Text (Final)

Supplemental Figure Legends

Supplemental Figures (6)

-Figure S1 relates to Figure 1.
-Figure S2 relates to Table 1.
-Figure S3 relates to Figure 3.
-Figure S4 relates to Figure 4.
-Figure S5 relates to Figure 5.
-Figure S6 relates to a point in the Discussion.

Supplemental Table (1)

A complement to the Materials and Methods section.

Supplemental Materials and Methods

A complement to the Materials and Methods section.

File 2 : Wu et al Seq

-Sequence file. 3' RACE sequences on the Intermediate RNA species (relates to Figure 3 D).

Supplemental Figure Legends

Table S1 (relates to the Materials and Methods section)**.** Primer sequences for northern analyses, qRT-PCR, translation and stability assays, and cloning.

Fig. S1. (Relates to Fig. 1) miR-35 quantification in *C. elegans* embryonic extracts and fractions. (A) miR-35 real-time (qRT)-PCR analysis. Total RNA was isolated from fractions of wild-type (N2) *C. elegans* embryonic extract not filtered (NF) or filtered fractions (1-5). A standard curve was made with different concentrations of miR-35 template primer (fM). miR-35 concentration per reaction was multiplied by the dilution factor to obtain miR-35 concentration per fraction (nM). Dilution factor: RNA concentration from the stock divided by 0.0025 (final concentration in qRT-PCR reaction). (B) miR-35 northern blot analysis. A standard curve was made with different amounts of miR-35 DNA primer (pg) to determine the concentration of miR-35 in the middle embryo (me) lysate from wild-type (N2). 5S rRNA was used as a loading control for RNA. (C) miR-35 concentration (nM) per fraction from the data obtained in (A) and (B). (D) Northern blot analysis of miR-35-42 family members on 2'-*O*-Me depletions. Extracts prepared from wild-type (N2) *C. elegans* embryos were incubated with either α-miR-35 or α-let-7 2'-*O*-Me to pull down miRISC, and unbound fractions were probed for miR-35, miR-37 and miR-41. 5S rRNA was used as a loading control.

Fig. S2. (Relates to Table 1) Characterization of miR-35-RISC pulldown from *C. elegans* embryos. Western blot analysis on affinity-purified miRISC to confirm results obtained by MudPIT analysis. N2 embryonic extracts were incubated with 2'-*O*-Me, as

indicated. Proteins were probed with polyclonal antibodies against ALG-1/2, DCR-1, AIN-1, RDE-4, and GFP. * and ** indicate known non-specific bands. Bottom panel: western blot analysis of DCP-2 on affinity-purified miRISC. Wild-type embryonic extracts were incubated with either no 2'-*O*-Me (beads only), α-miR-35, or α-let-7 2'-*O*-Me. Proteins were probed with polyclonal antibodies against ALG-1/ALG-2 and DCP-2.

Fig. S3. (Relates to Fig. 3) (A) Time-course of RL 6xmiR-52 deadenylation in wild-type embryos. Full-length reporters contain a poly(A) tail of 87 nucleotides. Images are representative of three independent experiments. (B) Time-course of mRNA stability of RL 6xmiR-52 reporters lacking a poly(A) tail in the absence or presence of specific (α-miR-52) or non-specific (α-miR-1) 2'-*O*-Me. Images are representative of a triplicate experiment conducted in the same wild-type embryonic extract. Values represent the average from the triplicate experiment, and error bars indicate standard deviation. Quantification of the mRNA half-deadenylation time ($t_{d1/2}$) and half-life ($t_{1/2\ decay}$) was obtained using ImageJ.

Fig. S4. (Relates to Fig. 4) Translation of RL in *C. elegans* embryos prepared from *alg-2(ok304); alg-1 RNAi*. Luciferase activity from each fraction was measured following 3-hours translation incubation.

Fig. S5. (Relates to Fig. 5) (A) Northern blot analysis of candidate miRNAs affecting the 3'UTR of *egl-1* and *toh-1* mRNAs. RNA was prepared from wild-type (N2) or mid-

development embryo (me). Primers complementary to the probe (0.5 and 1.5 pg or 5 and 25 pg in the case of miR-60) were used as positive controls. (B) Time-course of RL *egl-1* 3'UTR and RL *toh-1* 3'UTR deadenylation in N2 embryo extract. Reporter mRNAs were incubated in the presence or absence of 50 nM of α-miR-35, α-miR-58, α-miR-81, α-miR-86 and α-miR-87 or the negative control α-miR-1. (C) Time-course of RL *egl-1* 3'UTR translation in N2 embryo extract. The reporter mRNA was incubated with 50 nM of 2'-*O*-Me, as indicated. α-miR-1 served as a negative control. Values represent averages from a triplicate experiment conducted in the same extract, and error bars indicate standard deviation. (D) Time-course of RL reporter mRNAs deadenylation fused to various copies of miR-52 binding sites (1x-6x). (E) Time-course of RL 3xmiR-35 reporter mRNAs deadenylation. For all the 3xmiR-35 reporters, miR-35 binding sites were separated by five nucleotides, with the exception of RL 3xmiR-35 spaced, in which the miR-35 binding sites are separated by 50 nucleotides. The size of the linker (sequence between the miR-35 sites and the poly(A) tail) are as follows: 161 nts (RL 3xmiR-35 and RL 3xmiR-35 spaced), 261 nts (RL 3xmiR-35 LL), and 32 nts (RL 3xmiR-35 SL). Images in D and E are representative of three independent experiments. Values for time of half-deadenylation ($t_{d1/2}$) were obtained by measuring the intensity of the bands using ImageJ.

Fig. S6. (Relates to a point in the Discussion section) qRT-PCR analysis of the expression levels of *egl-1* and *toh-1* mRNA. Total RNA from embryonic preparations was isolated from wild-type (N2) and *alg-2(ok304); alg-1 RNAi*. *egl-1* and *toh-1* mRNA levels were normalized against actin mRNA. qRT-PCR results are presented as the

mean from triplicate independent preparations and error bars represent standard deviation.

Table S1. (Complement to the Materials and Methods section) Primer sequences for northern analyses, qRT-PCR, translation and stability assays, and cloning.

Name	Sequence
Starfire probes	
α-miR-35	5'-ACTGCTAGTTTCCACCCGGTGA/3StarFire/-3'
α-miR-52	5'-AGCACGGAAACATATGTACGGGTG/3StarFire/-3'
α-miR-58	5'-ATTGCCGTACTGAACGATCTCA/3StarFire/-3'
α-miR-60	5' ACTAGAAAATGTGCATAATA/3StarFire/-3'
α-miR-86	5'-GACTGTGGCAAAGCATTCACTTA/3StarFire/-3'
α-miR-87	5'ACACCTGAAACTTTGCTCAC/3StarFire/-3'
α-miR-230	5' TCTCCTGGTCGCACAACTAATAC/3StarFire/-3'
α-miR-232	5' TCACCGCAGTTAAGATGCATTTA/3StarFire/-3'
qRT-PCR	
Universal primer	5'-CATGATCAGCTGGGCCAAGA-3'
miR-35 specific primer	5'-CATGATCAGCTGGGCCAAGAACTGCTA GTT-3'
miR-35 LNA	5'-T+CACCGGGTGGAAAC-3'
2'-*O*-Me oligos	
α-miR-1	5'-UCUUCCUCCAUACUUCUUUACAUUCCAACCUU-3'
α-miR-35	5'-UUAAUACUGCUAGUUUCCACCCGGUGAUUAAU-3'
α-miR-52	5'-UUAAUAGCACGGAAACAUAUGUACGGGUGUUAAU-3'
α-miR-58	5'-UUAAUUGCCGUACUGAACGAUCUCAUUAAU-3'
α-miR-81	5'-UUAAUACUGGCUUUCACGAUGAUCUCAUUAAU-3'
hsa-miR-16	5'-UUAAUCGCCAAUAUUUACGUGCUGCUAUUAAU-3'
α-miR-86	5'-UUAAUGACUGUGGCAAAGCAUUCACUUAUUAAU-3'
α-miR-87	5'-UUAAUACACCUGAAACUUUGCUCACUUAAU-3'
miR-35 targets 3'UTR cloning	
c34h3.1 fwd	5'-ATAAACTAGTGCAATGCTTGATTCTACCACA-3'
c34h3.1 rev	5'-TATTGCGGCCGCTAATGGAATCTGTGAGCAACG-3'
hlh-11 fwd	5'-ACTAGTGCCTGACTTTTGACAAATGTAG-3'
hlh-11 rev	5'-GCGGCCGCATTGGTACTCTTGTCTCAGTGG-3'

nhl-2 fwd	5’-ATAAACTAGTGGAGGTTACCCCAATTCCTAT-3’
nhl-2 rev	5’-TATTGCGGCCGCGGGCGAGCTGAAATTCAAATT-3’
r05h11.2 fwd	5’-ATAAACTAGTATTGAATACTTATAGACCTCAAG-3’
r05h11.2 rev	5’-TATTGCGGCCGCTCTAACCGTCTGAATATTATCTG-3’
spn-4 fwd	5’-ATAAACTAGTTCAGTTCAACTGATACGCCC-3’
spn-4 rev	5’-TATTGCGGCCGCTATGGCGAAGCACTTCATTTG-3’
toh-1 fwd	5’-ACTAGTATTCATTTTCTAGTTCTTCTACTC-3’
toh-1 rev	5’-GCGGCCGCAAGACTCAAATGTTTCATTGGG-3’
y71f9b.8 fwd	5’-ATAAACTAGTATTTTCAGGCTTTCAAGCCCA-3’
y71f9b.8 rev	5’-TATTGCGGCCGCTTTATAGTTAATAAATTTATTTGATTTA-3’

Cloning

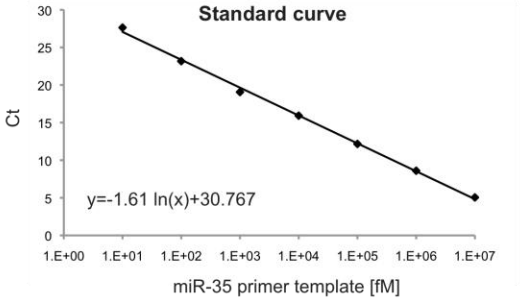
miR-52 1X fwd	5’-CTAGAGATTTTTCCCAGCAGCGAAAATGTACGGGTGAATTCGC-3’
miR-52 1X rev	5’-GGCCGCGAATTCACCCGTACATTTTCCGTGCTGGGAAAAATCT-3’
miR-52 2X fwd	5’ CTAGA A GCA CGG AAA ATG TAC GGG TG CTCGAG A GCA CGG AAA ATG TAC GGG TG GC-3’
miR-52 2X rev	5’-GGCCGC C ACC CGT ACA TTT TCC GTG CT CTCGAG C ACC CGT ACA TTT TCC GTG CT T-3’
miR-52 3X fwd	5’-CTAGA A GCA CGG AAA ATG TAC GGG TG CTCGAG A GCA CGG AAA ATG TAC GGG TG CTCGAG A GCA CGG AAA ATG TAC GGG TG GC-3’
miR-52 3X rev	5’-GGCCGC C ACC CGT ACA TTT TCC GTG CT CTCGAG C ACC CGT ACA TTT TCC GTG CT CTCGAG C ACC CGT ACA TTT TCC GTG CT T-3’
miR-52 4X fwd	5’-AATTC A GCA CGG AAA ATG TAC GGG TG CTCGAG A GCA CGG AAA ATG TAC GGG TG CTCGAG A GCA CGG AAA ATG TAC GGG TG G-3’
miR-52 4X rev	5’-AATTC C ACC CGT ACA TTT TCC GTG CT CTCGAG C ACC CGT ACA TTT TCC GTG CT CTCGAG C ACC CGT ACA TTT TCC GTG CT G-3’
miR-35 short linker fwd	5’-ATAAGCTGCAATAAACAAGTTG-3’
miR-35 short linker rev	5’-CTA AAG GGA AGC GGC CGC-3’
miR-35 long linker fwd	5’-GCGGCCGCTTCCCTTTAG-3’
miR-35 long linker rev	5’-GCGGCCGCAATAAAGCATTTTTTTCACTGCA-3’
miR-35 1X fwd	5’-CTAGTACTGCTAGTTTCCACCCGGTGAGC-3’
miR-35 1X rev	5’-GCCCGCTCACCGGGTGGAAACTAGCAGTA-3’
miR-35 2X fwd	5’-CTAGAACTGCTAGCCACCCGGTGAG

	AATTCACTGCTAGCCACCCGGTGAGC-3’
miR-35 2X rev	5’-GGCCGCTCACCGGGTGGCTAGCAG TGAATTCTCACCGGGTGGCTAGCAGTT-3’
miR-35 3X fwd	5’-CTAGAACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCC GGTGATTAATACTGCTAGCCACCCGGTGAGC-3’
miR-35 3X rev	5’-GGCCGCTCACCGGGTGGCTAGCAGTATTAATCACCGGGT GGCTAGCAGTATTAATCACCGGGTGGCTAGCAGTT-3’
miR-35 4X fwd	5’-AATTGACTGCTAGCCACCCGGTGATTAATACTGCTAGCCA CCCGGTGATTAATACTGCTAGCCACCCGGTGATTAATG-3’
miR-35 4X rev	5’-AATTCATTAATCACCGGGTGGCTAGCAGTATTAATCACC GGGTGGCTAGCAGTATTAATCACCGGGTGGCTAGCAGTC-3’

A

qRT-PCR

Fractions	Ct mean	Dilution Factor	miR-35 conc. [nM]
NF	16.33 ± 0.23	1062	**8.38 ± 1.16**
1	16.78 ± 0.23	487.5	**2.91 ± 0.40**
2	16.22 ± 0.10	808.3	**6.81 ± 0.43**
3	16.26 ± 0.23	1006	**8.31 ± 1.18**
4	16.60 ± 0.09	819.2	**5.41 ± 0.30**
5	16.42 ± 0.33	429.0	**3.23 ± 0.78**



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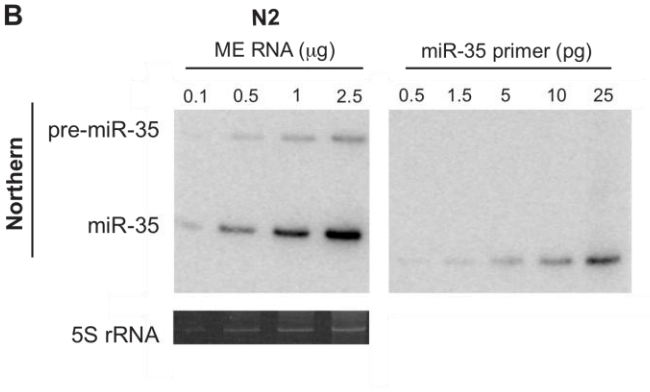

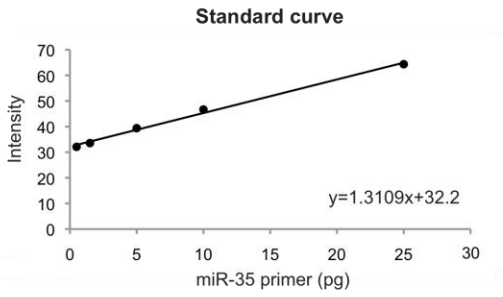

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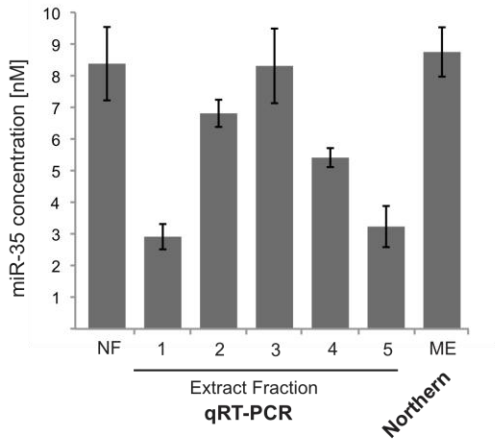

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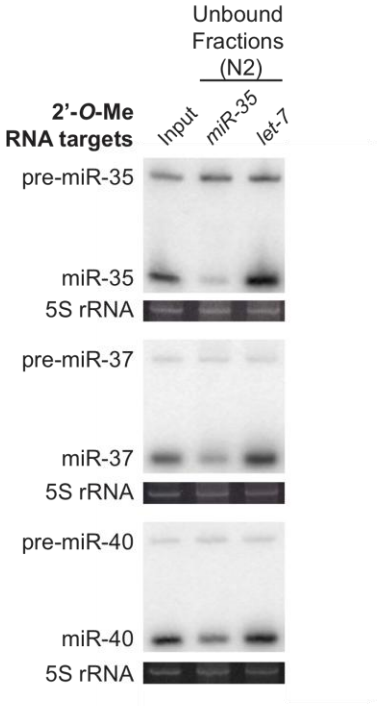
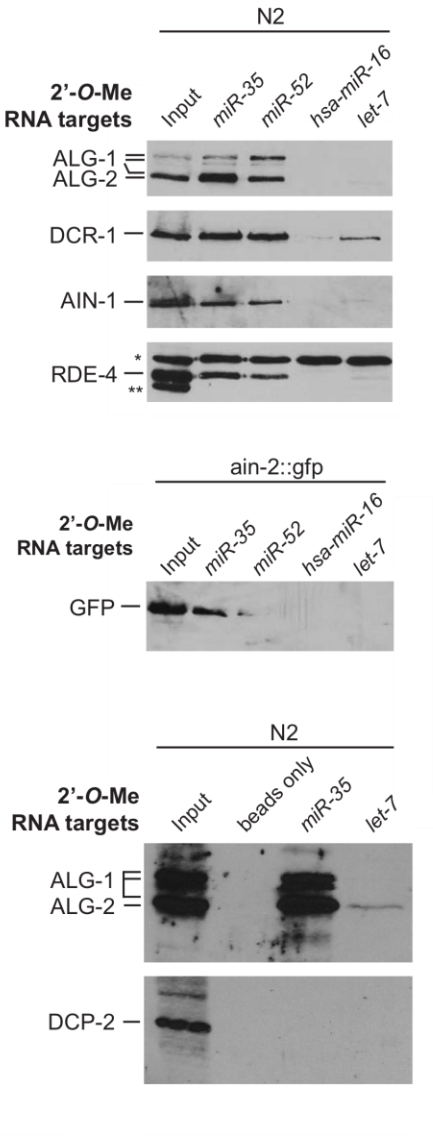
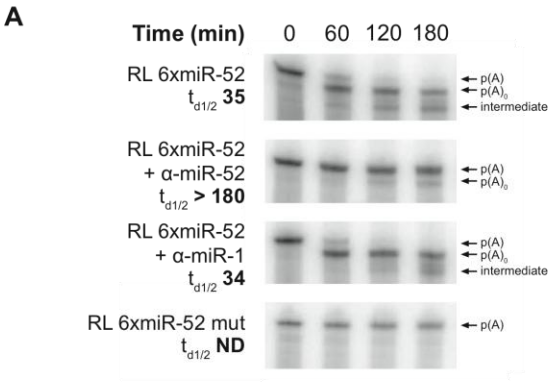
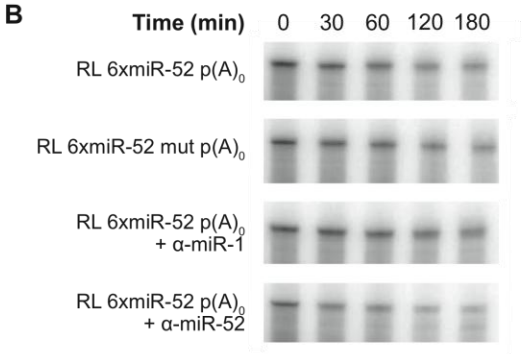


Wu et al. Fig. S1

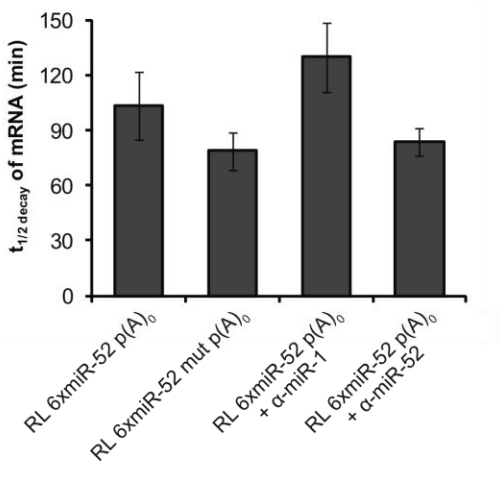


Wu et al. Fig. S2



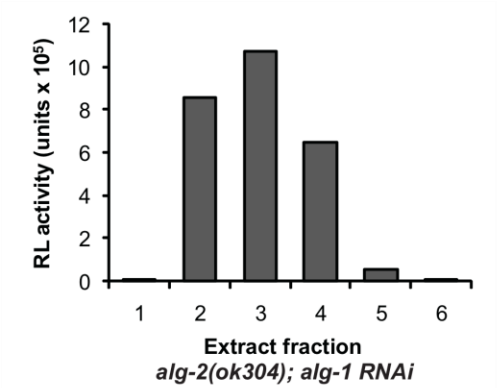


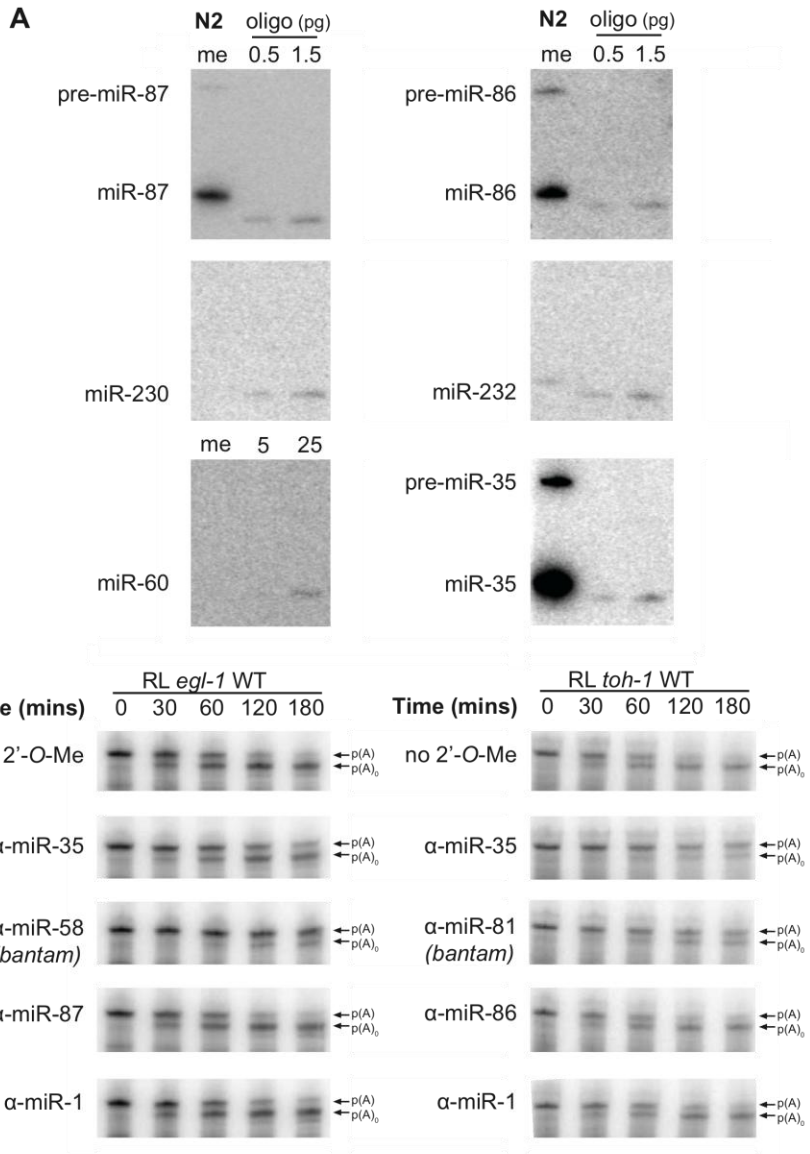
mRNA	2'-*O*-Me	$t_{1/2\ decay}$ (min)
RL 6xmiR-52 $p(A)_0$	-	103 ± 18
RL 6xmiR-52 mut $p(A)_0$	-	79 ± 11
RL 6xmiR-52 $p(A)_0$	α-miR-1	130 ± 19
RL 6xmiR-52 $p(A)_0$	α-miR-52	84 ± 7



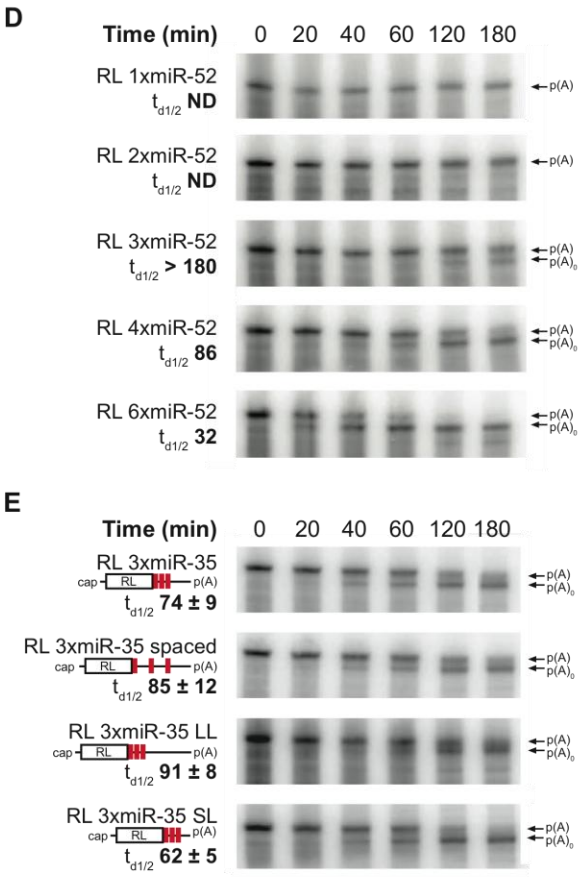
Wu et al. Fig. S3

Wu et al. Fig. S4



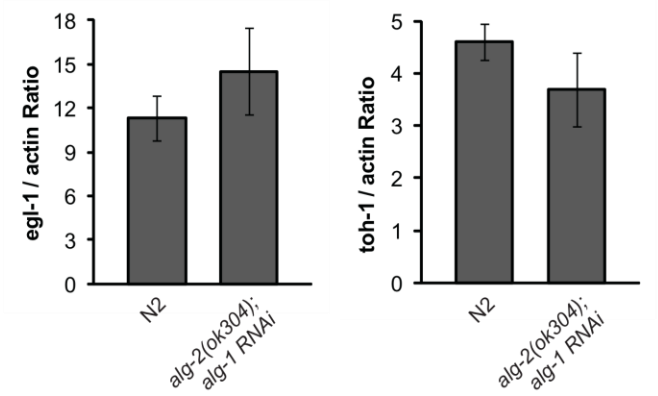


Wu et al Fig. S5



Wu et al Fig. S5

Wu et al. Fig. S6



Supplemental Materials and Methods

Construction of reporters

To generate RL reporters containing miR-35 sites, annealed primers were inserted into XbaI-NotI sites of pCI neo RL p(A). miR-35 targets 3'UTR: 3'UTR sequences were amplified by PCR from genomic DNA or cDNA isolated from C. elegans embryos using primers listed in Table S1 and cloned into XbaI/NotI in pCI neo truncated RL, in which the RL cds region between NheI and BsaBI (position 1-764) was removed. For c34h3.1 3'UTR reporter, RL cDNA contained only region 491-936. The sequences of 6xmiR-35 mutant, 6xmiR-52, 6xmiR-52 mutant, egl-1 3'UTR (wild-type, miR-35 mutant, bantam mutant, miR-35 + bantam mutant), and toh-1 3'UTR (miR-35 mutant, bantam mutant, miR-35 and bantam mutant) were purchased from IDT, as pIDTSMART-KAN clones and were subcloned into pCI neo RL in XbaI-NotI sites.

Embryonic extract Preparation

C. elegans embryonic pellets were homogenized in hypotonic buffer [10 mM HEPES-KOH pH 7.4, 15 mM KCl, 1.8 mM $Mg(OAc)_2$, 2 mM DTT] with a pre-chilled Kontes dounce homogenizer. The extract was then centrifuged twice at 13,200 rpm for 10 minutes at 4°C. The supernatant was loaded onto a Column-Prep (BioRad) stacked with Sephadex G-25 Superfine beads (volume of beads was four-times the volume of the supernatant, Amersham Bioscience) and pushed into the matrix with 1 supernatant volume of isotonic buffer (30 mM HEPES-KOH pH 7.4, 100 mM KOAc, 1.8 mM $Mg(OAc)_2$, 2 mM DTT). Multiple elutions (5-7) were gathered and protein concentrations

were determined by Bradford. The average concentration of active fractions ranged from 10-30 mg/mL.

In vitro Translation Assays

Reactions were typically set up as follows: each 12.5 ul reaction contained 5 ul embryonic extract, 0.1 mM spermidine, 60 uM amino acids, 36 mM HEPES-KOH (pH 7.4), 2 mM $Mg(OAc)_2$, 65 mM KOAc, 0.1 ug/ul calf liver tRNA, 0.096 U/ul RiboLock RNase Inhibitor (Fermentas), 16.8 mM creatine phosphate, 81.6 ng/ul creatine phosphokinase, 0.8 mM ATP, and 0.2 mM GTP). Reactions were incubated with mRNA (1 nM final) at 17ºC for 0 to 3 hours, as indicated. Luciferase activities were analyzed with the Dual-Luciferase® Reporter Assay System (Promega). To assay for miRNA activity, reactions were pre-incubated with 50 nM (except where indicated) 2'-*O*-Me oligonucleotides (Dharmacon) prior to mRNA addition for 30 minutes at 17°C. The 2'-*O*-Me miRNA inhibitors were designed as antisense oligonucleotides to the mature miRNAs according to Wormbase registry (www.wormbase.org).