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The *mir-84* and *let-7* paralogous microRNA genes of *Caenorhabditis elegans* direct the cessation of molting via the conserved nuclear hormone receptors NHR-23 and NHR-25

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The *let-7* microRNA (miRNA) gene of *Caenorhabditis elegans* controls the timing of developmental events. *let-7* is conserved throughout bilaterian phylogeny and has multiple paralogs. Here, we show that the paralog *mir-84* acts synergistically with *let-7* to promote terminal differentiation of the hypodermis and the cessation of molting in *C. elegans*. Loss of *mir-84* exacerbates phenotypes caused by mutations in *let-7,* whereas increased expression of *mir-84* suppresses a *let-7* null allele. Adults with reduced levels of *mir-84* and *let-7* express genes characteristic of larval molting as they initiate a supernumerary molt. *mir-84* and *let-7* promote exit from the molting cycle by regulating targets in the heterochronic pathway and also *nhr-23* and *nhr-25*, genes encoding conserved nuclear hormone receptors essential for larval molting. The synergistic action of miRNA paralogs in development may be a general feature of the diversified miRNA gene family.

KEY WORDS: miRNAs, microRNAs, *mir-84*, *let-7*, Heterochronic pathway, Molting, Nuclear hormone receptors, NHR-23, NHR-25

INTRODUCTION

MicroRNAs (miRNAs) constitute a large class of small (~22 nt) noncoding RNAs present across eukaryotic phylogeny. In *Caenorhabditis elegans*, miRNAs were discovered through genetics (Johnston and Hobert, 2003; Lee et al., 1993; Reinhart et al., 2000), cloning (Lau et al., 2001; Lim et al., 2003) and bioinformatic prediction (Ambros et al., 2003; Grad et al., 2003; Lim et al., 2003). Similar approaches revealed hundreds of miRNAs in plants, fungi and other metazoans (Bartel, 2004). Most of the few metazoan miRNAs studied to date negatively regulate the expression of protein-coding genes by binding imperfectly complementary sites in the 3′ untranslated region (UTR) of the target mRNA and inhibiting translation (Lee et al., 1993; Olsen and Ambros, 1999; Wightman et al., 1993). However, the mechanism of this translational block remains unclear. Also, some miRNAs previously thought to act primarily by blocking translation cause some degradation of their target transcripts (Bagga et al., 2005).

The *let-7* and *lin-4* miRNAs were discovered as mutations that alter the normally invariant cell lineage of *C. elegans*. Mutations in *let-7* cause cell divisions normally restricted to the last larval stage to recur in adults (Reinhart et al., 2000). Similarly, mutation of *lin-4* causes the reiteration of cell division patterns appropriate for the first larval stage, such that vulval and hypodermal tissues characteristic of adults never form (Ambros and Horvitz, 1984; Chalfie et al., 1981). By contrast, mutations in the coding regions of target genes of *let-7* and *lin-4* cause the precocious execution of later larval or adult-specific programs during early larval stages. The *let-7* miRNA is robustly expressed at the L4 stage, when *let-7* binds to the *lin-41* and *hbl-1* mRNAs, causing LIN-41 and HBL-1 protein levels to decline (Abrahante et al., 2003; Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000). Freed of repression by LIN-41 and HBL-1, the transcription factor LIN-29 then directs the larval-to-adult transition in the epidermis, marked by fusion of the lateral seam cells, synthesis of an adult cuticle and the cessation of molting (Bettinger et al., 1996; Rougvie and Ambros, 1995).

Many miRNAs are members of paralogous families (Grad et al., 2003; Lim et al., 2003), suggesting the importance of knowing whether paralogous miRNAs typically act in the same or different pathways and whether they share targets. The *C. elegans* genome specifies three paralogs of *let-7*: *mir-48*, *mir-84* and *mir-241*, all of which are expressed in a temporally regulated manner (Lau et al., 2001; Lim et al., 2003). Genetic analysis revealed that *let-7* paralogs function redundantly to specify patterns of cell division during larval development (Abbott et al., 2005).

The life cycle of *C. elegans* includes four molts, when animals synthesize a new cuticle and shed their old one. Mutations in *let-7*, *lin-4* or *lin-29* cause animals to continue molting after reproductive maturity. Conversely, mutations in particular precocious heterochronic genes cause animals to synthesize an adult cuticle and exit the molting cycle prematurely (Ambros, 1989; Jeon et al., 1999). Although the heterochronic pathway impacts the number of molts, the molecular mechanism by which heterochronic genes affect the molting cycle has not yet been described.

Molting is the hallmark of the ecdysozoan clade, which includes nematodes and insects (Aguinaldo et al., 1997). In insects, pulses of the steroid hormone ecdysone control transitions between life stages by activating stage-specific transcriptional cascades involving several nuclear hormone receptors, including ECR and USP, which together form the receptor for 20-hydroxyecydsone, as well as DHR3 and βFTZ-F1 (Riddiford et al., 2003). For example, the prepupal pulse of ecdysone induces expression of *DHR3*, the product of which in turn promotes expression of *βFTZ-F1* (Lam et al., 1997; White et al., 1997). Abrogation of the function of *βFTZ-F1* causes a defect in the prepupal-to-pupal transition (Broadus et al., 1999). Intriguingly, expression of *let-7* in *Drosophila* correlates with pulses of ecdysone (Bashirullah et al., 2003; Sempere et al., 2002; Sempere et al., 2003).

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The *C. elegans* genes *nhr-23* and *nhr-25* encode orphan nuclear hormone receptors orthologous, respectively, to DHR3 and βFTZ-F1, which are related to mammalian ROR/RZR/RevErb and SF-1, respectively. Both receptors are essential for completion of the larval molts (Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 2001), suggesting that particular functions of *nhr-23/DHR3* and *nhr-25/βFTZ-F1* might be conserved and, further, that regulation by steroid hormones might be a common feature of molting in *C. elegans* and *Drosophila.* However, a steroid hormone regulating molting of *C. elegans* has not yet been identified and the genome lacks orthologs of *ECR* or *USP* (Sluder and Maina, 2001).

Here, we show that *mir-84* works together with *let-7* to direct the terminal differentiation of the epidermis and cessation of the molting cycle. We show that genes normally expressed only before the larval molts are also expressed in *let-7 mir-84* mutants as they enter a supernumerary molt. Moreover, we show that *mir-84* and *let-7* control the molting cycle by regulating known targets in the heterochronic pathway as well as the nuclear hormone receptor genes *nhr-23* and *nhr-25*.

MATERIALS AND METHODS

C. elegans strains and culture

Cultivation and genetic manipulation of *C. elegans* were performed using standard techniques (Sulston and Hodgkin, 1998). The *mir-84(tm1304)* deletion allele was generated by the laboratory of S. Mitani, and outcrossed to wild-type (N2) *C. elegans* six to eight times before analysis. GR1431 was generated via eight crosses to N2.

The *mir-84* gene was PCR-amplified from genomic DNA using Taq polymerase (Roche) and primers GH21 5′-AAGTTGACTGACATGACAACCGAC-3′ and GH32 5′-TTGACACAAAGGCAAGAGCTTG-3′. The *mir-84::gfp* reporter gene was generated via single-end overlap extension PCR (Hobert, 2002), fusing the *mir-84* promoter sequence and *gfp* from vector pPD95.75 (A. Fire). The primers used were GH32, GH107 5′-TATTCATCATACGTCTGCCTGTGCATGCCTGCAGGTCGACTAGAG-3′, GH108 5′-CTCTAGTCGACCTGCAGGCATGCACAGGCAGACGTATGATGAATA-3′, and CAW32 5′-CCGCTTACAGACAAGCTGTGACCG-3′. For both constructs, three independent PCR reactions were combined to ensure that much of the product lacked unwanted mutations. To generate *mgEx671*, the *mir-84* gene was injected into N2 animals at a concentration of 15 ng/μl along with 50 ng/μl of plasmid DNA specifying the co-injection marker *tub-1::gfp*, kindly provided by Ho Yi Mak. Transgenic animals were irradiated with ultraviolet light to generate four independent lines in which the transgene integrated into a chromosome. *mgIs45* and *mgIs47* animals were outcrossed three or more times to N2 before analysis. To generate *mgEx674*, the *mir-84::gfp* fusion gene was injected into N2 animals at 10 ng/μl along with 25 ng/μl of plasmid DNA specifying the co-injection marker *ttx-3::rfp*, provided by Ho Yi Mak. Transgenic animals expressing *mir-84::gfp* were cultivated at 15°C, whereas other animals were typically cultivated at 20°C.

The *mlt-10p::gfp-pest* and *nas-37p::gfp-pest* fusion genes were previously described (Frand et al., 2005). To generate *mgIs49,* the *mlt-10p::gfp-pest* fusion gene was injected into wild-type (N2) animals at 10 ng/μl along with 50 ng/μl plasmid DNA specifying the co-injection marker *ttx-3::gfp* (Hobert et al., 1997), and 20 ng/μl pBluescript. Transgenic animals were irradiated with ultraviolet light to integrate the transgene into a chromosome. One integrant was backcrossed four times to N2 to generate GR1395.

Construct 4271, specifying *nhr-23::gfp,* was provided courtesy of J. Rall and colleagues (Kostrouchova et al., 1998). Plasmid pCG9, specifying *nhr-25::gfp,* was a kind gift from C. Gissendanner and A. Sluder (Gissendanner and Sluder, 2000). To generate the extrachromosomal arrays *mgEx728[nhr-23::gfp]* and *mgEx729[nhr-25::gfp]*, the plasmids were injected into wild-type (N2) animals at a concentration, respectively, of 10 or 20 ng/μl, along with plasmid pRF4, specifying the co-injection marker *rol-6(su1006),* to a final DNA concentration of 100 ng/μl.

The following strains were used in this study.

N2: wild type
RG365: *him-1(e879)* I; *veIs13[col-19::gfp; rol-6(su1006)]* V
SP231: *mnDp1(X;V)*/+ V; *unc-3(e151) let-7(mn112)* X
JR672: *wIs54[scm::gfp]* V
GR1395: *mgIs49[mlt-10p::gfp-pest; ttx-3::gfp]*
GR1368: *mgEx656[nas-37p::gfp-pest; pha-1(+)]*
GR1425: *mgIs46[mir-84++; tub-1::gfp]*; *wIs54[scm::gfp]* V
GR1426: *mgIs45[mir-84++; tub-1::gfp]* I; *let-7(mn112) unc-3(e151)* X
GR1427: *mgEx674[mir-84::gfp; ttx-3::rfp]*
GR1428: *mgIs45[mir-84++; tub-1::gfp]* I
GR1429: *veIs13[col-19::gfp; rol-6(su1006)]* V; *mir-84(tm1304)* X
GR1430: *wIs54[scm::gfp]* V; *mir-84(tm1304)* X
GR1431: *mir-84(tm1304)* X
GR1432: *let-7(mg279)* X
GR1433: *let-7(mg279) mir-84(tm1304)* X
GR1434: *wIs54[scm::gfp]* V; *let-7(n2853)* X
GR1435: *wIs54[scm::gfp]* V; *let-7(n2853) mir-84(tm1304)* X
GR1436: *mgIs49[mlt-10p::gfp-pest; ttx-3::gfp]* IV; *let-7(mg279)* X
GR1437: *mgIs49[mlt-10p::gfp-pest; ttx-3::gfp]* IV; *mir-84(tm1304)* X
GR1438: *mgIs49[mlt-10p::gfp-pest; ttx-3::gfp]* IV; *let-7(mg279) mir-84(tm1304)* X
GR1439: *mgIs47[mir-84++; tub-1::gfp]*; *let-7(mg279) mir-84(tm1304)* X
GR1440: *mgIs47[mir-84++; tub-1::gfp]*; *let-7(mg279)* X
GR1441: *mgIs47[mir-84++; tub-1::gfp]*
GR1442: *mgIs47[mir-84++; tub-1::gfp]; mgIs49[mlt-10p::gfp-pest; ttx-3::gfp] IV; let-7(mg279) mir-84(tm1304)* X
GR1443: *mgEx656[nas-37p::gfp-pest; pha-1(+)]; let-7(mg279) mir-84(tm1304)* X
GR1444: *veIs13[col-19::gfp; rol-6(su1006)]* V; *let-7(mg279) mir-84(tm1304)* X
GR1445: *veIs13[col-19::gfp; rol-6(su1006)]* V; *let-7(mg279)* X
GR1446: *mgIs45[mir-84++]* I; *lin-29(n333) sqt-1(sc13)* II; *mgIs49[mlt-10p::gfp-pest]* IV
GR1447: *mgIs45[mir-84++]*/+ I; *lin-29(n333) sqt-1(sc13)* II; *mgIs49[mlt-10p::gfp-pest]* IV
GR1448: *mgEx728[nhr-23::gfp; rol-6(su1006)]*
GR1449: *mgEx729[nhr-25::gfp; rol-6(su1006)]*
GR1450: *mgEx729[nhr-25::gfp; rol-6(su1006)]; let-7(mg279) mir-84(tm1304)*
GR1451: *mgEx728[nhr-23::gfp; rol-6(su1006)]; let-7(mg279) mir-84(tm1304)*

Microscopy

Images were captured on a Zeiss Axioplan microscope equipped with a Hamamatsu ORCA-ER digital camera and Openlab software (Improvision).

RNAi

RNAi was performed essentially as described (Fraser et al., 2000), except that our nematode growth medium (NGM) contained 8 mmol/l isopropyl-β-D-thiogalactopyranoside and 25 μg/ml carbenicillin. Bacterial clones expressing double-stranded RNA were obtained from J. Ahringer (Fraser et al., 2000; Kamath et al., 2003) and M. Vidal (Rual et al., 2004). In the case of *lin-28*, we cloned *C. elegans* genomic DNA corresponding to nucleotides 3766 to 4098 of cosmid F02E9 (Accession number: emblZ81494) into the same vector and bacterial strain (Rual et al., 2004).

Northern analysis

RNA extractions and northern blots were performed essentially as described (Lee et al., 1993; Reinhart et al., 2000). We used a Starfire-labeled oligonucleotide probe (Integrated DNA Technologies) with sequence complementary to *mir-84* (5′-TACAATATTACATACTACCTCA-3′) and incubated blots at 44°C.

Western analysis

Protein extractions and immunoblots were performed as described (Reinhart and Ruvkun, 2001). Approximately 4 μg total protein were loaded per lane and transferred to Hybond ECL membrane (Amersham). We obtained anti-GFP monoclonal antibody and E7 β-tubulin monoclonal antibody,

respectively, from Clontech and the Developmental Studies Hybridoma Bank at the University of Iowa. The Western Lightning ECL kit (Perkin-Elmer) and X-Omat Blue XB-1 or X-Omat AR film (Kodak) were used to detect signal.

RESULTS

mir-84 acts together with *let-7* to promote the cessation of molting

To explore the function of microRNAs paralogous to *let-7*, we studied the *mir-84* gene of *C. elegans*. Of three known paralogs, *mir-84* is most similar in sequence to *let-7*, sharing 17 out of 22 nucleotides (Fig. 1A). The *mir-84* gene is expressed from the first larval stage through adulthood (Abbott et al., 2005; Esquela-Kerscher et al., 2005), consistent with a role in postembryonic development. We obtained a strain bearing a 654 bp chromosomal deletion that includes the *mir-84* gene from the Mitani lab of the Japanese National BioResources Project (Fig. 1B). No *mir-84* could be detected by northern analysis in total RNA prepared from the *mir-84(tm1304)* mutant (Fig. 1C), verifying that *tm1304* eliminates expression of the gene. To test whether loss of *mir-84* caused phenotypes associated with *tm1304*, a transgene carrying many copies of the wild-type gene, including 989 bp 5′ and 813 bp 3′ of the mature *mir-84* miRNA, was integrated into the genome. The abundance of mature *mir-84* miRNA increased in wild-type animals carrying any one of four independent chromosomal arrays (Fig. 1D).

Given the sequence similarity between *mir-84* and *let-7*, we expected that these miRNAs might target the same or similar mRNAs to control the timing of developmental events in particular tissues. We therefore asked whether loss of *mir-84* would enhance *mg279*, a partial loss-of-function mutation in *let-7*. The level of mature *let-7* miRNA is diminished in *let-7(mg279)* mutants because a deletion of 27 nucleotides upstream of the *let-7* precursor hairpin impedes processing of the primary transcript (Bracht et al., 2004; Reinhart et al., 2000). We anticipated that some *let-7(mg279)* animals would initiate a supernumerary molt after reproductive maturity, because other mutations in *let-7* cause a supernumerary, fifth molt (Reinhart et al., 2000).

We observed the molting behavior of ten individual *let-7(mg279) mir-84(tm1304)* double mutants, *let-7(mg279)* and *mir-84(tm1304)* single mutants, and wild-type animals for one day following the fourth molt. All the *let-7(mg279) mir-84(tm1304)* double mutants became immobile and ceased pharyngeal pumping, behaviors characteristic of lethargus, a period of inactivity that precedes every larval molt. By contrast, only two *let-7(mg279)* mutants and no *mir-84(tm1304)* or wild-type animals entered lethargus during this time. Interestingly, *let-7(mg279) mir-84(tm1304)* and *let-7(mg279)* mutants remained lethargic for over 6 hours (data not shown), whereas wild-type larvae reduce activity for only 2 hours before ecdysis (Singh and Sulston, 1978). Most miRNA mutants that became lethargic also initiated a supernumerary molt but were unable to completely shed the cuticle (Fig. 2A). The mutants died shortly thereafter, when they ceased to lay eggs and their progeny hatched internally. At the end of this experiment, half the *let-7(mg279) mir-84(tm1304)* animals were dead, whereas all the single mutants were alive.

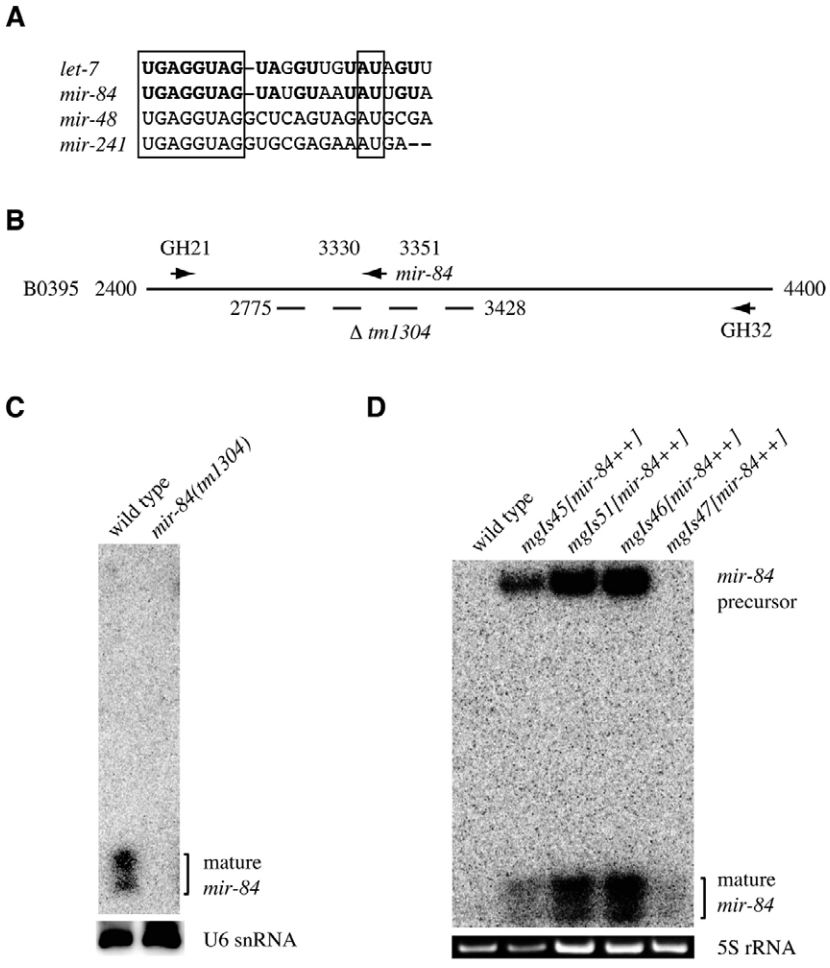


Fig. 1. Genetic analysis of *mir-84*. (**A**) Sequence alignment of the *let-7* family members of *C. elegans*. Residues identical between *mir-84* and *let-7* are shown in bold. Boxes indicate residues conserved among all *let-7* paralogs. (**B**) The *mir-84* genomic region. Numbers correspond to cosmid B0395 (Accession number: emb|Z68131). The dashed line indicates the extent of deletion *tm1304*. Arrows indicate mature *mir-84* and related primers. (**C**,**D**) Northern blots showing *mir-84* levels in early L4-stage larvae of the indicated genotypes. A shorter exposure time was used in D than in C. Levels of 5S rRNA, stained by ethidium bromide, or U6 snRNA, provide loading controls.

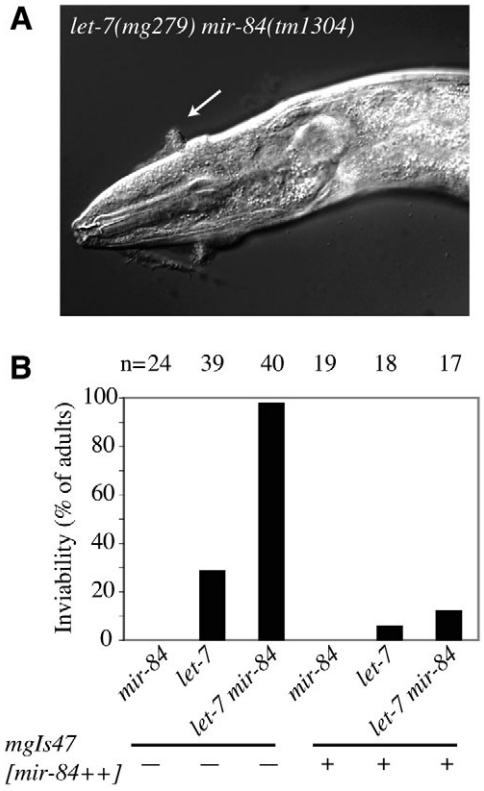


Fig. 2. *mir-84* acts synergistically with *let-7* to promote the cessation of molting. (**A**) Nomarski image of a *let-7(mg279) mir-84(tm1304)* adult. The arrow indicates partly shed cuticle. (**B**) Inviability of adults of the indicated genotypes.

In subsequent experiments, we used lethality caused by the internal hatching of progeny as an indicator of a supernumerary molt. Fig. 2B shows that 98% (*n*=40) of *let-7(mg279) mir-84(tm1304)* double mutants died within 72 hours of the fourth molt at 22°C, compared with 28% (*n*=39) of *let-7* single mutants. We observed no lethality in *mir-84(tm1304)* single mutant adults. Further, restoring expression of *mir-84* by introducing the *mgIs47[mir-84(++)]* transgene rescued the viability of *let-7(mg279) mir-84(tm1304)* mutants. The loss of *mir-84* thus accounts for enhancement of *let-7(mg279)*, and the 1.8 kb DNA contained in *mgIs47* is sufficient to confer the function of *mir-84* in the cessation of molting. Together, the data show that *mir-84* and *let-7* work synergistically to inhibit lethargus and shedding of the cuticle during the adult stage.

We asked whether *mir-84* and *let-7* regulate particular genes expressed during larval molting, including the metalloprotease gene *nas-37* and the novel gene *mlt-10. nas-37* specifies a collagenase essential for ecdysis that probably degrades the pre-molt cuticle (Davis et al., 2004; Frand et al., 2005). Missense alleles of *mlt-10* prevent ecdysis (A.R.F. and G.R., unpublished), but the function of the gene is not yet understood. A transcriptional fusion gene between green fluorescent protein (*gfp*) and *mlt-10* or *nas-37* is expressed in epithelial cells before each of the four larval molts, but is not expressed in adults that no longer molt (Frand et al., 2005).

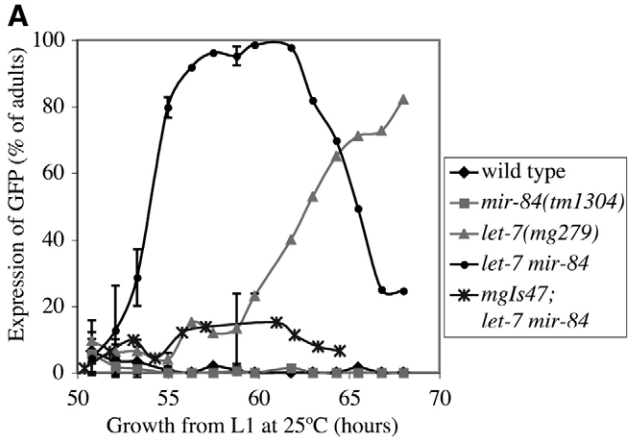
We monitored expression of the *mlt-10p::gfp-pest* reporter in populations of animals cultivated at 25°C for 68 hours following release from starvation as L1 larvae. Animals expressed a pulse of GFP and then completed the fourth molt after approximately 42 hours of cultivation. The *let-7(mg279) mir-84(tm1304)* double mutants expressed an extra pulse of GFP, at levels comparable to those of wild-type animals late in the fourth larval stage, as judged by visual inspection and also by western analysis (Fig. 3). The majority of double mutants expressed GFP after 54 hours of cultivation, about the same time they began to lay eggs. Many *let-7(mg279)* adults also expressed GFP, but later, such that the majority of animals became fluorescent after 63 hours of cultivation. By contrast, none of the *mir-84(tm1304)* or wild-type animals was observed to express GFP after the fourth molt (Fig. 3A). Expression of *mir-84* from the *mgIs47* transgene restored repression of the *mlt-10* reporter to *let-7(mg279) mir-84(tm1304)* adults (Fig. 3A). *let-7(mg279) mir-84(tm1304)* adults also expressed the *gfp* reporter for *nas-37*, consistent with a supernumerary molt (Fig. 3D). Loss of *mir-84* thus promotes expression of genes characteristic of larval molting in adults with reduced levels of *let-7*.

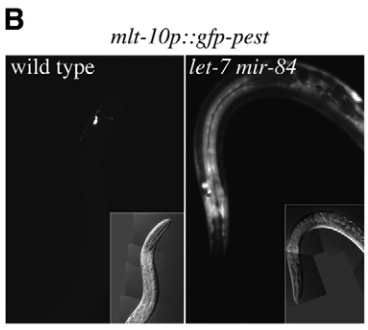
mir-84 and *let-7* promote the cessation of molting via the heterochronic pathway

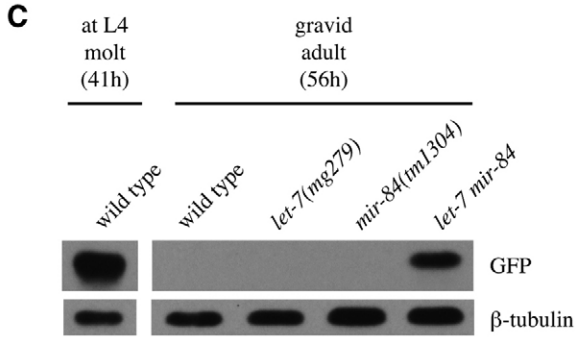
We expected *mir-84* and *let-7* to promote exit from the molting cycle by regulating known targets in the heterochronic pathway. Inactivation of any one of five precocious heterochronic genes, *lin-14*, *lin-28*, *lin-42*, *lin-41* or *hbl-1*, is sufficient to suppress mutations in *let-7*. We found that RNA-interference (RNAi) of *lin-42*, *hbl-1* or *lin-41* fully suppressed the supernumerary pulse of expression of *mlt-10p::gfp-pest* as well as the inviability of *let-7(mg279) mir-84(tm1304)* adults (Fig. 4). Inactivation of *lin-14* or *lin-28* likewise abrogated expression of the *gfp* reporter in *let-7(mg279) mir-84(tm1304)* mutants, but only when animals were fed the corresponding bacterial clones continuously for two generations (Fig. 4). RNAi of the *lin-14* and *lin-28* genes might be less effective in a single generation because *lin-14* and *lin-28* are downregulated early in larval development by *lin-4* (Feinbaum and Ambros, 1999; Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993). Thus, *mir-84* and *let-7* act through the heterochronic pathway to prevent molting in the adult stage. Further, we identify *mlt-10* and *nas-37* as targets of the heterochronic pathway, consistent with our previous report that *mlt-10p::gfp-pest* is expressed in adults that continue molting due to inactivation of *lin-29* (Frand et al., 2005), the transcription factor gene farthest downstream in the heterochronic pathway.

mir-84 and let-7 repress molting via the conserved nuclear hormone receptor genes *nhr-23* and *nhr-25*

We hypothesized that *let-7* and related miRNAs ensure the cessation of molting by directly or indirectly repressing genes that otherwise provoke a molt, including the conserved nuclear hormone receptor genes, *nhr-23* and *nhr-25*, that are key regulators of the larval molting cycle (Gissendanner and Sluder, 2000; Kostrouchova et al., 2001). We therefore asked whether *nhr-23* and *nhr-25* were required for *let-7(mg279) mir-84(tm1304)* mutants to enter the supernumerary molt. Fig. 5 shows that inactivation of either *nhr-23* or *nhr-25* by RNAi restored viability and repression of *mlt-10p::gfp-pest* to the vast majority of *let-7(mg279) mir-84(tm1304)* adults. Likewise, among *let-7(mg279) mir-84(tm1304)* mutants suppressed by RNAi of *nhr-25* or *nhr-23,* respectively, only 1 of 20 or 0 of 16 expressed *nas-37p::gfp-pest* as gravid adults. By contrast, 37% (*n*=83) of mutants fed control bacteria expressed the *nas-37* reporter gene (data not shown). Double mutants fed *nhr-23* or *nhr-25* dsRNA typically remained active and did not shed their cuticle, in stark







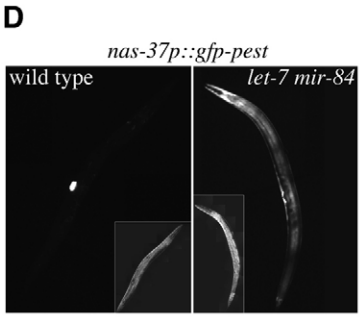


Fig. 3. *let-7 mir-84* adults express *gfp* reporters associated with molting. (**A-D**) Animals were cultivated at 25°C following release from starvation at the early L1 stage. (A) Prevalence of fluorescence from *mlt-10p::gfp-pest*, as detected with a Zeiss SV-6 microscope. Each sample contained 52 or more animals. Data were collected in three experiments to cover all time points. Error bars indicate the standard deviation from the mean in cases where two populations of animals were observed at the same time point. (B) Fluorescence from *mlt-10p::gfp-pest* in adults cultivated for 56 hours. (C) Western blot showing GFP in protein extracts from late L4-stage or gravid adults cultivated, respectively, for 41 or 56 hours. Each strain carried the *mgIs49[mlt-10p::gfp-pest]* transgene. Levels of β-tubulin provide a loading control. (D) Fluorescence from *nas-37p::gfp-pest* in adults.

contrast to animals fed control bacteria not expressing dsRNA for a worm gene. Thus, inactivation of either *nhr-23* or *nhr-25* was sufficient to block initiation of the supernumerary molt in *let-7(mg279) mir-84(tm1304)* mutants.

The model that *mir-84* and *let-7* act via regulation of nuclear hormone receptors predicts an increase in the levels of NHR-23 and NHR-25 in adults with less of the miRNAs. Indeed, we found that fluorescence from a *gfp* fusion gene containing the promoter and first six exons of *nhr-23* (Kostrouchova et al., 1998) was approximately fourfold brighter in the hypodermal nuclei of *let-7(mg279) mir-84(tm1304)* adults than in wild-type animals (Fig. 5C). The miRNAs probably regulate *nhr-23* indirectly, considering that the 3′ UTR of *nhr-23* lacks obvious binding sites for the *let-7* family and that this particular *gfp* reporter lacks the native 3′ UTR of *nhr-23*. One possibility is that transcription of *nhr-23* is repressed by LIN-29. A similar *gfp* reporter for *nhr-25*, also lacking the native 3′ UTR (Gissendanner and Sluder, 2000), showed only a modest increase in expression in *let-7(mg279) mir-84(tm1304)* mutants compared with wild-type gravid adults (data not shown).

To address the possibility that *let-7* and related miRNAs target the *nhr-25* message, we searched the 3′ UTR of the *nhr-25* gene for binding sites using the computer program RNAhybrid (Rehmsmeier et al., 2004). We identified one site apt to form a helix that includes the first seven nucleotides of *mir-84* and lacks G:U base-pairs or bulged nucleotides within that seed region (Fig. 6), features characteristic of high-quality miRNA binding sites (Doench and Sharp, 2004; Lall et al., 2006). Two additional sites were predicted to form helices with *let-7* or *mir-241* and *mir-48* that contain G:U base-pairs and bulged nucleotides within the seed region, features present in experimentally validated *let-7* binding sites in *lin-41*

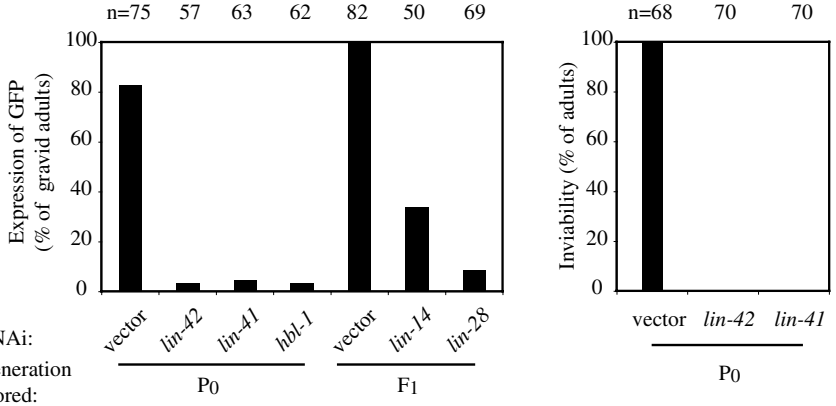


Fig. 4. Inactivation of precocious heterochronic genes prevents the supernumerary molt of *let-7 mir-84* mutants. Synchronized populations of L1 larvae were fed bacteria expressing dsRNA corresponding to the indicated genes and cultivated at 25°C. Animals in the P_0 generation were scored for fluorescence from *mlt-10p::gfp-pest* 56 hours later and then for viability the next day. To score F_1 animals, progeny were collected, synchronized as L1 larvae, and then fed the appropriate bacterial clone for an additional 55 hours.

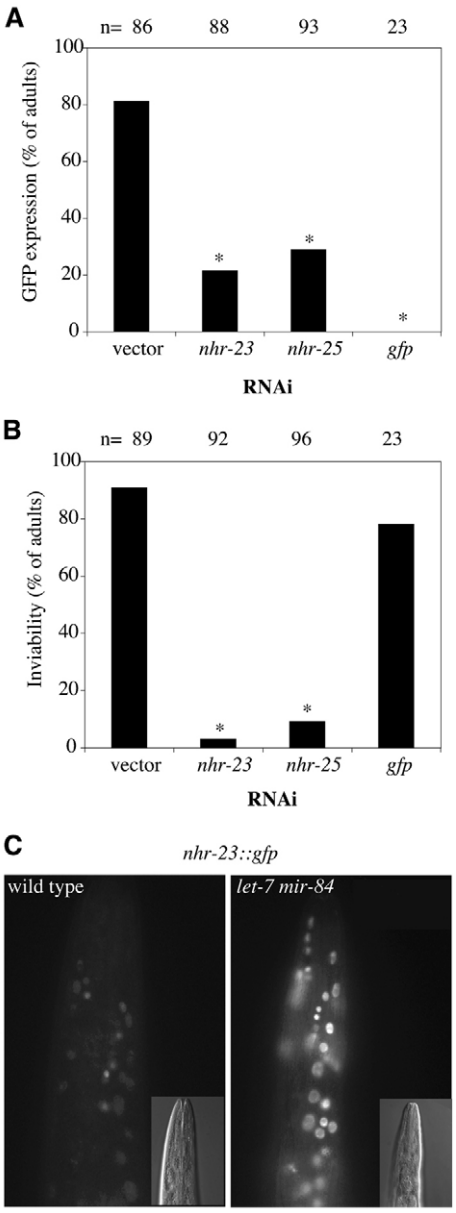


Fig. 5. Inactivation of *nhr-23* or *nhr-25* blocks the supernumerary molt of *let-7 mir-84* mutants. (A,B) Synchronized populations of *let-7(mg279) mir-84(tm1304)* animals were cultured until the fourth larval stage at 20°C. Individuals were then fed bacteria expressing dsRNA and observed several times over the next 2 days. The combined results of three independent experiments are shown. Asterisks indicate a significant difference from the control sample ($P \leqslant 0.001$, chi-square test). (**A**) Prevalence of detectable fluorescence from *mlt-10p::gfp-pest*. (**B**) Prevalence of death. (**C**) Fluorescence from *nhr-23::gfp* in the nuclei of epithelial cells near the head of *let-7 mir-84* or wild-type adults.

(Vella et al., 2004b; Vella et al., 2004a). All three sites are highly conserved among the 3′ UTRs of *nhr-25* from the nematodes *C. briggsae*, *C. remanei* and *C. elegans*, consistent with interaction with the *let-7* paralogs in vivo (Fig. 6B). The *let-7* family of miRNAs might thereby directly target the *nhr-25* message, reinforcing transcriptional downregulation of *nhr-25* during the adult stage (Gissendanner and Sluder, 2000).

We considered that *mir-84* and *let-7* might target additional genes that promote molting and therefore examined the predicted 3′ UTRs (Hajarnavis et al., 2004) of 47 genes identified as essential for completion of the larval molts through a genome-wide RNAi screen (Frand et al., 2005 and Table 1 within). We identified potential binding sites for *let-7* and related miRNAs in *alg-1* and *pan-1*, the latter consistent with work by Lall and colleagues (Lall et al., 2006) (see Fig. S1 in the supplementary material). We are exploring the significance of these sites to the regulation of molting.

As a complementary strategy to identify genes acting downstream of the *let-7* paralogs in genetic pathways regulating the molting cycle, we asked whether inactivation of additional genes required for the larval molts (Frand et al., 2005) would prevent the supernumerary molt of miRNA mutants. RNAi of the ribosomal protein gene *rpl-27*, *rpl-31* or *rpl-32* restored viability and repression of the *mlt-10p::gfp-pest* reporter to many *let-7(mg279) mir-84(tm1304)* adults, suggesting that protein synthesis is essential for initiation or progression of the supernumerary molt (see Table S1 and Table S2 in the supplementary material). RNAi of 89 other genes did not significantly suppress both indicators of the supernumerary molt, at the threshold of $P \leqslant 0.001$ in chi-square tests. The *rpl* genes are unlikely to serve as direct targets of *let-7* or paralogous miRNAs, because their 3′ UTRs lack obvious binding sites and contain no more than 47 nucleotides. Also, *let-7(mg279) mir-84(tm1304)* mutants suppressed by RNAi of an *rpl* gene were smaller and less active than those suppressed by RNAi of *nhr-23* or *nhr-25,* suggesting that NHR-23 and NHR-25 are the main effectors of *let-7* and *mir-84* in the regulation of molting.

Overexpression of *mir-84* suppresses mutations in *lin-29*

Because we identified potential binding sites for the *let-7* family members among genes essential for molting, we predicted that increased expression of *mir-84* would suppress mutations in *lin-29,* bypassing the canonical heterochronic pathway. We examined the molting phenotypes caused by a probable null allele, *lin-29(n333)* (Bettinger et al., 1996; Rougvie and Ambros, 1995), in the presence or absence of excess *mir-84,* comparing *mgIs45[mir-84++]*; *lin-29(n333); mgIs49[mlt-10p::gfp-pest]* animals to segregants from an *mgIs45* heterozygote (GR1447). Individuals expressing *mlt-10p::gfp-pest* were selected late in the fourth larval stage and observed several times over the next 29 hours of cultivation at 25°C. In total, 57% (17/30) of *lin-29(n333)* mutants carrying the *mir-84++* transgene expressed GFP as adults, whereas all (30/30) animals lacking *mgIs45* expressed an extra pulse of GFP ($P \leqslant 0.001$, chi-square test). Moreover, only 21% (7/30) of *mir-84++* animals completed a supernumerary molt, whereas 86% (25/29) of animals lacking *mgIs45* molted, indicated by shedding of the cuticle ($P \leqslant 0.001$, chi-square test). The observation that overexpression of *mir-84* can prevent or delay the supernumerary molt of *lin-29(n333)* mutants supports the view that *mir-84* directly targets particular mRNAs, the products of which otherwise provoke a supernumerary molt.

mir-84 is expressed in the lateral hypodermal seam cells

To determine where and when *mir-84* is expressed, we fused the *gfp* gene and the *unc-54* 3′ UTR to the putative *mir-84* promoter, a 989 bp sequence 5′ of the mature miRNA. Transgenic *mgEx674[mir-84::gfp]* animals expressed GFP in the lateral hypodermal seam cells (Fig. 7) and other cells (see Fig. S2 in the supplementary material). The *mir-84::gfp* reporter was expressed in seam cells

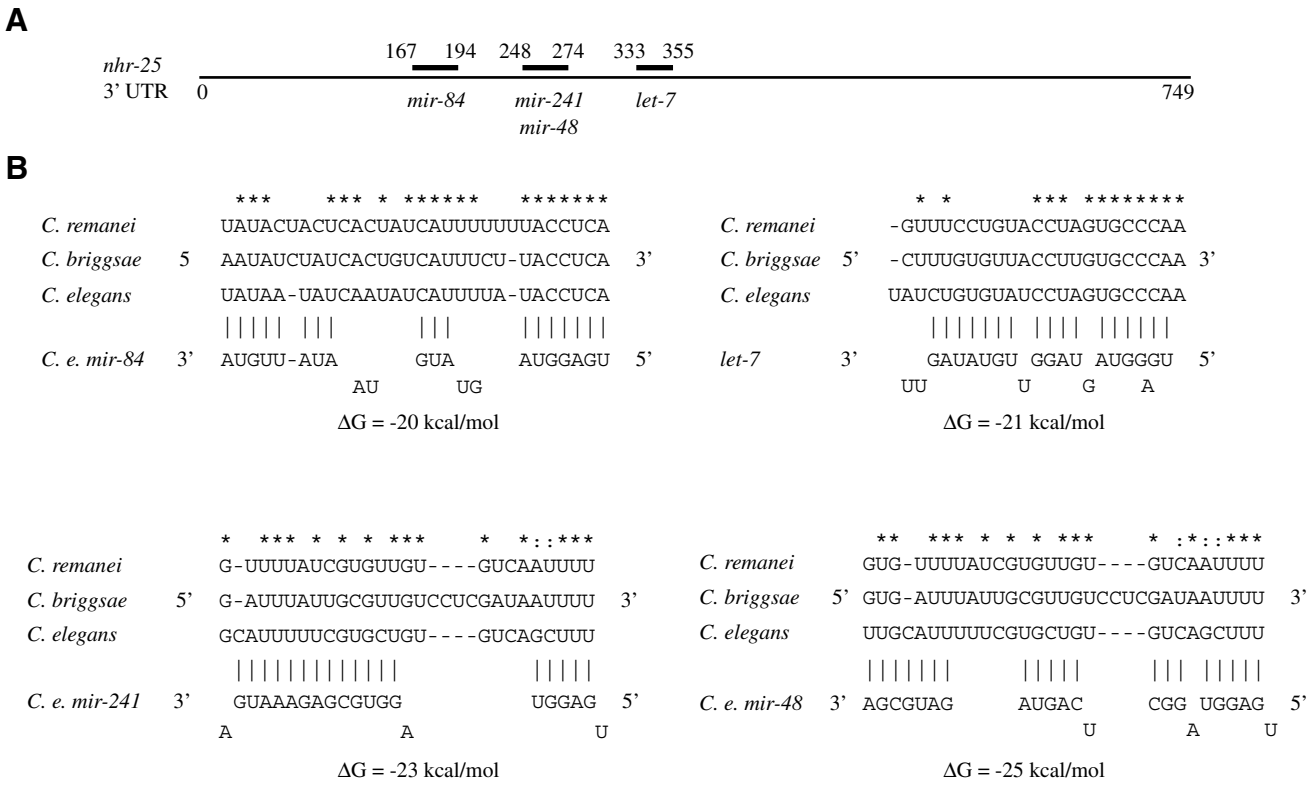


Fig. 6. Potential binding sites for *mir-84* and paralogs in the 3′ UTR of *nhr-25*. (**A**) The 3′ UTR of *C. elegans nhr-25,* corresponding to nucleotides 35690 through 36438 of cosmid F11C1 (Accession number: emb|Z54270). Lines denote potential binding sites for *let-7* and related miRNAs, as indicated. (**B**) Vertical lines indicate potential base-pairing between the 3′ UTR of *C. elegans nhr-25* and the *let-7*-like miRNA, for each site shown in A. The predicted free energy of folding is indicated. Sequence alignments show conservation of the putative binding sites in the 3′ UTRs of predicted *nhr-25* orthologs from *C. briggsae* (Accession number: emb|CAAC01000078) and *C. remanei* (contig 0.60, nucleotides 49497 through 50100). Asterisks indicate nucleotides perfectly conserved among all three species, including seed sequences for the predicted *mir-84* and *let-7* sites. Colons indicate substitutions within seed sequences of the predicted *mir-48* and *mir-241* binding site compatible with G:U base-pairing.

during early larval stages in some transgenic animals (Fig. 7B), although expression was more prevalent in L3-stage and older animals (Fig. 7A; see Fig. S2A in the supplementary material). Our results thus suggest a broader temporal expression pattern for *mir-84* than previously described by Esquela-Kerscher and colleagues (Esquela-Kerscher et al., 2005), who observed expression of a *mir-84::gfp* fusion gene in the seam cells beginning only in the L4 stage. We saw a similar pattern of *mir-84::gfp* expression in three independent transgenic lines (data not shown). Further, we saw no obvious difference in expression between this particular *mir-84::gfp* fusion gene and one in which 8.1 kb of sequence upstream of *mir-84* was fused to yellow fluorescent protein, kindly provided by A. Yoo and I. Greenwald (Fig. 7C; see Fig. S2F in the supplementary material). Consistent with synergistic functions for *mir-84* and *let-7,* mutations in *let-7* impact the development of many tissues that express *mir-84::gfp*, including the seam cells and vulva, and a *let-7::gfp* fusion gene is expressed in the seam cells beginning at the L4 larval stage (Johnson et al., 2003).

mir-84 acts synergistically with *let-7* to promote differentiation of epithelial cells

Given that *mir-84* regulates termination of molting and is expressed in epithelial cells that synthesize the cuticle, we anticipated that *mir-84* might promote the terminal differentiation of particular epithelial cells, including the lateral seam cells and the major body hypodermal syncytium, hyp7. Seam cells divide in a stem-cell like fashion at the larval-to-larval molts, but terminally differentiate, fuse and secrete a cuticular structure called alae at the larval-to-adult molt (Sulston and Horvitz, 1977). To examine the role of *mir-84* in the seam-cell lineage, we used the temperature-sensitive mutation *let-7(n2853)* to sensitize the genetic background and an *scm::gfp* fusion gene (Terns et al., 1997) to visualize the nuclei of seam cells. *let-7(n2853) mir-84(tm1304)* young adults had an average of 22.4±2.7 (n=17) seam cells when cultivated at 20°C (Table 1), whereas *let-7(n2853)* young adults had 19.2±3.0 (n=23) seam cells, a modest but significant difference (P<0.001, Student's t-test). By contrast, wild-type adults had 16.1±0.2 (n=17) seam cells, similar to all animals

Table 1. Seam cell nuclei in *mir-84* and *let-7* mutants

	Wild type	*mir-84(tm1304)*	*let-7(n2853)*	*let-7(n2853) mir-84(tm1304)*
Late L4	16.0±0.5 (n=17)	15.9±0.6 (n=20)	15.9±0.5 (n=16)	15.9±0.9 (n=21)
Young adult	16.1±0.2 (n=17)	16.1±0.4 (n=14)	19.2±3.0 (n=23)	22.4±2.7* (n=17)

The number of seam cell nuclei observed in *wIs54[scm::gfp]* animals of the indicated stage and genotype. For L4 animals, nuclei were counted after the end of cell divisions. For young adults, nuclei were counted before egg production. Mean number of nuclei and s.d. are shown. Asterisk indicates a significant difference from *let-7* adults (P<0.001, Student's t-test).

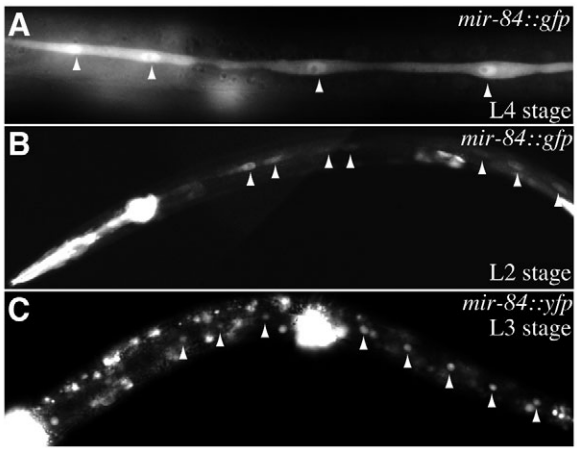


Fig. 7. Expression of *mir-84* fusion genes. (**A**) Fluorescence from *mir-84::gfp* in the seam cells of an L4-stage larva. (**B**) Fluorescence from *mir-84::gfp* in the seam cells, pharynx and somatic gonad of an L2-stage larva. (**C**) Fluorescence from *mir-84::yfp* in the seam cells of an L3-stage larva. Arrowheads indicate seam cells.

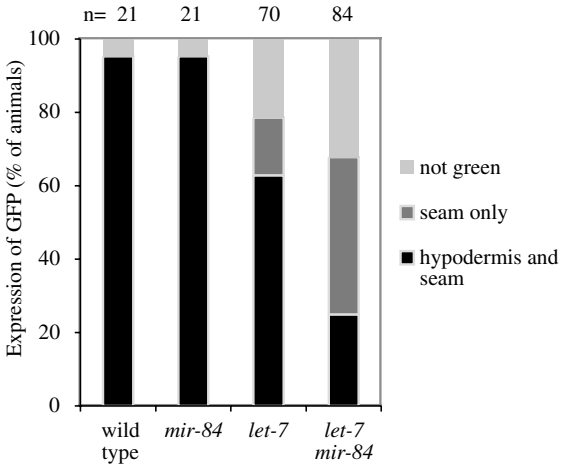


Fig. 8. *mir-84* and *let-7* promote full expression of *col-19::gfp* in adults. Loss of *mir-84* exacerbates the failure of *let-7(mg279)* mutants to express *col-19::gfp* in both the hypodermis and seam cells. Gravid adults in mixed-stage populations were scored using a Zeiss SV-6 microscope.

observed at the L4 stage. We also examined the cuticle of young adults. Animals with reduced levels of *let-7* due to the mutation *mg279* form less distinct alae than wild-type animals (Reinhart et al., 2000). The alae of *let-7(mg279) mir-84(tm1304)* double mutants were even less prominent than those of *let-7(mg279)* animals (data not shown).

To examine the role of *mir-84* and *let-7* in differentiation of the hypodermis, we used a *gfp* reporter for the cuticle collagen gene *col-19*. The fusion gene is expressed only in the adult stage, in both the hypodermis and seam cells (Abrahante et al., 1998). Only 25% (n=84) of *let-7(mg279) mir-84(tm1304)* young adults expressed GFP in both the hypodermis and seam cells, whereas 63% (n=70) of *let-7(mg279)* mutants expressed GFP in both tissues, a significant difference ($P \leqslant 0.001$, chi-square test) (Fig. 8). Similarly, Abbott and colleagues (Abbott et al., 2005) show that *mir-48(n4097); mir-84(n4037)* double mutants fail to express a *col-19::gfp* reporter in the hypodermis, even though the mutants express that particular *col-19::gfp* in seam cells. Together, our observations indicate that loss of *mir-84* exacerbates defects in the terminal differentiation of the seam cells and hypodermis caused by mutations of *let-7*.

mir-84 overexpression suppresses *let-7* lethality

Given the similarities between the nucleotide sequences and the spatial and temporal expression patterns of *mir-84* and *let-7*, we expected that *mir-84* could functionally substitute for *let-7*. Animals with a null mutation in *let-7* burst at the vulva during the L4 stage, and therefore rarely have progeny (Reinhart et al., 2000). We found that 93% (n=15) of *let-7(mn112)* mutants overexpressing *mir-84* from the *mgIs45* transgene survived and produced progeny, whereas only 3% (n=31) of *let-7(mn112)* animals produced progeny in the absence of auxiliary *mir-84* (Fig. 9). Rescue of the null allele of *let-7* required robust expression of *mir-84*, because *mgIs47*, which drives a lower level of *mir-84* expression than *mgIs45* (Fig. 1D), failed to suppress *let-7(mn112)* (data not shown). However, the *mgIs47* transgene did reduce lethality caused by *let-7(mg279)* (Fig. 2B). Thus, *mir-84* can substitute for *let-7* when abundant. Alternatively, suppression of the *let-7* null allele by *mgIs45* might be attributable to precocious developmental events caused by overexpression of *mir-84* (G.D.H. and G.R., PhD thesis, Harvard University, 2005) (Johnson et al., 2005), considering that mutations in precocious heterochronic genes also suppress *let-7* mutations (Slack et al., 2000). Similar to our findings with *mir-84,* increased expression of the *let-7* paralog *mir-48* also suppresses lethality caused by the loss of *let-7* (Li et al., 2005).

DISCUSSION

Here, we show that *mir-84* functions synergistically with the paralogous miRNA *let-7* to promote the transition from larval to adult developmental programs, including the terminal differentiation of particular epithelial cells and the cessation of molting. Our findings suggest that the *let-7* family of miRNAs work in a combinatorial mode to repress particular targets. Indeed, animals that lack all three paralogs of *let-7*, but express *let-7* itself, fail to repress a reporter for the *let-7* target gene *hbl-1* or exit the molting cycle at the appropriate time (Abbott et al., 2005).

Fig. 10 shows a genetic model for the function of *mir-84* and *let-7* in epithelial differentiation, as related to the molting cycle. The *let-7* miRNA targets *lin-41* mRNA (Slack et al., 2000) and also *hbl-1* mRNA, in combination with paralogous miRNAs (Abbott et al., 2005; Abrahante et al., 2003; Lin et al., 2003). During early larval development, LIN-41 and HBL-1 together repress production of the zinc-finger transcription factor LIN-29 (Abrahante et al., 2003; Rougvie and Ambros, 1995; Slack et al., 2000). Expression of *let-7* and related miRNAs late in larval development represses *lin-41* and *hbl-1,* thereby activating LIN-29. LIN-29 promotes expression of *col-19* and possibly other collagen genes characteristic of an adult cuticle and also represses expression of *col-17* and possibly other collagen genes characteristic of larval cuticle (Bettinger et al., 1996; Liu et al., 1995; Reinhart et al., 2000; Rougvie and Ambros, 1995). LIN-29 is likely to regulate additional genes that control the molting cycle that have not yet been identified.

Here, we show that inactivation of either one of the nuclear hormone receptor genes *nhr-23* or *nhr*-25 is sufficient to prevent the aberrant supernumerary molt caused by reduced levels of *mir-84* and *let-7*. NHR-23 and NHR-25 thus serve as key downstream effectors of the miRNAs in regulation of the molting cycle (Fig. 10). One

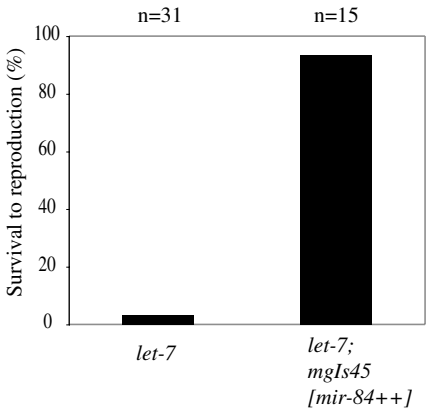


Fig. 9. Overexpression of *mir-84* rescues a null allele of *let-7*. Graph shows the percent of *let-7(mn112)* animals that survived and produced progeny in the presence or absence of *mgIs45[mir-84++]*. We compared GR1426 to animals derived from SP231 that displayed uncoordinated movement.

model is that LIN-29, or a transcription factor regulated by LIN-29, represses *nhr-23* and *nhr-25* following the fourth molt. Accordingly, GFP expression from an *nhr-23* reporter gene increases fourfold in the hypodermis of *let-7 mir-84* adults. The relationship between *nhr-23* and *nhr-25* in *C. elegans* remains to be determined; however, DHR3 stimulates transcription of *βFTZ-F1* in flies (Lam et al., 1997; White et al., 1997).

The identification of sites in the 3′ UTR of *nhr-25* that are complementary to *let-7* family members and are also conserved in other nematodes suggests that the *let-7* family targets the *nhr-25* message to negatively regulate production of NHR-25 in adults (Fig. 10). Consistent with this model, increasing the abundance of *mir-84* partly suppresses the supernumerary molt caused by a probable null mutation in the *lin-29* gene. Also, in preliminary experiments we have detected RNA species attributable to cleavage of the *nhr-25* message upon binding of *let-7*-like miRNAs in extracts from wild-type adults, using the method of Bagga et al. (Bagga et al., 2005). Steroid hormones and co-factors probably also regulate activity of NHR-23 and NHR-25 during the life cycle.

Regulation by miRNAs thus converges on transcription factors upstream in the genetic networks regulating molting. NHR-23 coordinates several aspects of larval molting by promoting expression of genes required for patterning the new cuticle and ecdysis, including, respectively, the collagen gene *dpy-7* and the collagenase gene *nas-37* (Frand et al., 2005; Kostrouchova et al., 1998; Kostrouchova et al., 2001). Here, we show that inactivation of either *nhr-23* or *nhr-25* abrogates the reiterated expression of *gfp* reporters for *mlt-10* and *nas-37* caused by mutation of *let-7* and *mir-84*. NHR-25 might promote expression of the corresponding genes during larval development, even though RNAi of *nhr-25* is not sufficient to abrogate expression of the *gfp* reporters in wild-type larvae (Frand et al., 2005). Interestingly, inactivation of *nhr-23* or *nhr-25* causes an earlier blockade in the molting program in *let-7 mir-84* adults than in wild-type larvae, such that the mutant adults do not enter lethargus or attempt to ecdyse. Parallel pathways might drive early steps of molting during larval development.

Intriguingly, adults with reduced levels of *mir-84* and *let-7* are unable to shed their cuticle to complete the supernumerary molt. One possibility is that particular genes required for ecdysis are not induced. Whereas the hypodermis and seam cells retain some larval character in *let-7 mir-84* adults, other cells, perhaps particular neurons or specialized epithelia, might be fully differentiated and therefore unable to coordinate with the molting program. Consistent with this idea, *let-7 mir-84* adults spend an atypically long time in lethargus, suggesting a failure to exit the behavioral program. Alternatively, particular structural features of the fifth cuticle might be physically incompatible with shedding the exoskeleton.

Considering an aberrant ecdysis as the terminal phenotype of *let-7 mir-84* mutants, it is intriguing to speculate that the *let-7* family and possibly other miRNAs regulate aspects of the larval molting cycle. Indeed, increased expression of either *mir-84* or *let-7* causes some larvae to arrest development, trapped inside partly shed cuticle, indicating that levels of *let-7*-like miRNAs can impact molting of larvae (G.D.H. and G.R., unpublished).

Mechanisms that set the pace of the molting cycle are not well understood, although physiologic cues such as nutritional status (Ruaud and Bessereau, 2006) and environmental cues such as temperature impact the duration of larval stages. Interestingly, *let-7* and *let-7 mir-84* mutants initiate the supernumerary molt in synchrony, rather than in a stochastic fashion, relative to the time of hatching. Thus, a timing mechanism for molting persists in these particular miRNA mutants.

The *let-7* gene is perfectly conserved throughout bilaterian phylogeny (Pasquinelli et al., 2000), and vertebrate genomes specify many miRNAs homologous to *let-7* (Lagos-Quintana et al., 2001). Vertebrate *let-7* and protein-coding genes orthologous to targets of *let-7* identified in *C. elegans* play crucial roles in development (Kloosterman et al., 2004; Moss and Tang, 2003). Moreover, reduced expression of human *let-7* correlates with shortened survival in lung cancer patients (Takamizawa et al., 2004), and *let-7* might regulate the *RAS* oncogene (Johnson et al., 2005). The possibility of functional conservation among homologs of *let-7* in humans and worms intimates the importance of understanding how *let-7* and its paralogs function in *C. elegans*. Our work shows how analysis of double mutants can reveal how the many miRNAs that form paralogous families work together to regulate their targets.

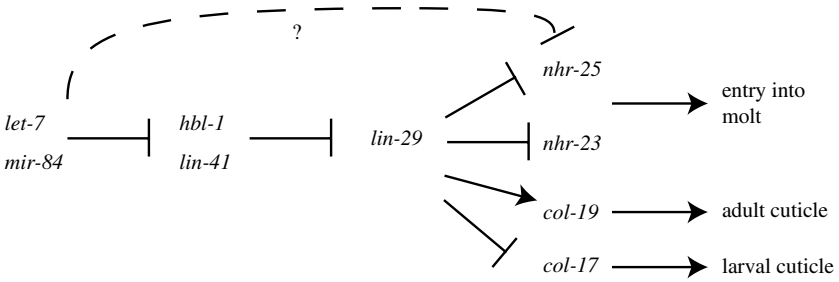


Fig. 10. A genetic model for the cessation of molting. We propose that *let-7* and *mir-84* act through the heterochronic pathway to repress key regulators of molting, including the conserved nuclear hormone receptor genes *nhr-23* and *nhr-25*. *let-7* and paralogous miRNAs might also target *nhr-25* mRNA. Positive regulation is denoted by an arrowhead and negative regulation by a perpendicular line.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/23/4631/DC1

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Table S1. Expression of *mlt-10p::gfp-pest* in *let-7(mg279) mir-84(tm1304)* adults upon RNAi against genes implicated in molting

RNAi	Fluorescent (n)	Total (n)	% Fluorescent	Control Value	% Fluorescent (Normalized)	Chi-square	*P*-value
vector 1	30	55	55	55	100	N.A.	N.A.
vector 2	35	54	65	65	100	N.A.	N.A.
vector 3	19	25	76	76	100	N.A.	N.A.
vector 4	23	25	92	92	100	N.A.	N.A.
vector 5	81	99	82	82	100	N.A.	N.A.
vector 6	40	59	68	68	100	N.A.	N.A.
gfp 1	0	73	0	55	0	52.01	0.001
gfp 2	0	35	0	92	0	23.20	0.001
gfp 3	0	68	0	68	0	67.29	0.001
rpl-27	1	10	10	68	15	11.85	0.001
rpl-32	6	45	13	82	16	72.77	0.001
rpl-31	13	88	15	82	18	83.77	0.001
nhr-23	13	66	20	82	24	62.34	0.001
nhr-23	16	73	22	55	40	14.50	0.001
Y37D8A.21	38	106	36	82	44	44.42	0.001
vha-9	19	65	29	65	45	15.07	0.001
bli-1	17	55	31	65	48	12.56	0.001
ZK430.8	16	46	35	65	54	8.97	0.010
pod-2	14	36	39	65	60	5.85	0.025
nhr-25	7	15	47	76	61	3.55	0.100
pan-1	26	61	43	65	66	5.66	0.025
acn-1	20	40	50	76	66	4.33	0.050
nas-36	29	79	37	55	67	4.19	0.050
ran-4	24	52	46	65	71	3.74	0.100
Y48B6A.3	30	65	46	65	71	4.14	0.050
vha-16	54	92	59	82	72	12.30	0.001
stc-1	44	94	47	65	72	4.47	0.050
rps-6	43	72	60	82	73	10.21	0.010
lrp-1	13	32	41	55	74	1.57	1.000
rme-8	24	48	50	65	77	2.29	0.200
unc-52	29	58	50	65	77	2.51	0.200
F40G9.1	29	57	51	65	78	2.21	0.200
rps-32	25	42	60	76	78	1.89	0.200
F53B8.1	29	56	52	65	80	1.92	0.200
F20G4.1	22	42	52	65	81	1.51	1.000
Y51H1A.3	53	79	67	82	82	5.12	0.025
vha-8	20	26	77	92	84	2.19	0.200
mlt-10	19	41	46	55	84	0.63	1.000
kin-2	27	42	64	76	85	1.00	1.000
mlt-9	11	17	65	76	85	0.63	1.000
lir-1	30	54	56	65	85	0.97	1.000
Y65B4A.6	32	44	73	82	89	1.52	1.000
lrp-1	69	94	73	82	90	1.97	0.200
K04A8.6	31	50	62	68	91	0.40	1.000
fbn-1	49	82	60	65	92	0.35	1.000
F53G12.4	32	63	51	55	92	0.17	1.000
let-805	38	63	60	65	93	0.25	1.000
nhr-6	24	47	51	55	93	0.12	1.000
est-1	48	63	76	82	93	0.75	1.000
mlt-11	45	63	71	76	94	0.19	1.000
Y54E10BR.5	44	57	77	82	94	0.49	1.000
imb-3	55	71	77	82	94	0.49	1.000
C23G10.10	42	54	78	82	95	0.36	1.000
Y47D3B.1	40	51	78	82	96	0.25	1.000
R06A4.9	15	23	65	68	96	0.05	1.000
ntl-1	45	72	63	65	96	0.07	1.000
F49C12.12	24	27	89	92	97	0.14	1.000
ifc-2	36	49	73	76	97	0.06	1.000
M03F4.7	38	51	75	76	98	0.02	1.000
mec-7	63	78	81	82	98	0.03	1.000
ptr-23	39	60	65	65	100	0.00	1.000
C23F12.1	16	21	76	76	100	0.00	1.000
alg-1	26	34	76	76	101	0.00	1.000
ptr-4	39	51	76	76	101	0.00	1.000
adt-2	36	47	77	76	101	0.00	1.000

dmd-5	33	50	66	65	102	0.02	1.000
Y53F4B.22	99	118	84	82	102	0.16	1.000
nsf-1	22	39	56	55	103	0.03	1.000
W03F9.10	82	97	85	82	103	0.26	1.000
F38A1.8	29	37	78	76	103	0.05	1.000
mlt-8	35	52	67	65	104	0.07	1.000
bli-5	34	43	79	76	104	0.09	1.000
lin-66	47	55	85	82	104	0.33	1.000
let-92	24	30	80	76	105	0.13	1.000
vrs-2	65	75	87	82	106	0.74	1.000
D1054.15	84	104	81	76	106	0.28	1.000
C37C3.3	38	47	81	76	106	0.23	1.000
T25B9.10	45	51	88	82	108	1.03	1.000
crs-2	30	41	73	68	108	0.33	1.000
F25H8.6	53	60	88	82	108	1.20	1.000
lev-11	38	43	88	82	108	0.95	1.000
M03F8.3	41	50	82	76	108	0.38	1.000
npp-20	36	49	73	68	108	0.41	1.000
T23F2.1	23	28	82	76	108	0.30	1.000
qua-1	81	115	70	65	108	0.54	1.000
npp-6	26	31	84	76	110	0.54	1.000
F56C9.12	39	43	91	82	111	1.81	0.200
skp-1	60	71	85	76	111	0.92	1.000
noah-2	29	34	85	76	112	0.82	1.000
T19A5.3	23	30	77	68	113	0.76	1.000
nas-37	24	28	86	76	113	0.81	1.000
bli-4	18	29	62	55	113	0.44	1.000
pas-7	80	86	93	82	113	5.12	0.025
psa-1	44	57	77	68	114	1.28	1.000
pas-1	28	36	78	68	114	1.09	1.000
bli-3	53	84	63	55	115	1.01	1.000
gei-16	94	98	96	82	117	9.87	0.010
noah-1	39	60	65	55	118	1.31	1.000
T01C3.1	61	67	91	76	120	3.63	0.100
K12H4.4	52	57	91	76	120	3.47	0.100
mua-6	12	13	92	76	121	1.51	1.000
emo-1	48	57	84	68	124	4.27	0.050
ZC13.3	33	39	85	68	124	3.50	0.100
pbs-5	59	69	86	65	132	7.20	0.010
nhr-41	29	38	76	55	139	4.59	0.050

Populations of GR1348 (*let-7(mg279) mir-84(tm1304) mgIs49[mlt-10p::gfp pest]*) animals were synchronized as L1 larvae, fed *E. coli* OP50 until the 4th larval stage, and then fed bacteria expressing dsRNA corresponding to the indicated genes. As a control, worms were fed bacteria not expressing dsRNA against a worm gene. Animals were examined for any detectable fluorescence approximately 18 hours later, when the majority of control animals expressed GFP. Values represent combined results from two independent experiments. Clones that yielded ≤ 80% normalized fluorescence with a *P*-value >0.001 were tested again (not shown). Only RNAi of *nhr-25* significantly suppressed ($P \leq 0.001$) both GFP expression and lethality in the additional test.

Table S2. Viability of *let-7(mg279) mir-84(tm1304)* mutant adults following inactivation of genes implicated in molting

RNAi	Viable (n)	Total (n)	% Viable	Chi-square	*P*-value
vector 1	6	117	5	N.A.	N.A.
gfp 1	2	137	1	2.78	0.100
vector 2	2	124	2	N.A.	N.A.
gfp 2	1	59	2	0.00	1.000
nhr-23	63	63	100	155.92	0.001
nhr-23	141	148	95	237.36	0.001
nhr-25	18	21	86	80.48	0.001
rpl-32	27	60	45	57.33	0.001
rpl-27	5	15	33	28.15	0.001
rpl-31	17	67	25	27.41	0.001
Y37D8A.21	19	81	23	25.43	0.001
rps-6	10	60	17	15.03	0.001
vha-16	14	89	16	14.86	0.001
rps-32	8	52	15	4.98	0.050
nsf-1	4	38	11	1.38	1.000
pas-1	4	39	10	6.25	0.025
vha-9	5	49	10	1.44	1.000
noah-1	5	53	9	1.12	1.000
F20G4.1	3	33	9	0.72	1.000
mua-6	1	11	9	0.31	1.000
pod-2	3	35	9	0.57	1.000
noah-2	3	35	9	0.57	1.000
bli-1	4	50	8	0.51	1.000
pan-1	4	51	8	0.47	1.000
lev-11	3	39	8	3.69	0.100
acn-1	3	43	7	0.20	1.000
F25H8.6	3	49	6	2.54	0.200
stc-1	5	82	6	0.09	1.000
T01C3.1	4	69	6	0.04	1.000
let-92	2	39	5	0.00	1.000
ntl-1	3	64	5	0.02	1.000
emo-1	2	43	5	1.26	1.000
C37C3.3	2	43	5	0.02	1.000
nas-36	3	66	5	0.03	1.000
W03F9.10	3	67	4	1.40	1.000
F53G12.4	2	45	4	0.03	1.000
T25B9.10	2	46	4	1.09	1.000
F49C12.12	1	23	4	0.02	1.000
alg-1	2	47	4	0.06	1.000
Y48B6A.3	2	49	4	0.08	1.000
C23G10.10	2	51	4	0.86	1.000
M03F8.3	2	52	4	0.13	1.000
K12H4.4	2	53	4	0.15	1.000
imb-3	2	59	3	0.59	1.000
Y51H1A.3	2	61	3	0.54	1.000
qua-1	3	93	3	0.46	1.000
mlt-11	2	65	3	0.42	1.000
bli-3	2	70	3	0.55	1.000
npp-20	1	37	3	0.19	1.000
D1054.15	3	111	3	0.88	1.000
mlt-10	1	38	3	0.41	1.000
F56C9.12	1	40	3	0.13	1.000
lin-66	1	40	3	0.13	1.000
T23F2.1	1	40	3	0.48	1.000
K04A8.6	2	82	2	0.18	1.000
Y65B4A.6	1	42	2	0.10	1.000
let-805	1	42	2	0.02	1.000
mlt-8	1	43	2	0.59	1.000
F40G9.1	1	43	2	0.03	1.000
crs-2	1	44	2	0.08	1.000
ZK430.8	1	44	2	0.63	1.000
adt-2	1	47	2	0.74	1.000
bli-5	1	48	2	0.78	1.000
mec-7	1	49	2	0.04	1.000
lir-1	1	49	2	0.82	1.000
psa-1	1	50	2	0.03	1.000
ifc-2	1	50	2	0.85	1.000

skp-1	1	62	2	1.33	1.000
vrs-2	1	66	2	0.00	1.000
lrp-1	1	109	1	0.22	1.000
pas-7	0	81	0	1.32	1.000
Y47D3B.1	0	53	0	0.86	1.000
est-1	0	57	0	0.93	1.000
gei-16	0	88	0	1.43	1.000
Y54E10BR.5	0	47	0	0.77	1.000
Y53F4B.22	0	71	0	1.16	1.000
ZC13.3	0	30	0	0.49	1.000
T19A5.3	0	27	0	0.44	1.000
R06A4.9	0	21	0	0.34	1.000
lrp-1	0	27	0	1.44	1.000
nhr-6	0	38	0	2.03	0.200
nhr-41	0	33	0	1.76	0.200
bli-4	0	36	0	1.92	0.200
dmd-5	0	39	0	2.08	0.200
rme-8	0	50	0	2.66	0.200
ran-4	0	41	0	2.19	0.200
F53B8.1	0	43	0	2.29	0.200
pbs-5	0	45	0	2.40	0.200
ptr-23	0	37	0	1.97	0.200
fbn-1	0	63	0	3.34	0.100
unc-52	0	50	0	2.66	0.200
F38A1.8	0	31	0	1.66	0.200
npp-6	0	32	0	1.71	0.200
kin-2	0	44	0	2.34	0.200
ptr-4	0	41	0	2.19	0.200
M03F4.7	0	40	0	2.13	0.200
C23F12.1	0	17	0	0.91	1.000
nas-37	0	24	0	1.29	1.000
mlt-9	0	10	0	0.54	1.000
vha-8	0	31	0	1.66	0.200

Viability of the identical animals described for Table S1 was assessed by visual inspection approximately 45 hours after transfer to *E. coli* expressing dsRNA. Values for particular gene inactivations represent the combined results from two independent experiments. In this table, values reported for vector 1 and vector 2 include the combined results from multiple independent samples reported separately in Table S1 as, respectively, vector 1, 2, 3, and 4, or vector 5 and 6. Note that RNAi of a very small number of these genes, including Y37D8A.21, did not reproducibly interfere with molting of larvae, and those particular genes were therefore not discussed in Frand et al., 2005.