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

Gabriel D. Hayes, Alison R. Frand and Gary Ruvkun*

The let-7 microRNA (miRNA) gene of Caenorhabditis elegans controls the timing of developmental events. let-7 is conserved throughout bilateral 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

miRNAs (miRNAs) constitute a large class of small (~22 nt) noncoding RNA species that regulate gene expression 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 cause the reiteration of cell division patterns appropriate for the last larval stage (Bettinger et al., 1996; Rougvie and Ambros, 1995). The let-7 and lin-4 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). 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-hydroxyecdysone, as well as DHR3 and BFTZ-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 BFTZ-F1 (Lam et al., 1997; White et al., 1997). Abrogation of the function of BFTZ-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 ecdyson (Bashirullah et al., 2003; Sempere et al., 2002; Sempere et al., 2003).
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, 2002; 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 (Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 2001) are related to mammalian ROR/RZR/RevErb and SF-1, respectively. We also 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′-AAGTTGACTGACATGACAACCCGAC3′ and GH32 5′-TTGACACAAAGGCAAGGCTGTG-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 pDH95.75 (A. Fire). The primers used were GH32, GH1107 5′-TATTCTACATCGCTGCTGCTGCTGCAGTCTGAC-3′, GH108 5′-CTCTAGTCGACCTGACCGACCCAGCAACAGGCAGAAGCCTGATAAGCTG-3′, and CAW32 5′-CGCTTACAGAAGCTGTGACCCG-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 integrate the transgene into a chromosome. *mgIs45* and *mgIs47* animals were outcrossed three more times to N2 before analysis. To generate *mgIs674*, 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-10::gfp-pest* and *nas-37::gfp-pest* fusion genes were previously described (Frand et al., 2005). To generate *mgIs49*, the *mlt-10::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::rfp* (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-25::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 pHFR4, 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

| Accession | Description |
|-----------|-------------|
| RG365     | him-1(e879) I; vels13[col-19]; gfp; rol-6(su1006) V |
| SP231     | mdp1(X;V) plasmid DNA |
| JR672     | wls54(scn::gfp) V |
| GR1395    | mgls49[mlt-10::gfp-pest; ttx-3::gfp] |
| GR1368    | mgEx556[nas-37::gfp-pest; pha-1(+)] |
| GR1425    | mgls46[mir-84++; tub-1::gfp]; wls54(scn::gfp) V |
| GR1426    | mgls45[mir-84++; tub-1::gfp]; mgls49[mlt-10::gfp-pest; ttx-3::gfp] IV; let-7(mg79) |
| GR1427    | mgls49[mlt-10::gfp-pest; ttx-3::gfp] IV; mir-84(tm1304) X |
| GR1428    | mgls49[mlt-10::gfp-pest; ttx-3::gfp] IV; let-7(mg79) mir-84(tm1304) X |
| GR1439    | mgIs47[mir-84++; tub-1::gfp]; let-7(mg79) |
| GR1440    | mgls47[mir-84++; tub-1::gfp] |
| GR1441    | mgls47[mir-84++; tub-1::gfp] |
| GR1442    | mgls49[mlt-10::gfp-pest; ttx-3::gfp] IV; let-7(mg79) mir-84(tm1304) X |
| GR1443    | mgEx565[nas-37::gfp-pest; pha-1(+)]; let-7(mg79) mir-84(tm1304) X |
| GR1444    | vels13[col-19]; gfp; rol-6(su1006) V; let-7(mg79) mir-84(tm1304) X |
| GR1445    | vels13[col-19]; gfp; rol-6(su1006) V; let-7(mg79) |
| GR1446    | mgls45[mir-84++; tub-1::gfp]; let-7(mg79) |
| GR1447    | mgls45(mir-84++; tub-1::gfp); lin-29(n333) sqt-1(sc13) II; mgls49[mlt-10::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(mg79) mir-84(tm1304) |
| GR1451    | mgEx728[nhr-23::gfp; rol-6(su1006)]; let-7(mg79) mir-84(tm1304) |

### Microscopy

Images were captured on a Zeiss Axiosplan 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-26, we cloned *C. elegans* genomic DNA corresponding to nucleotides 3760 to 4098 of cosmid F02E9 (Accession number: embZ81494) 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,
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.
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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 mgls47[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 mgls47 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 mltr-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 mltr-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 mltr-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 mltr-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 mgls47 transgene restored repression of the mltr-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 mltr-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 mltr-10 and nas-37 as targets of the heterochronic pathway, consistent with our previous report that mltr-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 mltr-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. 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.
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’ H11032 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’ H11032 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...
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≤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 mglS45[mir-84++; lin-29(n333); mglS49[mlt-10p::gfp-pest]] animals to segregants from an mglS45 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 mglS45 expressed an extra pulse of GFP (P=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 mglS45 molted, indicated by shedding of the cuticle (P=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 miRNAs, 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 mglEx674[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...
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 synergetic 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  | 15.9±0.6       | 15.9±0.5     | 15.9±0.9                   |
| Young adult | 16.1±0.2 | 16.1±0.4       | 19.2±3.0     | 22.4±2.7                   |

The number of seam cell nuclei observed in wls54[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).
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<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 mgs45 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 mgs47, which drives a lower level of mir-84 expression than mgs45 (Fig. 1D), failed to suppress let-7(mn112) (data not shown). However, the mgs47 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 mgs45 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
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 let-7 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 let-7 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 mlr-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. 9. Overexpression of mir-84 rescues a null allele of let-7.](image)

Graph shows the percent of let-7(mn112) animals that survived and produced progeny in the presence or absence of mgl45[mir-84++]. We compared GR1426 to animals derived from SP231 that displayed uncoordinated movement.

![Fig. 10. A genetic model for the cessation of molting.](image)

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
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