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The expression of the Alzheimer's *Amyloid Precursor Protein-like* gene is regulated by developmental timing microRNAs and their targets in *Caenorhabditis elegans*

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by the accumulation of dense plaques in the brain, resulting in progressive dementia. A major plaque component is the β-amyloid peptide, which is a cleavage product of the amyloid precursor protein (APP). Studies of dominant inheritable familial AD support the hypothesis that APP is critical for AD development. On the other hand, the pathogenesis of amyloid plaque deposition in AD is thought to be the result of age-related changes with unknown mechanisms. Here we show that the *Caenorhabditis elegans* homolog of *APP*, *APP-like-1* (*apl-1*), functions with and is under the control of molecules regulating developmental progression. In *C. elegans*, the timing of cell fate determination is controlled by the heterochronic genes, including *let-7* microRNAs. *C. elegans apl-1* shows significant genetic interactions with *let-7* family microRNAs and *let-7*-targeted heterochronic genes, *hbl-1*, *lin-41* and *lin-42*. *apl-1* expression is upregulated during the last larval stage in hypodermal seam cells which is transcriptionally regulated by *hbl-1*, *lin-41* and *lin-42*. Moreover, the levels of the *apl-1* transcription are modulated by the activity of *let-7* family microRNAs. Our works places *apl-1* in a developmental timing pathway and may provide new insights into the time-dependent progression of AD.

Keywords

Alzheimer's disease; Amyloid precursor protein; *apl-1*; *Caenorhabditis elegans*; developmental timing; heterochronic genes; *let-7*; microRNA; molting

Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by the accumulation of dense plaques and neurofibrillary tangles in the brain, resulting in progressive dementia. A major plaque component is the β-amyloid peptide (Aβ), which is a cleavage product of the

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amyloid precursor protein (APP). Studies of dominant inheritable familial AD support the hypothesis that APP is critical for AD development (Hardy and Selkoe, 2002). On the other hand, while the pathogenesis of amyloid plaque deposition in AD is thought to be the result of age-related changes (Goedert and Spillantini, 2006), chronological aspects of amyloid accumulation are largely unknown.

The best understood genes controlling timing during development are the heterochronic genes of the nematode *Caenorhabditis elegans*. Heterochronic genes control the appropriate temporal execution of stage-specific programs of cell division and differentiation through the four larval stages, L1–L4, of *C. elegans* (Ambros and Horvitz, 1984; Ambros, 2000; Banerjee and Slack, 2002; Rougvie, 2005). For example, one of the heterochronic genes, *let-7*, is expressed from the late larval stages and promotes the transition from L4 to adult (Reinhart et al., 2000). *Let-7* encodes a microRNA (miRNA) and binds with imperfect complementarity to the 3′UTR of its targets, such as *lin-41* and *hbl-1*, to prevent their translation (Slack et al., 2000; Abrahante et al., 2003; Lin et al., 2003; Rougvie, 2005).

Here we report that the *C. elegans APP-like-1* (*apl-1*) gene functions with and is under the control of the heterochronic genes, including the miRNA *let-7*. *apl-1* shows significant genetic interactions with *let-7* family miRNAs and their developmental timing target genes including *hbl-1*, *lin-41* and *lin-42*. Moreover, *apl-1* expression shows a temporal change in hypodermal cells that is controlled by these developmental timing regulators. To our knowledge, this study provides the first indication that *APP*-related genes could be under the control of molecules regulating timed events.

Materials and Methods

Nematode strains and culture

C. elegans strains were grown under standard conditions. Strains were grown at 20°C for all experiments in this study. The mutant strains used were as follows: wild-type N2 Bristol, *hbl-1 (ve18)* (Abrahante et al., 2003), *hbl-1(mg285)* (Lin et al., 2003), *lin-29(n546)* (Ambros and Horvitz, 1984), *lin-41(ma104)* (Slack et al., 2000), *let-7(n2853)* (Reinhart et al., 2000), *mir-48 (n4097);mir-84(n4037)* (Abbott et al., 2005). *YnIs79* is a strain overproducing *apl-1* (Hornsten et al., 2007). To visualize seam cell junctions, we utilized an integrated array (*syIs78*) carrying *ajm-1::gfp* (MH27/GFP). To visualize both nuclei and junctions of seam cells, another integrated strain *wIs79* containing both *ajm-1::gfp* and *scm-1::gfp* was used. *MaIs105*, a strain expressing the adult stage specific transgene *col-19::gfp* (Abrahante et al., 1998; Abbott et al., 2005), was kindly provided by C. Hammel and V. Ambros.

The *apl-1::gfp::unc-54* reporter constructs were generated via single end overlap extension PCR, fusing the 7.0 kb *apl-1* promoter sequence and *gfp* from vector pPD95.70 (A. Fire). We used animals carrying integrated *apl-1::gfp* constructs to observe *apl-1* expression for all experiments described in this manuscript. The details of the constructions are described in Supplemental Materials.

RNAi experiments

Gene knockdown was achieved through RNAi by feeding as described (Timmons and Fire, 1998; Fraser et al., 2000; Kamath et al., 2003). Except for the experiment to obtain the data shown in Figs. 1E and 1F, synchronized populations of L1 larvae were fed bacteria expressing dsRNA corresponding to the target genes. In the experiment shown in Figs. 1E and 1F, synchronized L1 larvae of *mir-48(n4097);mir-84(n4037)* were grown on NGM plates containing *E. coli* OP50 bacterial lawns until 36-hours after hatching at 20°C. Then, these early L4 animals were put on RNAi plates. In mock RNAi experiments, bacteria carrying a control

empty vector were used. RNAi vectors used in this study are described in Supplemental Materials.

Observation of worms

Staging of L4 animals was by relative positions of their gonadal distal tip cells to the vulva: early, mid and late L4 animals were defined as animals showing 0–1/4, 1/4–1/2 and >1/2 gonadal turns, respectively. Microscopy images were acquired using a Axioplan II microscope (Carl Zeiss) equipped with a AxioCam MRm CCD camera (Carl Zeiss). We used Image J software (Abramoff et al., 2004) to quantify a mean level of GFP signals inside each nucleus of a seam cells. To observe GFP signals in seam cells of the *apl-1::gfp* transgenic lines, we observed all seam cells except the few cells surrounding the head and pharyngeal regions, because the non-seam expression of *apl-1::gfp* in these regions is strong (Supplemental Fig. S3) and interferes with evaluation of the seam cell GFP expression. Therefore, “GFP in all seam cells” described in Figs. 3 and 4 means the *apl-1::gfp* animals showing GFP expression in all seam cells except the cells in the head region.

Miscellaneous

Methods of reverse transcription-PCR and X-gal staining are described in Supplemental Materials.

Results

Loss of apl-1 function partially suppresses phenotypes associated with loss of let-7 family miRNAs

Loss-of-function (lf) mutants of *let-7* reiterate earlier seam cell fates and die by bursting through the vulva at adults (Figs. 1A, C) (Reinhart et al., 2000). The *let-7* mutant phenotype appears to be caused by over-expression of several target genes, as knocking down expression of *let-7* targets partially suppresses the *let-7* bursting phenotype (Reinhart et al., 2000; Slack et al., 2000; Abrahante et al., 2003; Lin et al., 2003; Grosshans et al., 2005; Lall et al., 2006). During the course of an RNA interference (RNAi)-based screen to identify suppressors of *let-7*, we found that the *let-7* vulval bursting phenotype was suppressed by postembryonic reduction of the *C. elegans APP-like* gene, *apl-1* (Daigle and Li, 1993) (Figs. 1B, C). This suppression is specific to *apl-1*, as RNAi constructs targeting different regions of the *apl-1* gene suppressed *let-7* bursting, but mock RNAi did not (Fig. 1C and Supplemental Fig. S1A). *let-7(lf)* causes retarded development of specialized hypodermal cells called seam cells, which results in additional seam cell divisions and a defect in secretion of adult-specific cuticular structures (alae) (Reinhart et al., 2000). As shown in Table 1, *apl-1(RNAi)* partially rescued these seam cell defects in young *let-7(lf)* adults.

This suppression was not just a consequence of not having a normal vulva through which to burst, as the vulval development in the *apl-1(RNAi)* animals appears normal (data not shown). *Apl-1(RNAi)* adult animals were small (Supplemental Fig. S2B), but the suppression of the *let-7* bursting phenotype by *apl-1(RNAi)* was not caused by the smaller body size, as the *let-7* bursting phenotype was not suppressed by *sma-1(RNAi)* or *dpy-7(RNAi)* (83%, n = 408, and 89%, n = 441, showing the bursting phenotype, respectively), which also caused smaller body shape (Johnstone et al., 1992; McKeown et al., 1998). In addition, we did not observe a significant suppression of the *let-7* bursting phenotype by RNAi against *sel-12*, the *C. elegans* homolog of *presenilin* (Levitan and Greenwald, 1995), and *feh-1*, the *C. elegans* homolog of *Fe65* (Zambrano et al., 2002) (92%, n = 312 and 94%, n = 253 showing the bursting phenotype, respectively). This demonstrates the specificity of the *let-7* suppression by *apl-1*, and suggests that the suppression might not be mediated by the presenilin-dependent proteolytic processing of *apl-1* or the *Fe65*-dependent intracellular regulation of *apl-1*.

It should be noted that postembryonic RNAi of *apl-1* by feeding synchronized L1 wild-type animals exhibited a ~40% reduction of *apl-1* mRNA (Supplemental Fig. S2A) and therefore created a partial loss of *apl-1* function, whereas complete or strong loss of *apl-1* function caused early larval lethality and a molting defect (Zambrano et al., 2002; Hornsten et al., 2007). Since the early lethality in strong *apl-1* mutants hampers an examination of *apl-1* function after the first molt, this partial loss of *apl-1* function by RNAi proved to be crucial to identifying novel, non-lethal roles for *apl-1*.

Two other *let-7*-related miRNAs, *mir-48* and *mir-84*, are similar in sequence (Lim et al., 2003). A *mir-48(lf);mir-84(lf)* mutant displays a penetrant extra molting phenotype that results in a double cuticle in the adult (Figs. 1D, F) (Abbott et al., 2005; Hayes et al., 2006). *Apl-1* RNAi suppressed the molting phenotype in the *mir-48(lf);mir-84(lf)* mutant (Figs. 1E, F). Interestingly, *apl-1(RNAi)* suppressed the cuticle defect when applied in either the first (L1) or fourth (L4) larval stage (Fig. 1F). These results suggest that phenotypes caused by loss of *let-7* family miRNAs are at least partially due to excessive activity of *apl-1* in the late larval and/or adult stages.

apl-1 itself may not be a canonical heterochronic gene

While *apl-1(RNAi)* greatly suppressed the *let-7* mutant phenotypes, *apl-1(RNAi)* animals alone exhibited no obvious heterochronic phenotype on alae formation (Supplemental Fig. S2C) and seam cell fusion (Supplemental Fig. S2D). We did not find any other major abnormality up to the L4 stage when the animals took on a small appearance (Supplemental Fig. S2B). In addition to the small body size of the *apl-1(RNAi)* adults described above, we observed that a small percentage of *apl-1(RNAi)* animals failed to shed their larval cuticle at the L4 molt (Fig. 2B and Supplemental Fig. S1B). In addition, an *apl-1* overexpressor strain *ynIs79* (Hornsten et al., 2007) did not show any heterochronic defect in alae formation either (0% of L4 animals showing any precocious alae, n = 37; 99% of adult animals showing complete alae, n = 65), suggesting that *apl-1* itself should not be classified as a canonical heterochoronic gene.

apl-1 genetically interacts with hbl-1, lin-41 and lin-42, which are downstream of let-7

We examined whether *apl-1* also acts with other heterochronic genes that are epistatic to *let-7*, including *hbl-1*, *lin-41*, and *lin-42*, which promote larval development and prevent precocious adult differentiation (Rougvie, 2005). Indeed, *apl-1(RNAi);hbl-1(lf)*, *apl-1 (RNAi);lin-41(lf)* and *apl-l(RNAi);lin-42(lf)* double mutants showed a growth defect during the L4 stage and typically could not shed their larval cuticle at the L4 molt (Figs. 2A, B, and Supplemental Fig. S1B), while each of the single mutants only rarely showed the phenotype (Fig. 2B and Supplemental Fig. S1B). Besides the synthetic effect of *apl-1* and the heterochronic genes on molting, *apl-1(lf)* did not appear to modify the penetrance of precocious seam cell differentiation in these mutants (Supplemental Figs. S2D–F). This suggests that *apl-1* acts with these heterochronic genes specifically in the regulation of molting at the larval-to-adult switch or that the partial loss of *apl-1* function was not sufficient to overcome these cell fate defects.

In contrast, *apl-1* did not genetically interact with *lin-29*, one known gene downstream of *hbl-1*, *lin-41* and *lin-42* in adult seam cell differentiation (Ambros and Horvitz, 1984; Ambros, 2000; Rougvie, 2005). *Apl-1(RNAi)* did not modify retarded alae formation of the *lin-29* mutant (0% alae formation of *lin-29* mutants on either mock RNAi (n = 23) or *apl-1* RNAi (n = 27). The extra molting phenotype of *lin-29* (Ambros and Horvitz, 1984) was also not affected by *apl-1* RNAi (data not shown).

apl-1 expression is temporally regulated in seam cells

The spatial and temporal expression pattern of *apl-1* was evaluated by following the expression of an *apl-1* promoter-driven *gfp* construct fused with the heterologous *unc-54* 3′UTR (*apl-1::gfp::unc-54*). *Apl-1* was expressed in several types of cells throughout development (Supplemental Figs. S3A, B), which is consistent with data from *in situ* RNA hybridization (Supplemental Figs. S3C–E) and a previously reported GFP-reporter assay with a shorter *apl-1* promoter (Hornsten et al., 2007). Notably, we observed strong GFP expression in seam cells (Fig. 3B), whose development is well known to be regulated by the heterochronic genes *let-7*, *hbl-1*, *lin-41* and *lin-42* (Rougvie, 2005) and which have an essential role in the *C. elegans* molting process (Frand et al., 2005). Furthermore, *apl-1* expression in seam cells was temporally regulated (Figs. 3A–C). The GFP signal in seam cells was not detected (or in rare cases, weakly detected) from L1 to early L4 stages (Figs. 3A, C), frequently detected from the mid L4 stage, and almost always detected with high-level expression at late L4 and adult stages (Figs. 3B, C). This temporal expression profile of *apl-1* was observed in two independent integrated lines of *apl-1::gfp::unc-54* (Supplemental Fig. S4). No temporal change of *apl-1* expression was apparent in any other cells (data not shown).

apl-1 expression in seam cells is indirectly repressed by let-7 family miRNAs

One of the simplest interpretations of available genetic data is that the heterochronic genes that genetically interact with *apl-1* regulate *apl-1* expression in seam cells. We first examined whether *apl-1* was a direct target of *let-7* family miRNAs. Although weak potential binding sites for *let-7* family miRNAs were predicted in the *apl-1* 3′UTR (Supplemental Fig. S5) (Enright et al., 2003; John et al., 2004; Lall et al., 2006), we did not observe any difference in the temporal expression profile between *apl-1::gfp* constructs fused with either the native *apl-1* 3′UTR or the unregulated *unc-54* 3′UTR in seam cells (Fig. 3C). Additionally, a *lacZ* reporter expressed in seam cells detected no significant temporal down-regulation through the *apl-1* 3′UTR, as 35% (n = 1,421) and 29% (n = 1,344) of animals showing X-gal staining at L3 animals and young adults carrying *col-10::lacZ* constructs, respectively. These results suggest that *apl-1* is not likely to be a direct target of the *let-7* family miRNAs in seam cells.

Rather, we found that *apl-1* was transcriptionally up-regulated in hypodermal cells of *let-7* family mutants. *Apl-1::gfp::unc-54* GFP signals in seam cells, as well as other hypodermal cells (hyp7), were significantly elevated in the background of *let-7* family mutants (Figs. 3E–G) as compared to a wild type background (Figs. 3D, G), whereas timing of expression was not altered (Fig. 3C). Specifically, the level of GFP signal was elevated in the adult stage in the *let-7(lf)* background, while *mir-48(lf);mir-84(lf)* showed an elevation both in the late L4 and adult stages (Fig. 3G). This trend is consistent with the difference in expression profiles between *let-7* miRNA and miR-48; miR-84. Transcription of *let-7* miRNA begins in the L3 stage and is progressively upregulated during the L4 stage, while miR-48 and miR-84 reach half-maximal expression at the L3 stage (Abbott et al., 2005; Esquela-Kerscher et al., 2005). The effect of the *let-7* family miRNAs was specific for the *apl-1* promoter, as a GFP signal from a seam cell marker promoter (*scm-1::gfp*) was not elevated in either *let-7(lf)* or *mir-48(lf);mir-84(lf)* backgrounds (data not shown). These results lead us to hypothesize that the *apl-1* transcription in seam cells is regulated indirectly by *let-7* family miRNAs, perhaps through downstream targets of the *let-7* miRNAs.

apl-1 transcription is regulated by hbl-1, lin-41 and lin-42

The heterochronic regulators *hbl-1*, *lin-41* and *lin-42* are downstream of *let-7* (Rougvie, 2005). We found that *apl-1* expression was precociously observed at the L3 molt in *hbl-1*, *lin-41* and *lin-42* mutant seam cells (Figs. 4A–C, E), suggesting that these heterochronic genes normally repress the precocious expression of *apl-1* during the L4 stage. In addition, *hbl-1*, *lin-41* and *lin-42* also play roles in temporally maintaining *apl-1* expression later in

development, as the number of animals showing correct seam cell expression in the late L4 stage was significantly reduced in *hbl-1*, *lin-41* or *lin-42* mutants (Figs. 4D, E; data not shown). These temporal expression changes of *apl-1* by the heterochronic genes was observed in two independent integrated lines of *apl-1::gfp::unc-54* (Supplemental Fig. S4C). By contrast, the temporal expression of *apl-1* in seam cells was independent of *lin-29* (Fig. 4E), which is downstream of *hbl-1*, *lin-41* and *lin-42* in adult seam cell differentiation (Ambros, 2000; Rougvie, 2005). These results show that developmental timing regulators *hbl-1*, *lin-41* and *lin-42* control *apl-1* expression negatively in early larval stages and positively in the late L4 stage (Supplemental Fig. S6), consistent with the known, dual role of HBL-1 in seam cell development (Lin et al., 2003).

Discussion

The heterochronic pathway regulates the temporal expression of apl-1 in seam cells

Hornsten et al. recently reported that *apl-1* plays an essential role in *C. elegans* early larval development, including molting and morphogenesis (Hornsten et al., 2007). Along with the early function of *apl-1* in neuronal cells, our study shows a role for *apl-1* in hypodermal cells in later development, namely the transition from L4 to adult stages (also see Supplemental Fig. S6). We demonstrate that the heterochronic pathway temporally regulates *apl-1* expression in seam cells and propose that this modulates their developmental timing, although our data indicate that *apl-1* itself may not be a canonical heterochronic gene.

It is noteworthy that *apl-1* is the first identified gene that is transcriptionally regulated by *let-7* targets, including *hbl-1* and *lin-42* that encode orthologs of *Drosophila* transcription factors Hunchback and Period, respectively. While *lin-29* is a well known gene downstream of *let-7* and the *let-7* targets and plays an essential roles in promoting adult programs (Ambros, 2000; Rougvie, 2005), it is unlikely that *lin-29* is directly controlled by the *let-7* target transcription factors because the *lin-29* transcript is detected from the L1 stage onwards (Rougvie and Ambros, 1995). Therefore, an elucidation of the molecular mechanisms of the *apl-1* expression in seam cells provides a foothold to understand the transcriptional regulation of *let-7*-dependent terminal differentiation pathway in *C. elegans*.

Possible function of apl-1 in vivo

apl-1(RNAi) suppresses the extra cell division in seam cells and retarded alae formation in the *let-7* adult. Therefore, the down-regulation of the *apl-1* expreession is critical for promoting the adult program in seam cells. However, the actual physiological function of *apl-1* for adult determination is not revealed from our study nor from previous studies (Zambrano et al., 2002; Hornsten et al., 2007). In mammals, it has been speculated that APP has role in cell proliferation and differentiation. For example, mammalian APP has been proposed to have a receptor-like function on the cell surface (Gralle and Ferreira, 2007). Indeed, some extracellular APP ligands, such as F-spondin and the low-density lipoprotein receptor-related protein, have been identified, _olstering the hypothesis that APP might function as a receptor (Schmitz et al., 2002; Gralle and Ferreira, 2007). An intracellular signaling pathway of APP has also been proposed, especially in axonal outgrowth and vesicle transport in neuronal cells (Gralle and Ferreira, 2007; Grimm et al., 2007). In addition to the receptor-like function, the secretory N-terminal ectodomain of APP (sAPP), which is the product cleaved by secretases, is also proposed to be involved in different physiological processes in mammals. For example, sAPP can promote cell proliferation in several types of cells including epithelial cells (Schmitz et al., 2002; Grimm et al., 2007), which is reminiscent of our genetic data showing that *apl-1* is involved in *let-7*-dependent cell division in seam cells. Specifically, an extracellular domain of APL-1 is sufficient to rescue the *apl-1* null mutant lethality (Hornsten et al., 2007). Therefore, it can be speculated that *C. elegans* APL-1 could also serve as a receptor receiving

a ligand to regulate seam cell development and molting, or sAPL-1 could inhibit the proliferation and differentiation in seam cells. On the other hand, it is unlikely that seam cells produce the secretary APL-1 during development, as *sel-12(RNAi)* does not suppress the *let-7* bursting phenotype and *sel-12* expression of seam cells has not been reported. Further genetic characteristics of *apl-1* and the biochemical properties of APL-1 proteins will need to be investigated.

Our data show that *apl-1* has an important function in *C. elegans* molting. While a genome-wide RNAi-based screen has recently been performed to identify endocrine and enzymatic regulators of molting in *C. elegans* (Frand et al., 2005), *apl-1* was not identified there. The reason might be because the *apl-1(RNAi)* itself does not create a strong molting phenotype, and the phenotype is only highlighted when *apl-1(RNAi)* is combined with heterochoronic mutations, such *mir-48;mir-84* and *hbl-1*. The *let-7* mutants and some double mutants of *let-7* family miRNAs are well known to show an extra molting phenotype in the adult stage (Reinhart et al., 2000; Abbott et al., 2005), indicating that *let-7* family miRNAs repress some of the molting machinery in adult. Specifically a recent paper shows that *let-7* family miRNAs repress *nhr-23* and *nhr-25*, both of which encode nuclear hormone receptors essential for *C. elegans* molting (Hayes et al., 2006). *Apl-1* is an additional example of *let-7*-dependent repression in adults required for the molting process. It would be intriguing to examine molecular relationships among *apl-1* and other identified molting genes.

Implication for Alzheimer's disease

Our study provides the first evidence that *APP*-related genes can be under the transcriptional control of molecules regulating developmentally timed events. Besides the importance of the proteolytic regulation of APP to yield Aβ, it has been speculated that regulation of *APP* expression is associated with the development of AD, specifically supported by recent evidence that *APP* locus duplication is found in families with inheritable familial AD (Rovelet-Lecrux et al., 2006). In conjunction with the fact that heterochronic genes are involved in the aging process (Boehm and Slack, 2005) and the strong conservation of many *C. elegans* heterochronic genes in vertebrates (Rougvie, 2005), this study may provide new insights into the time-dependent progression of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, Horvitz HR, Ambros V. The *let-7* MicroRNA family members *mir-48*, *mir-84*, and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*. Dev Cell 2005;9:403–414. [PubMed: 16139228]

Abrahante JE, Daul AL, Li M, Volk ML, Tennessen JM, Miller EA, Rougvie AE. The *Caenorhabditis elegans hunchback*-like gene *lin-57/hbl-1* controls developmental time and is regulated by microRNAs. Dev Cell 2003;4:625–637. [PubMed: 12737799]

Abrahante JE, Miller EA, Rougvie AE. Identification of heterochronic mutants in Caenorhabditis elegans. Temporal misexpression of a collagen:green fluorescent protein fusion gene. Genetics 1998;149:1335–1351. [PubMed: 9649524]

Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. Biophotonics International 2004;11:36–42.

Ambros V. Control of developmental timing in *Caenorhabditis elegans*. Curr Opin Genet Dev 2000;10:428–433. [PubMed: 10889059]

Ambros V, Horvitz HR. Heterochronic mutants of the nematode *Caenorhabditis elegans*. Science 1984;226:409–416. [PubMed: 6494891]

Banerjee D, Slack F. Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. Bioessays 2002;24:119–129. [PubMed: 11835276]

Boehm M, Slack F. A developmental timing microRNA and its target regulate life span in *C. elegans*. Science 2005;310:1954–1957. [PubMed: 16373574]

Daigle I, Li C. *apl-1*, a *Caenorhabditis elegans* gene encoding a protein related to the human beta-amyloid protein precursor. Proc Natl Acad Sci USA 1993;90:12045–12049. [PubMed: 8265668]

Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in *Drosophila*. Genome Biol 2003;5:R1. [PubMed: 14709173]

Esquela-Kerscher A, Johnson SM, Bai L, Saito K, Partridge J, Reinert KL, Slack FJ. Post-embryonic expression of *C. elegans* microRNAs belonging to the *lin-4* and *let-7* families in the hypodermis and the reproductive system. Dev Dyn 2005;234:868–877. [PubMed: 16217741]

Frand AR, Russel S, Ruvkun G. Functional genomic analysis of *C. elegans* molting. PLoS Biol 2005;3:e312. [PubMed: 16122351]

Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature 2000;408:325–330. [PubMed: 11099033]

Goedert M, Spillantini MG. A century of Alzheimer's disease. Science 2006;314:777–781. [PubMed: 17082447]

Gralle M, Ferreira ST. Structure and functions of the human amyloid precursor protein: the whole is more than the sum of its parts. Prog Neurobiol 2007;82:11–32. [PubMed: 17428603]

Grimm MO, Grimm HS, Hartmann T. Amyloid beta as a regulator of lipid homeostasis. Trends Mol Med 2007;13:337–344. [PubMed: 17644432]

Grosshans H, Johnson T, Reinert KL, Gerstein M, Slack FJ. The temporal patterning microRNA *let-7* regulates several transcription factors at the larval to adult transition in *C. elegans*. Dev Cell 2005;8:321–330. [PubMed: 15737928]

Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297:353–356. [PubMed: 12130773]

Hayes GD, Frand AR, Ruvkun G. 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. Development 2006;133:4631–4641. [PubMed: 17065234]

Hornsten A, Lieberthal J, Fadia S, Malins R, Ha L, Xu X, Daigle I, Markowitz M, O'Connor G, Plasterk R, Li C. APL-1, a *Caenorhabditis elegans* protein related to the human beta-amyloid precursor protein, is essential for viability. Proc Natl Acad Sci USA 2007;104:1971–1976. [PubMed: 17267616]

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol 2004;2:e363. [PubMed: 15502875]

Johnstone IL, Shafi Y, Barry JD. Molecular analysis of mutations in the *Caenorhabditis elegans* collagen gene *dpy-7*. EMBO J 1992;11:3857–3863. [PubMed: 1396579]

Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 2003;421:231–237. [PubMed: 12529635]

Lall S, Grun D, Krek A, Chen K, Wang YL, Dewey CN, Sood P, Colombo T, Bray N, Macmenamin P, Kao HL, Gunsalus KC, Pachter L, Piano F, Rajewsky N. A genome-wide map of conserved microRNA targets in *C. elegans*. Curr Biol 2006;16:460–471. [PubMed: 16458514]

Levitan D, Greenwald I. Facilitation of *lin-12*-mediated signalling by *sel-12*, a *Caenorhabditis elegans* S182 Alzheimer's disease gene. Nature 1995;377:351–354. [PubMed: 7566091]

Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP. The microRNAs of *Caenorhabditis elegans*. Genes Dev 2003;17:991–1008. [PubMed: 12672692]

Lin SY, Johnson SM, Abraham M, Vella MC, Pasquinelli A, Gamberi C, Gottlieb E, Slack FJ. The *C. elegans hunchback* homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. Dev Cell 2003;4:639–650. [PubMed: 12737800]

McKeown C, Praitis V, Austin J. *sma-1* encodes a betaH-spectrin homolog required for *Caenorhabditis elegans* morphogenesis. Development 1998;125:2087–2098. [PubMed: 9570773]

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 2000;403:901–906. [PubMed: 10706289]

Rougvie AE. Intrinsic and extrinsic regulators of developmental timing: from miRNAs to nutritional cues. Development 2005;132:3787–3798. [PubMed: 16100088]

Rougviea E, Ambros V. The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *Caenorhabditis elegans*. Development 1995;121:2491–2500. [PubMed: 7671813]

Rovelet-Lecrux A, Hannequin D, Raux G, Le Meur N, Laquerriere A, Vital A, Dumanchin C, Feuillette S, Brice A, Vercelletto M, Dubas F, Frebourg T, Campion D. *APP* locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. Nat Genet 2006;38:24–26. [PubMed: 16369530]

Schmitz A, Tikkanen R, Kirfel G, Herzog V. The biological role of the Alzheimer amyloid precursor protein in epithelial cells. Histochem Cell Biol 2002;117:171–180. [PubMed: 11935293]

Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G. The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the LIN-29 transcription factor. Mol Cell 2000;5:659–669. [PubMed: 10882102]

Timmons L, Fire A. Specific interference by ingested dsRNA. Nature 1998;395:854. [PubMed: 9804418]

Zambrano N, Bimonte M, Arbucci S, Gianni D, Russo T, Bazzicalupo P. *feh-1* and *apl-1*, the *Caenorhabditis elegans* orthologues of mammalian Fe65 and beta-amyloid precursor protein genes, are involved in the same pathway that controls nematode pharyngeal pumping. J Cell Sci 2002;115:1411–1422. [PubMed: 11896189]

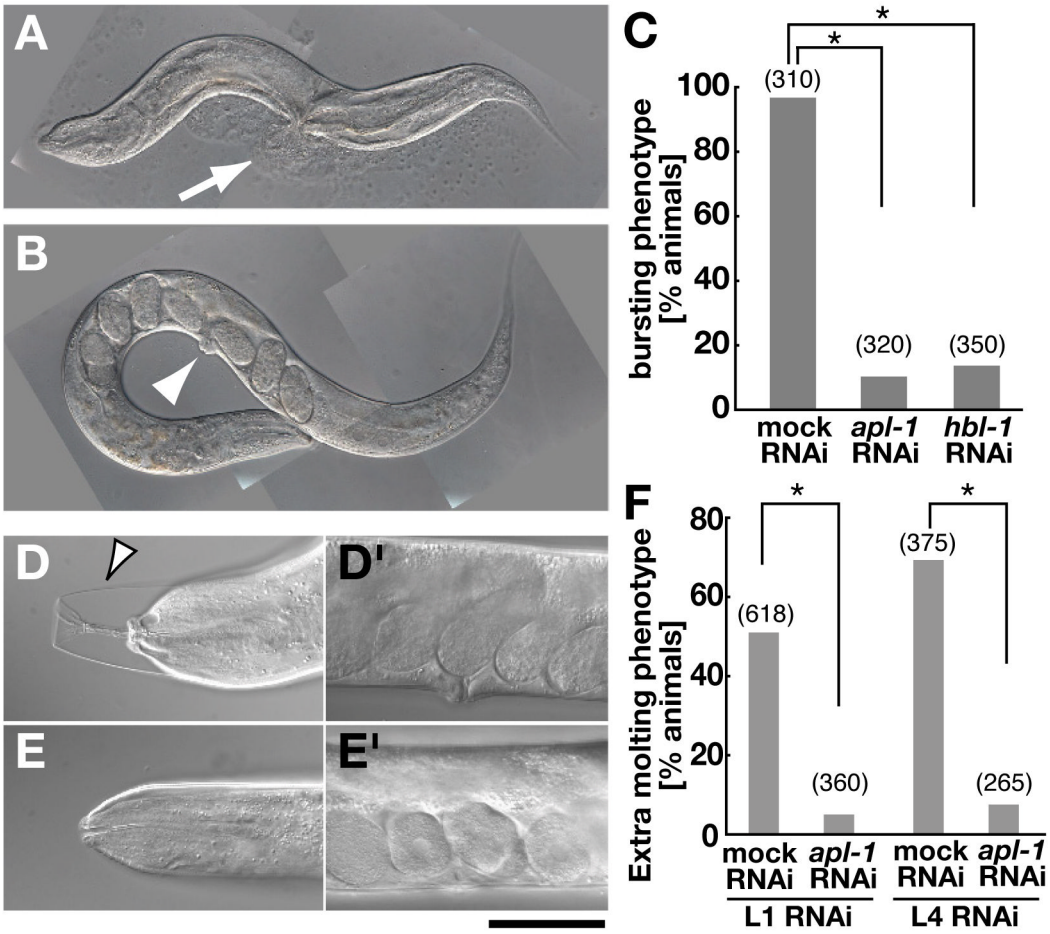

Fig. 1.

Loss of *apl-1* function suppresses phenotypes of *let-7* family member mutants. (A) *let-7 (n2853)* mutants on mock RNAi died at the L4 molt by bursting though the vulva (arrow) at the restrictive temperature (20 C). (B) In contrast, *let-7(n2853)* mutants on *apl-1* RNAi survived into adulthood, as indicated by the presence of oocytes and a functional vulva (arrowhead). (C) Percentage of *let-7(n2853)* animals with the bursting vulval phenotype. The vulval bursting phenotype of *let-7(n2853)* were scored at 6- to 12-hours post-L4 molt. *Hbl-1 (RNAi)* was used as a positive control. (D, E) DIC images of *mir-48(n4097);mir-84(n4037)* with embryos visible inside the animals (D′, E′) to show that the animals are in the adult stage. (D) Unshed cuticle surrounding the anterior region of the animal (arrowhead) was observed on mock RNAi. (E) The cuticle phenotype of *mir-48(n4097);mir-84(n4037)* was suppressed treating with *apl-1* RNAi from the L4 stage. (F) Percentage of *mir-48(n4097);mir-84 (n4037)* exhibiting the extra molting phenotype in adults. Synchronized populations of L1 or L4 larvae were used (see Materials and Methods).

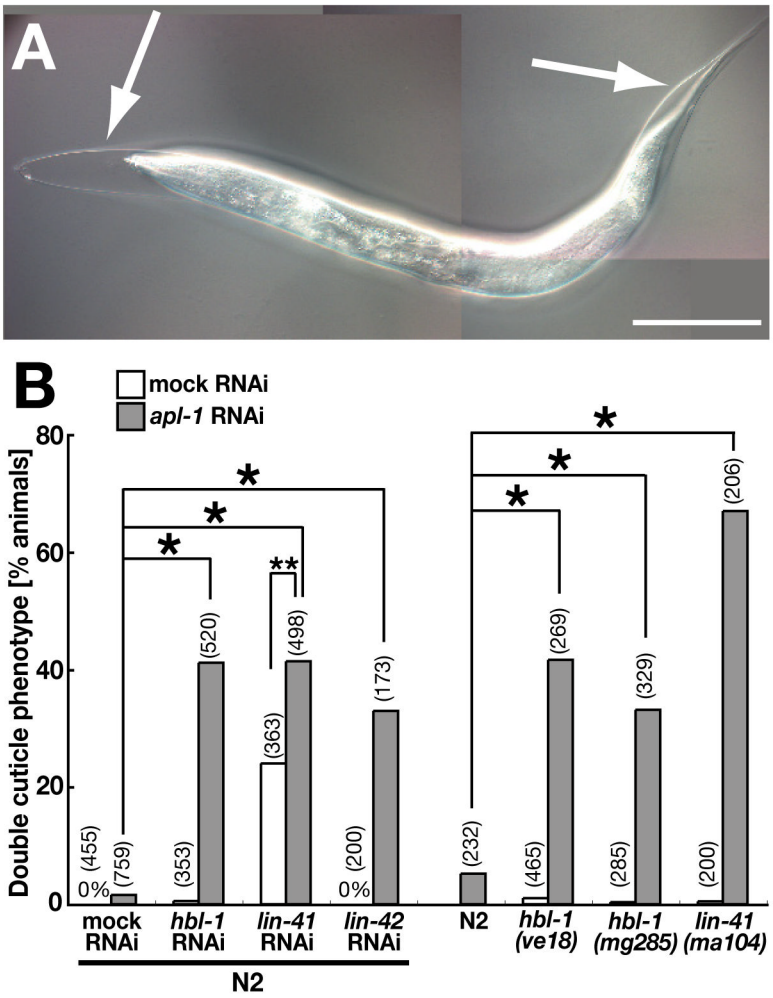

Fig. 2.

apl-1 genetically interacts with heterochronic genes downstream of *let-7* in the regulation of molting. (A) N2 larvae were fed both *apl-1* and *hbl-1* RNAi bacteria from the L1 stage. Unshed cuticle was observed at the L4 molt (arrows). (B) Quantification of the molting defect of double mutants of *apl-1* and other heterochronic mutants at the L4 molt. Synchronized L1 animals were used for the RNAi experiments. Data shown in the left half of the graph were obtained using two different RNAi clones. Each number in parentheses indicates *n* of observed animals in each sample. $*p < 0.001$ and $**p < 0.01$ chi-square test. Scale bar: 100 μm for A and B, 50 μm for D and E, and 117 μm for G.

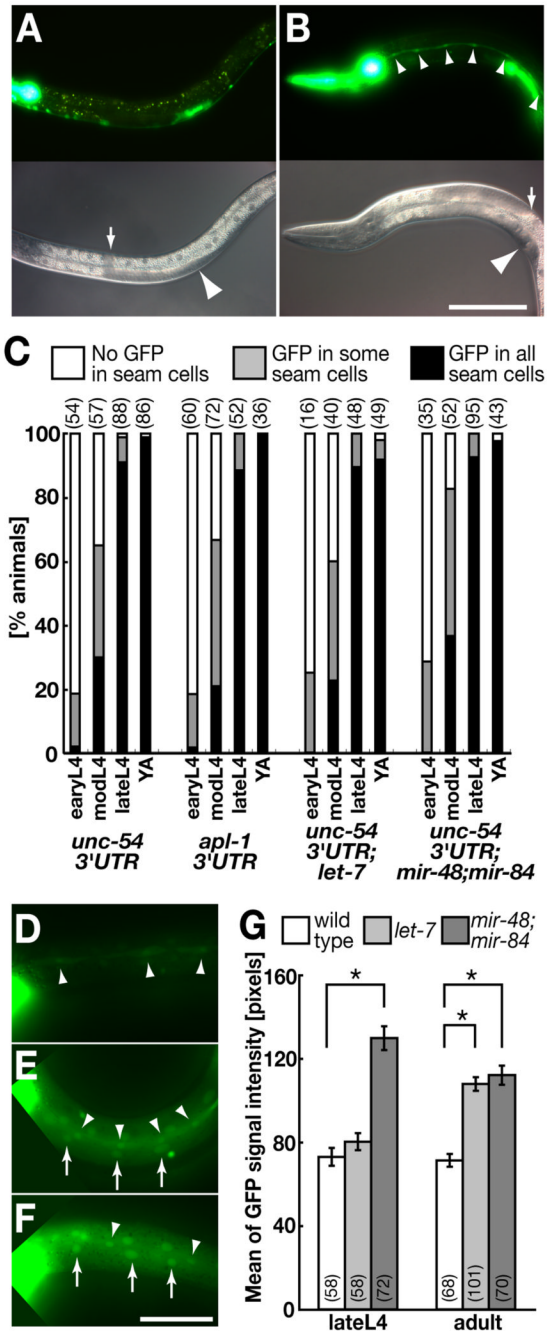

Fig. 3.

Level of *apl-1* expression in seam cells is indirectly regulated by *let-7* family microRNAs. (A, B) Strong GFP expression of *apl-1::gfp::unc-54* was observed in seam cells in late (B) but not early (A) L4 stages. Neuronal, pharyngeal and uterine cells showed constant GFP expression throughout development. Images A and B were taken for the same exposure time and processed identically. Arrows and arrowheads in lower DIC panels point to distal gonad tips and vulvas, respectively. Scale bar: 100 μm for A and B. (C) Temporal expression profiles in seam cells of *apl-1::gfp* constructs fused with 3′UTRs of either *unc-54* or *apl-1* in wild type, *let-7 (n2853)* or *mir-48(n4097);mir-84(n4037)* backgrounds. Staging of L4 animals were by relative positions of gonadal tips to vulva: early, mid and late L4 animals were defined as animals

showing 0–1/4, 1/4–1/2 and >1/2 gonadal turns, respectively. Each number in parentheses represents *n* of each sample. (D–F) GFP expression of *apl-1::gfp::unc-54* in wild type (D), *let-7(n2873)* (E) and *mir-48(n4097);mir-84(n4037)* (F) young adults. Arrows and arrowheads indicate GFP-expressing seam cells and hyp7 cells, respectively. Images D–F were taken for the same exposure time and processed identically. Scale bar: 50 μm for D–F. (G) Quantification of GFP levels (mean ± SEM) in seam cell nuclei of *apl-1::gfp::unc-54* in seam cells in wild type and *let-7* family mutants. **p* < 0.001 Student's *t*-test. Each number in parentheses indicates *n* of observed seam cells of each sample.

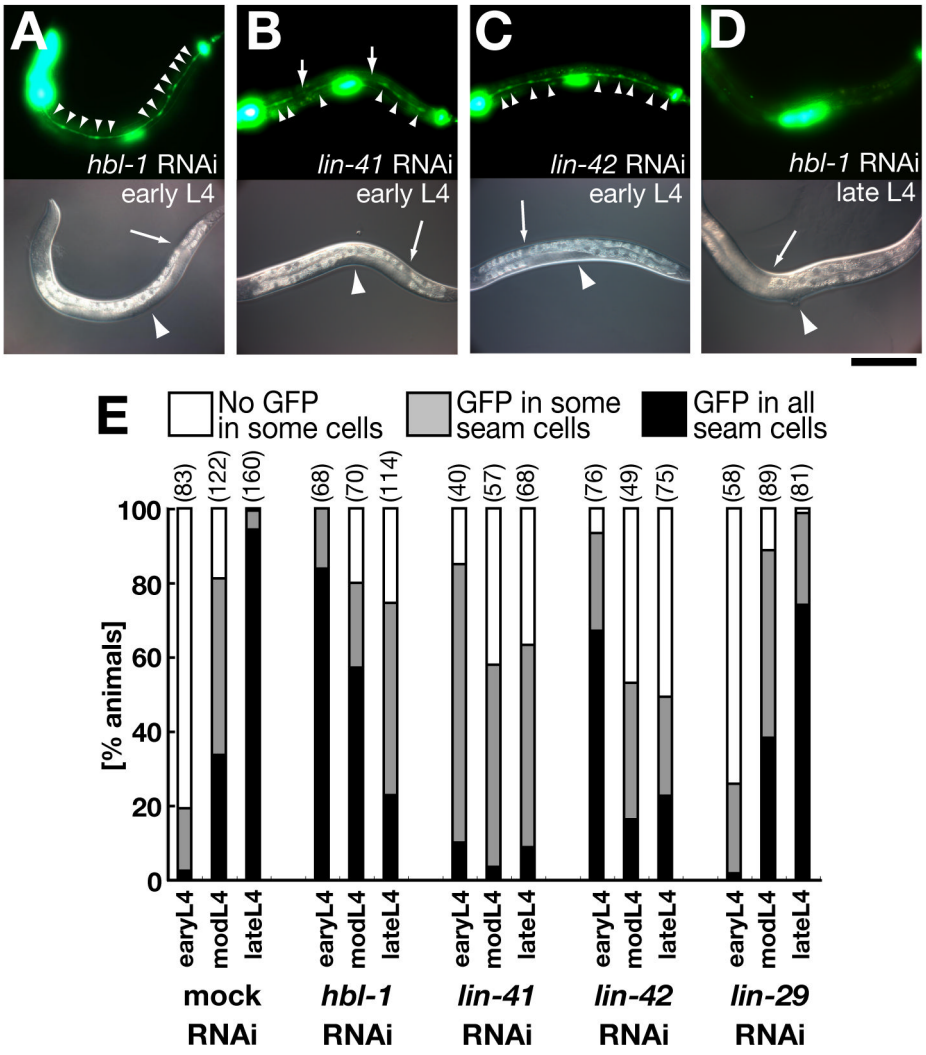

Fig. 4.

The dynamic expression of *apl-1* in seam cells is regulated by heterochronic genes. (A–D) Animals carrying integrated *apl-1::gfp::unc-54* constructs on RNAi plates of *hbl-1* (A, D), *lin-41* (B) and *lin-42* (C). (A–C) In the early L4 stage, all of these RNAi animals showed precocious GFP expression in seam cells (arrowheads). Note that seam cells without GFP were frequently observed in *lin-41(RNAi)* animals (Upper panel in B, arrowheads; also see E). (D) In contrast to the early L4 stage, the GFP signals in the late L4 stage were significantly repressed in the *hbl-1(RNAi)* animals. Arrows and arrowheads in lower DIC panels point to gonadal distal tip cells and vulvas, respectively. Images A–D were taken for the same exposure time and processed identically. Scale bar: 100 μm. (E) Temporal expression profiles of the *apl-1::gfp::unc-54* strain in the L4 stage with RNAi for several heterochronic genes. We used the same strain as animals shown in Fig. 3A and B. Note that *lin-41(RNAi)* showed precocious GFP expression in seam cells, but the fraction of animals showing GFP expression in all seam cells was reduced as compared with *hbl-1(RNAi)* and *lin-42(RNAi)*. Each number in parentheses indicates *n* of observed animals of each sample.

Table 1

apl-1 (RNAi) suppresses the retarded seam cell phenotype of *let-7*

Number of seam cell nuclei per side[a]		
let-7(n2853);wIs79; mock RNAi	20.1 ± 3.7 (*n* = 67)	
let-7(n2853);wIs79; apl-1(RNAi)	17.8 ± 2.6 (*n* = 50) *	
Adult alae formation per side[b]	Any alae	>50% alae
let-7(n2853); mock RNAi (*n* = 33)	45%	12%
let-7(n2853); apl-1(RNAi)(*n* = 35)	95% **	61% **

Number of seam cell nuclei and the presence of alae in *let-7(n2853)* were scored in young adult animals at a point where *let-7(n2853)* animals had not yet burst at 20 °C.

a * $p < 0.001$ as judged from Mann-Whitney's *U* test;

b ** $p < 0.01$ as judged from chi-square test.