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Developmental decline in neuronal regeneration by the progressive change of two intrinsic timers in *C. elegans*

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Abstract

Like mammalian neurons, *C. elegans* neurons lose axon regeneration ability as they age, but it is not known why. Here, we report that *let-7* contributes to a developmental decline in AVM axon regeneration. In older AVM axons, *let-7* inhibits regeneration by down regulating LIN-41, an important AVM axon regeneration-promoting factor. While *let-7* inhibits *lin-41* expression in older neurons through the *lin-41 3'UTR*, *lin-41* inhibits *let-7* expression in younger neurons through Argonaute ALG-1. This reciprocal inhibition ensures axon regeneration is only inhibited in older neurons. These findings show that a *let-7-lin-41* regulatory circuit, which was previously shown to control timing of events in mitotic stem cell lineages, is re-utilized in postmitotic neurons to control post-differentiation events.

We use *C. elegans* to study developmental decline in neuronal regeneration (Fig. 1A; *1*). As in vertebrates, advancing development leads to decreased axon regenerative capacity in *C. elegans* (Fig. 1C; *2-4*). The timing mechanism that controls developmental decline in neuronal regeneration is poorly understood (2-5). Since heterochronic genes are implicated in regulating developmental timing and aging in *C. elegans* (6), we hypothesized that they might regulate developmental decline in neuronal regeneration. The heterochronic pathway involves a number of microRNA-regulated post-transcriptional genetic circuits (7, 8), including an important interaction between the *let-7* microRNA and its direct target, *lin-41*, which encodes a tripartite motif (TRIM) protein (9, 10). We show here that *let-7* and *lin-41* function in postmitotic neurons to time their differentiation and post-differentiation events. Our study reveals that the intrinsic timing mechanism that controls developmental decline in neuronal regeneration depends on the progressive increase of *let-7* and the progressive

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decrease of *lin-41* in neurons, with *let-7/lin-41* reciprocal inhibition having a role in this process.

MicroRNA expression is either spatially restricted or temporally regulated in neuronal development (11-14). To explore the role of microRNAs in neuronal regeneration, we examined AVM axon regeneration in mutants defective in microRNA biogenesis, *dcr-1* and *alg-1 (15).* Although *dcr-1* hypomorphic mutant animals displayed normal AVM axon regeneration (fig. S1A), regenerating AVM neurons of adult *alg-1* mutants extended axons 2.5 times the length of those in adult wild-type animals (Fig. 1, B to D). In addition, regenerating axons of *alg-1* adults often displayed compact growth cones (fig. S1C). These regeneration phenotypes in adult *alg-1* mutants are reminiscent of those in wild-type animals at an earlier developmental stage (Fig. 1, B to D and fig. S1), suggesting that *alg-1* mutations may retard a normal developmental decline in axon regeneration. Consistent with this conclusion, we observe that although AVM axon regeneration in *alg-1* mutants is similar to that of wild-type animals at the L2 stage (Fig. 1, C and D), during advanced L3-YA stages, AVM axon regeneration is significantly reduced in wild-type animals, but is unchanged in *alg-1* mutants until day 4 of the adult stage (Fig. 1, C and D).

We hypothesize that ALG-1-dependent microRNAs are likely to be expressed late to contribute to the observed developmental decline in axon regeneration. Stem-loop RTPCR was used to globally survey mature microRNA expression in *C. elegans* (fig. S2A). Among 90 microRNAs surveyed (fig. S2), we identified 10 late-onset microRNAs whose expression coincides with the development decline in axon regeneration (Fig. 2A). Among the 10 late-onset microRNAs identified, only *let-7*, *mir-84*, and *mir-241* are expressed in AVM neurons (Fig. 2, B to H and fig. S3A). Out of those, only *let-7* maturation is affected in *alg-1* mutants (Fig. 2I and fig. S3B). In our assay using RNA preparations from staged animals, expression of the mature *let-7* microRNA is almost undetectable from embryonic to L2 stages and becomes apparent at L3 (Fig. 2A). To study specifically the expression of *let-7* in AVM, we developed a 2.9-kb *let-7* promoter reporter that stably expresses in AVM and whose expression levels in the whole animal at different stages correlate very well with the whole animal stem-loop RT-PCR results (Fig. 2A and fig S3C). This *let-7* reporter is expressed at relatively low levels in AVM at L1 and L2 stages (Fig. 2, C and J), but is significantly elevated from L3 onward (Fig. 2, F and J). This indicates that *let-7* is expressed at the right place and right time to contribute to the developmental decline in AVM axon regeneration. Several lines of evidence indicate that the effect of *alg-1* mutations on AVM axon regeneration can be attributed to *let-7* inactivation in AVM. First, *let-7* mutants display enhanced AVM axon regeneration to a similar extent to *alg-1* mutants (Fig. 2, K to M). Second, the effect of *alg-1* mutations on AVM axon regeneration is rescued by the *let-7* over-expression in AVM (Fig. 2M). Lastly, while the *alg-1* RNAi causes enhanced AVM axon regeneration, it does not further enhance regeneration in *let-7* mutants (Fig. 2M).

The temperature sensitive allele used in this study, *let-7(n2853ts)*, is a point mutation in the 5’ seed region of the *let-7* microRNA. *let-7(n2853ts)* animals have normal *let-7* activity at the permissive temperature of 15°C but have reduced *let-7* activity at the nonpermissive temperature of 20°C. In *let-7(n2853ts)* mutants at 20°C, AVM axons regenerated to twice the length of those regenerated in wild-type animals (Fig. 2M), suggesting that *let-7*

mutations recapitulate the *alg-1* mutant phenotype in enhancing AVM axon regeneration (Fig. 2M). *let-7(n2853ts)* likely represents a loss-of-function mutation in AVM at 20°C since *let-7(n2853ts)* mutants display a similar degree of AVM axon regeneration at 23°C as at 20°C (average axon regeneration is 105 μm at 23°C and 100 μm at 20°C, p=0.32). Like *alg-1* mutants, *let-7(n2853ts)* animals also display a retarded developmental decline in AVM axon regeneration (fig. S4, A and B). Regenerating AVM neurons in *let-7(n2853ts)* mutants not only extended longer axons, they also frequently displayed compact growth cone (fig. S1D), a phenotype shared by *alg-1* mutants (fig. S1C). Some regenerating axons in *let-7* mutants only stop outgrowth as they are about to enter the nerve ring (Fig. 2L). The effect of *let-7* mutations on AVM axon regeneration appears to be cell autonomous since expression of *let-7* in AVM significantly rescued the *let-7* mutant phenotype of enhancing AVM axon regeneration (Fig. 2M).

lin-4, like *let-7*, is a developmental timing microRNA and is expressed strongly in AVM (14). AVM axon regeneration in *lin-4* mutants is not significantly different from that in wild-type animals (fig. S5A), suggesting that different developmental timing microRNAs play different roles in AVM: *lin-4* is used to time AVM axon connectivity as reported previously (14); *let-7* is used to time developmental decline in AVM axon regeneration.

To determine if the effect of *let-7* on axon regeneration represents a prior role for *let-7* during development of the AVM to establish its regenerative potential, or rather, an acute requirement for *let-7* during the process of regeneration, we performed temperature shift experiments. We observed that *let-7(n2853ts)* animals shifted to the non-permissive temperature after injury exhibit better axon regeneration than wild-type animals (fig. S5). These data indicate that *let-7* has an immediate effect in regenerating AVM neurons to inhibit their regenerative capacity.

let-7 is expressed only weakly in AVM and ALM neurons at the L1 stage (Fig. 2J) when AVM and ALM axon development is underway, suggesting that a low level of *let-7* may be necessary for normal axon development. To test whether elevated *let-7* would inhibit AVM and ALM axon outgrowth in development, we forced early expression of *let-7* in AVM and ALM using a cell-specific *mec-4* promoter, which enables gene expression from the late embryonic stage onward. Forced early expression of *let-7* in AVM and ALM limits their axon outgrowth during development (fig. S6, A to G), suggesting that premature *let-7* expression closes early the window of plasticity for axon outgrowth.

hbl-1 and *lin-41* are critical direct targets of *let-7* in *C. elegans* developmental timing (10, 16). *hbl-1* encodes a transcription factor and LIN-41 is a member of TRIM family proteins. Analysis of the promoter activity of *hbl-1* and *lin-41* genes using promoter::GFP reporters showed that *lin-41* is expressed in AVM and ALM but *hbl-1* is not (Fig. 3A). Also, *lin-41* mutants (but not *hbl-1* mutants) display significantly reduced AVM axon regeneration (Fig. 3, C and E). *lin-41* mutations suppress the *let-7* mutant phenotype of enhancing AVM axon regeneration, suggesting that *lin-41* acts downstream of *let-7* in AVM (Fig. 3E). Consistent with a positive role for *lin-41* in promoting axon regeneration, over-expressing *lin-41* in AVM enhanced AVM axon regeneration (Fig. 3, D and E).

Consistent with a developmental down-regulation of *lin-41* in AVM by rising *let-7*, an engineered genomic reporter containing the 5', coding and 3' fragments of the *lin-41* gene showed a stage-dependent decline in AVM (Fig. 3B). This down-regulation of *lin-41* is likely via direct interaction of *let-7* with the *lin-41* 3' UTR, as the expression of a *GFP::lin-41* 3'UTR sensor in adult AVM was higher in *let-7* mutants than in wild-type animals (fig. S7), and ectopic expression of *let-7* in younger AVM was sufficient to repress *GFP::lin-41* 3'UTR (fig. S8).

lin-29, a zinc finger transcription factor of the C2H2 type (17), was known to be inhibited by *lin-41* (10). We show that *lin-29* mutations cause enhanced AVM axon regeneration but do not further enhance regeneration in *let-7* mutants (Fig. 3E). In addition, *lin-29* mutations suppress the *lin-41* mutant phenotype of reducing AVM axon regeneration (Fig. 3E). Further, the level of the LIN-29::GFP fusion protein in AVM at the L1 stage is significantly higher in *lin-41* mutants than in wild-type animals (Fig. 3F). Together, our results indicate that *let-7*/*lin-41* is acting via *lin-29* to control AVM axon regeneration.

A conserved MAPK pathway, including the DLK-1/MAPKKK and p38 kinase/PMK-3, controls axon regeneration in *C. elegans* (4). This pathway is negatively regulated by the conserved protein RPM-1 (4). We show that *dlk-1* and *pmk-3* mutations suppress the enhanced AVM axon regeneration of *let-7* mutants, suggesting that *let-7* may act upstream of *dlk-1* and *pmk-3* (fig. S9A). Further, *lin-41* mutations do not suppress the enhanced AVM axon regeneration of *rpm-1* mutants while *pmk-3* and *dlk-1* mutations do, suggesting that *lin-41* may act upstream of or in parallel to *rpm-1* (fig. S9A). Analysis of the RPM-1 level shows no difference between *lin-41* mutants and wild-type animals (fig. S9B), indicating that either LIN-41 is acting in parallel to RPM-1 or LIN-41 is acting through a post-translational mechanism to indirectly regulate RPM-1 activity but not its level.

In wild-type animals, mature *let-7* microRNA is expressed at low levels prior to the L3 stage (Fig. 2A and 4A). In *lin-41* mutants, *let-7* is precociously expressed at the L1 stage (Fig. 4A), indicating that *lin-41* prevents the early-onset expression of *let-7*. The expression intensity of a *Plet-7::GFP* reporter in the whole animal or in AVM is slightly lower instead of higher in *lin-41* mutants than in wild-type animals at L1-L3 stages (Fig. 4, B and C), suggesting that *lin-41* inhibits early expression of *let-7* post-transcriptionally.

Recent studies of mouse stem cells showed that mLin41 E3 ubiquitin ligase activity regulates Argonaute2 turnover (18). Our study shows that the ALG-1 Argonaute is required for *let-7* maturation in *C. elegans* (Fig. 2I). To test whether LIN-41 inhibits the early-onset expression of *let-7* through negative regulation of ALG-1, *alg-1* mutations were examined for their effects on *let-7* expression in *lin-41* mutants. In *lin-41; alg-1* double mutants, *let-7* levels were restored to late-onset expression (Fig. 4A). Since only the ectopic (early) expression but not the normal (late) expression of *let-7* was affected by *alg-1* mutations in *lin-41* mutants, this result suggests that LIN-41 may inhibit ALG-1 only at the early stage to repress early-onset *let-7* expression. Indeed, LIN-41 is no longer able to down-regulate *let-7* expression in the adult stage (Fig. 4D).

Consistent with this interpretation, the level of the ALG-1::GFP fusion protein in AVM at the L1 stage was significantly higher in *lin-41* mutants than in wild-type animals (Fig. 4E). Using a *lin-41* promoter construct that drives the FLAG tagged LIN-41 protein expression mainly in neurons (Fig. 3A), we tested whether immunoprecipitation (IP) of ALG-1 would co-precipitate the FLAG tagged LIN-41 protein. Co-IP of FLAG::LIN-41 with ALG-1 was observed (Fig. 4F and fig. S10A), suggesting that these two proteins may form a complex in neurons. The relatively low LIN-41::FLAG signal intensity in the ALG-1 IP suggests that only a subpopulation of LIN-41 is in complexes with ALG-1. Similarly, immunoprecipitation of GFP-tagged LIN-41 pulled down a subset of the ALG-1 protein population (fig. S10B). IP of small subsets of protein populations with components of microRNA-induced silencing complex has precedents (15, 19).

Even though *let-7* levels decline significantly by day 4 into the adult stage (fig. S11, A and B), AVM axon regeneration remained significantly enhanced in aged animals that either over-express *lin-41* or contain *let-7* mutations (fig. S11, C and D), suggesting that *let-7/ lin-41* pathway regulates axon regeneration even in aged AVM. Furthermore, *let-7* mutations significantly enhance while *lin-41* mutations significantly reduce axon regeneration in several neurons that co-express both genes, suggesting that the role of *let-7*/ *lin-41* in regulating axon regeneration can be extended beyond AVM neurons (fig. S12).

In this study, we show that *let-7* contributes to a developmental decline in AVM axon regeneration. We identify LIN-41 as an important AVM axon regeneration-promoting factor. *let-7* represses *lin-41* expression to inhibit AVM axon regeneration in older neurons. Our results suggest a negative regulatory loop between *let-7* and *lin-41* (Fig. 4G). In younger neurons, *lin-41* inhibits *let-7* expression through negative regulation of ALG-1. In older neurons, up-regulation of *let-7* overcomes the *lin-41* inhibition and in turn represses the *lin-41* expression through the *lin-41 3' UTR*. Like *C. elegans*, mammals also exhibit a developmental decline in axon regeneration. Our findings suggest that it may be possible to enhance axon regeneration after injury through therapeutic inhibition of the *let-7* microRNA in neurons, and thereby restore their youthful regenerative capacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and Notes

1. Chiu H, et al. Cell Adhes. Migr. 2011; 5:387.
2. Wu Z, et al. Proc. Natl. Acad. Sci. 2007; 104:15132. [PubMed: 17848506]
3. Gabel CV, et al. Development. 2008; 135:3623.
4. Hammarlund M, et al. Science. 2009; 323:802. [PubMed: 19164707]

5. Goldberg JL, et al. Science. 2002; 296:1860. [PubMed: 12052959]
6. Boehm M, Slack F. Science. 2005; 310:1954. [PubMed: 16373574]
7. Lee RC, et al. Cell. 1993; 75:843. [PubMed: 8252621]
8. Wightman B, et al. Cell. 1993; 75:855. [PubMed: 8252622]
9. Pasquinelli AE, et al. Nature. 2000; 408:86. [PubMed: 11081512]
10. Slack FJ, et al. Mol. Cell. 2000; 5:659. [PubMed: 10882102]
11. Johnston RJ, Hobert O. Nature. 2003; 426:845. [PubMed: 14685240]
12. Chang S, et al. Nature. 2004; 430:785. [PubMed: 15306811]
13. Hsieh YW, et al. PLoS Genet. 2012; 8:e1002864. [PubMed: 22876200]
14. Zou Y, et al. Sci. Signal. 2012; 5:ra43. [PubMed: 22692424]
15. Duchaine TF, et al. Cell. 2006; 124:343. [PubMed: 16439208]
16. Lin SY, et al. Dev. Cell. 2003; 4:639. [PubMed: 12737800]
17. Rougvie AE, Ambros V. Development. 1995; 121:2491. [PubMed: 7671813]
18. Rybak A, et al. Nat. Cell Biol. 2009; 11:1411. [PubMed: 19898466]
19. Hammell CM, et al. Cell. 2009; 136:926. [PubMed: 19269369]

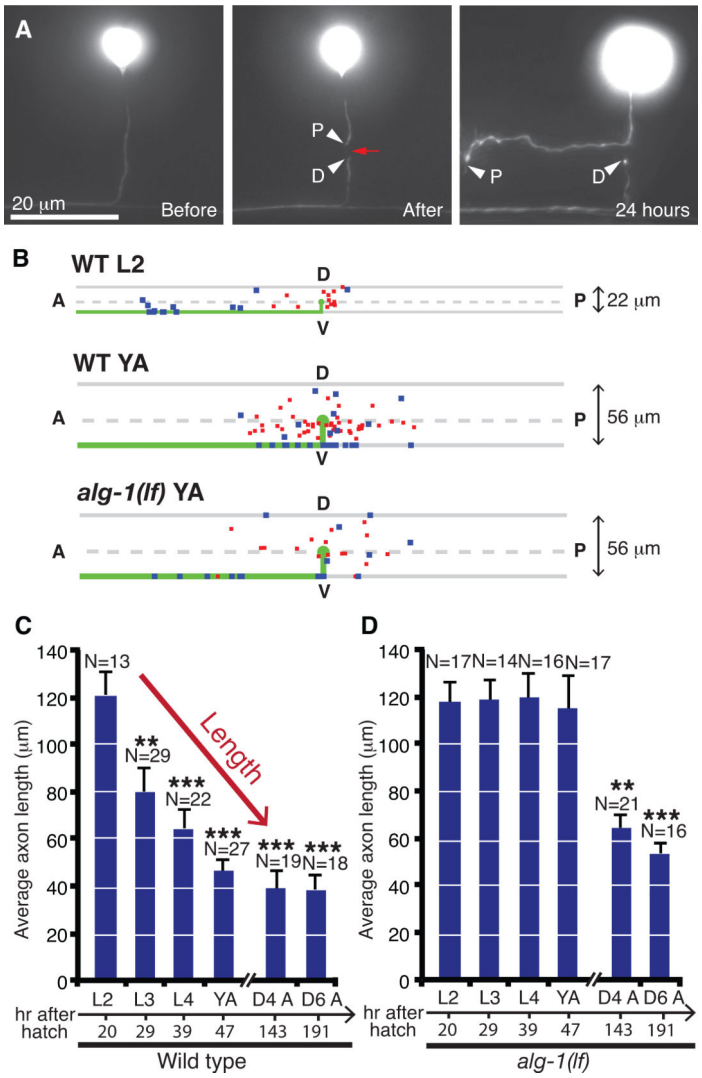

Fig. 1.

alg-1 mutations slow developmental decline in AVM axon regeneration. (**A**) AVM axon before, immediately after, and 24 hours after laser surgery in a wild-type animal. D (distal end) and P (proximal end) of severed axons. Red arrow points to the focus of laser plasma. Anterior is left, dorsal up. (**B**) Scatter plots showing the positions of regenerating axon termini in wild-type L2-stage animals, wild-type young adults, and *alg-1* young adults. In each scatter plot, the top line (dorsal nerve cord), the bottom line (ventral nerve cord), and the wild-type AVM morphology before surgery (green) are shown. (**C**, **D**) Bar charts of average AVM axon length in wild type (**C**) and *alg-1* mutants (**D**) at different stages. Asterisks indicate cases in which latter stage animals differ from L2-stage animals at P<0.001. Error bars indicate SEM.

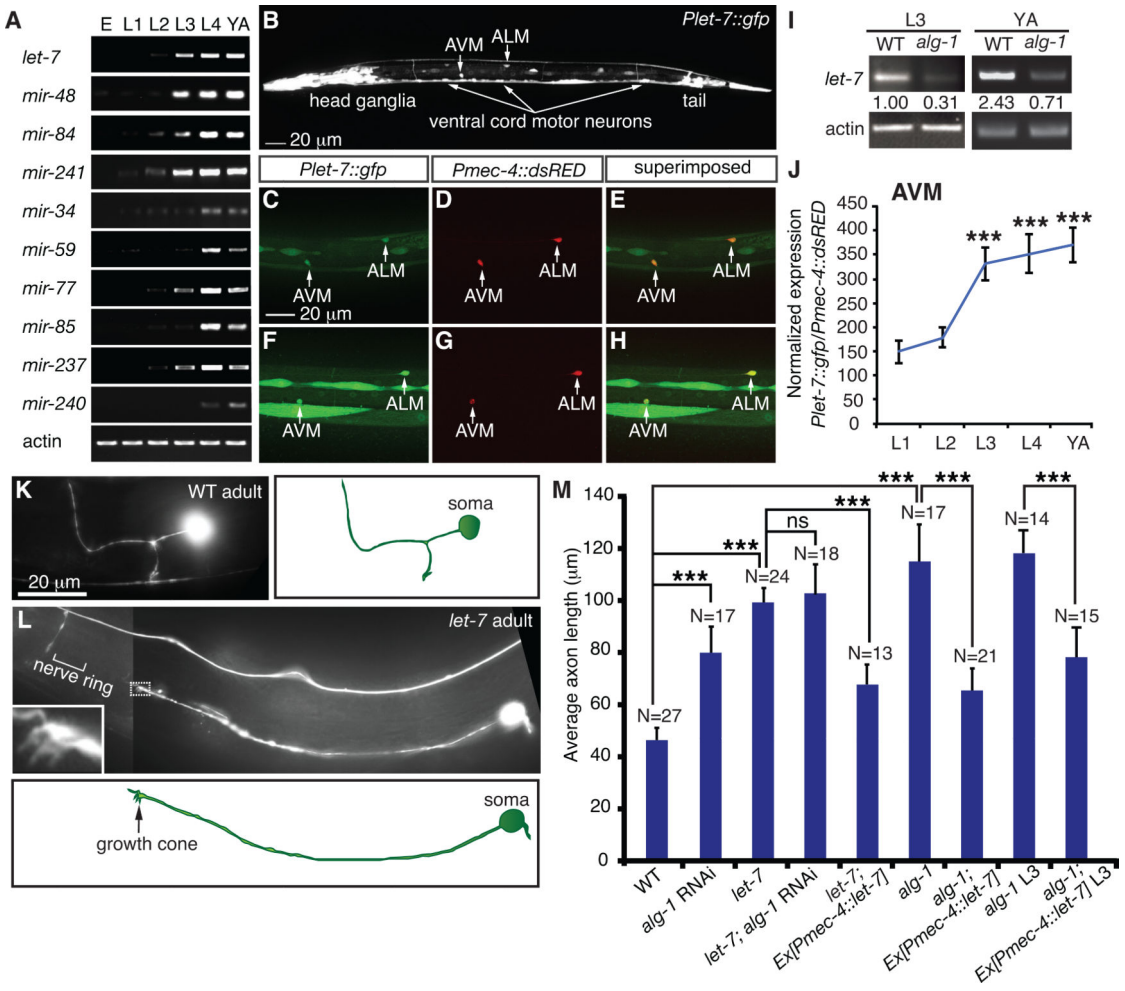

Fig. 2.

The *let-7* expression in AVM and ALM and the *let-7* mutant phenotype in enhancing AVM axon regeneration. (**A**) Stem-loop RT-PCR analysis of RNA isolated from populations of staged animals revealed late-onset expression of several mature microRNAs. (**B**) Broad *let-7* expression in neurons at L3. (**C-H**) The *let-7* promoter drives GFP expression in AVM and ALM touch neurons in L2-stage (**C-E**) and young adult (**F-H**) animals. The *Pmec-4::dsred* reporter labels AVM and ALM. (**I**) Mature *let-7* levels by stem-loop RT-PCR in *alg-1* mutants versus wild-type animals at L3 and YA stages. The value below each lane indicates amount of *let-7* normalized to actin. (**J**) Average fluorescent intensity of the *Plet-7::GFP* reporter normalized to the *Pmec-4::dsRED* in AVM. Regenerating AVM axons in wild-type adult animals (**K**) and adult *let-7(n2853)* mutants (**L**). The dashed box in (**L**) delineates the growth cone, whose blown up image is shown in the inset. (**M**) Bar chart of average AVM axon length. Asterisks indicate cases in which mutants differ from wild-type or a specific comparison is significantly different at P<0.001.

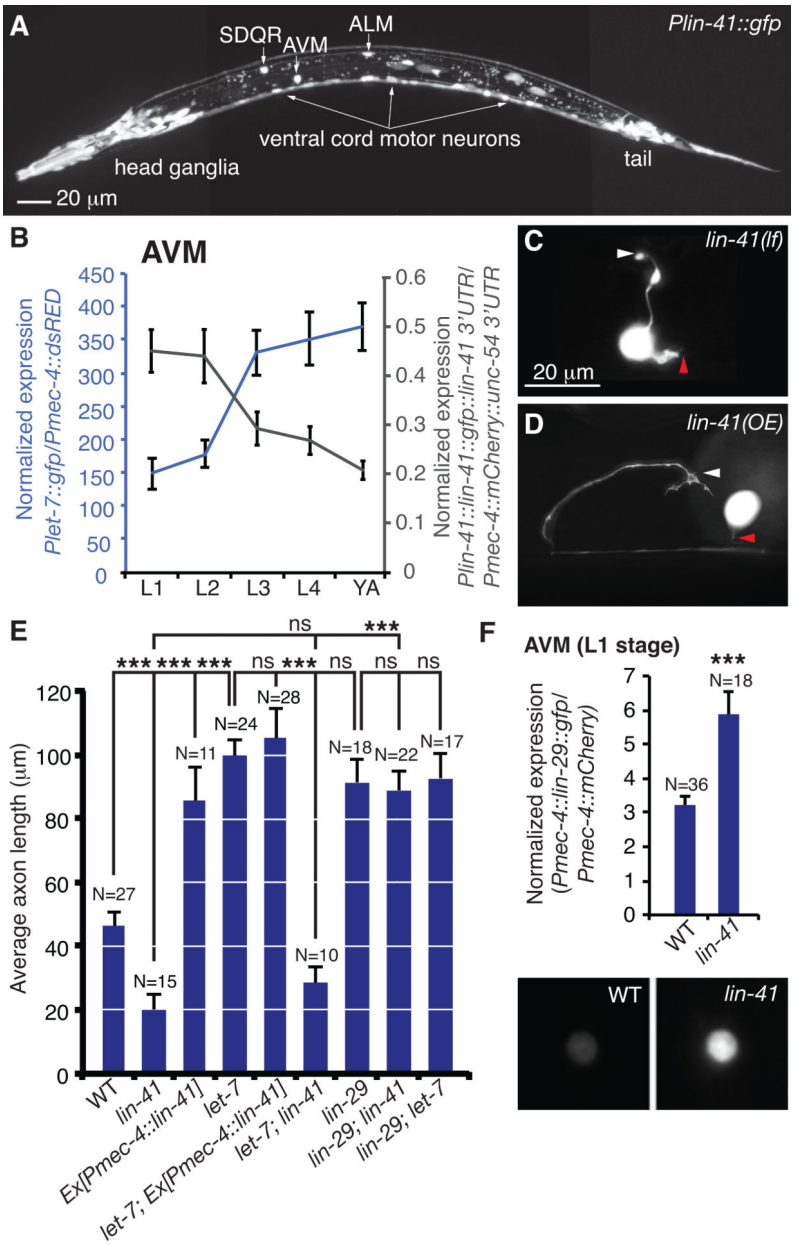

Fig. 3.

The *lin-41* expression study and the *lin-41* effect on AVM axon regeneration. (**A**) Broad *lin-41* expression in neurons at L2. (**B**) The *let-7* promoter reporter and the engineered *lin-41* genomic reporter are temporally regulated in AVM neurons. Typical regenerating AVM axons in adult *lin-41* mutants (**C**) and *Ex[Pmec-4::lin-41]* transgenic animals (**D**). Red arrowhead points to the axon break and white arrowhead points to the terminus of regenerating axons. Anterior is left, dorsal up. (**E**) Bar chart of average AVM axon length. Asterisks indicate cases in which mutants or transgenic animals significantly differ from wild-type or a comparison between mutants is significantly different at P<0.001 (***). (**F**) Analysis of the level of the LIN-29::GFP fusion protein in AVM in *lin-41* mutants versus

wild-type animals at L1. Images shown are expression of the LIN-29::GFP fusion protein in the AVM nucleus.

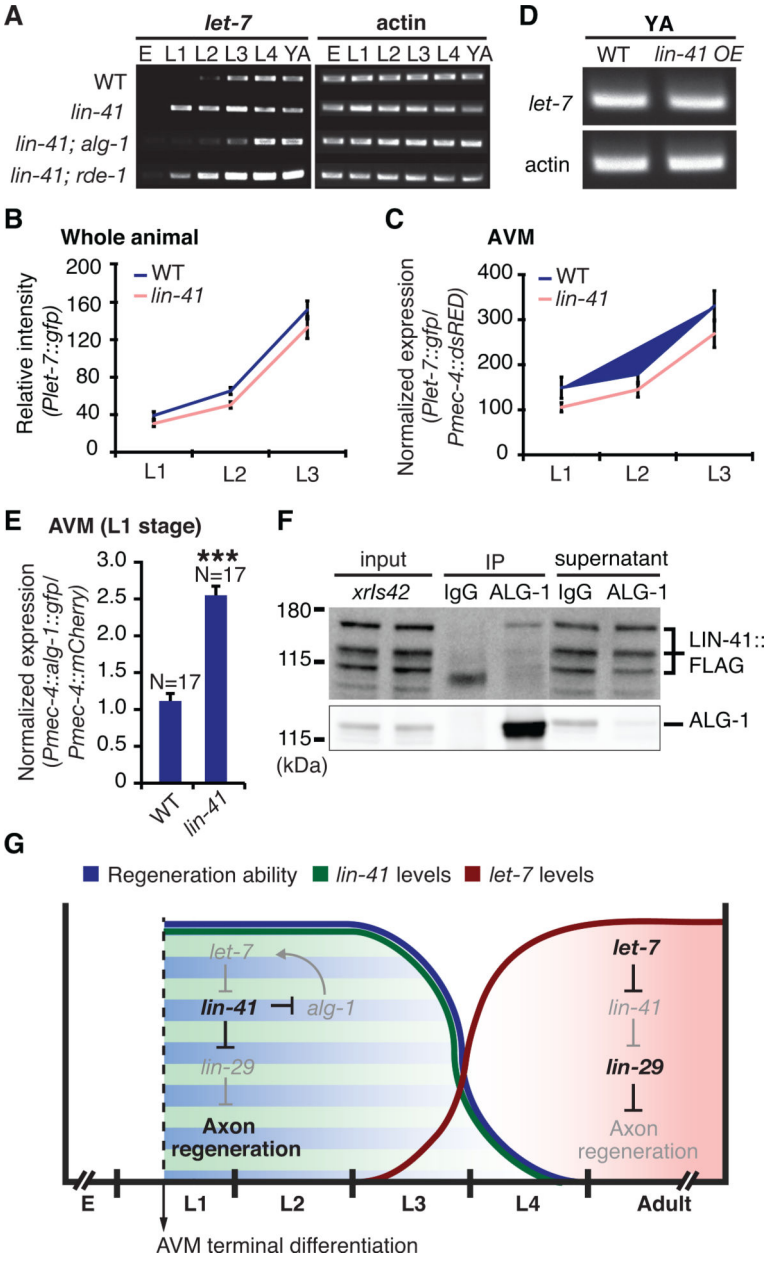

Fig. 4.

LIN-41 inhibits *let-7* expression through ALG-1 at the early stage. (**A**) Analysis of temporal expression of mature *let-7* microRNA in wild-type and *lin-41* mutants by the stem-loop RT-PCR assay. Analysis of the *let-7* promoter activity in the whole animal (**B**) and AVM (**C**) in *lin-41* mutants versus wild-type animals. (**D**) Stem-loop RT-PCR analysis of the mature *let-7* microRNA in wild-type and *Ex[Plin-41::lin-41]*. (**E**) Analysis of the expression of the ALG-1::GFP fusion protein in AVM in *lin-41* mutants versus wild-type animals at L1. (**F**) Immunoprecipitation of ALG-1 co-precipitates FLAG-tagged LIN-41. (**G**) Model of *let-7*-signaled developmental decline in AVM axon regeneration.