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RESEARCH ARTICLE

Regulation of nuclear-cytoplasmic partitioning by the *lin-28-lin-46* pathway reinforces microRNA repression of HBL-1 to confer robust cell-fate progression in *C. elegans*

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ABSTRACT

MicroRNAs target complementary mRNAs for degradation or translational repression, reducing or preventing protein synthesis. In *Caenorhabditis elegans*, the transcription factor HBL-1 (Hunchback-like 1) promotes early larval (L2)-stage cell fates, and the *let-7* family microRNAs temporally downregulate HBL-1 to enable the L2-to-L3 cell-fate progression. In parallel to *let-7*-family microRNAs, the conserved RNA-binding protein LIN-28 and its downstream gene *lin-46* also act upstream of HBL-1 in regulating the L2-to-L3 cell-fate progression. The molecular function of LIN-46, and how the *lin-28-lin-46* pathway regulates HBL-1, are not understood. Here, we report that the regulation of HBL-1 by the *lin-28-lin-46* pathway is independent of the *let-7*/*lin-4* microRNA complementary sites (LCSs) in the *hbl-1* 3′UTR, and involves stage-specific post-translational regulation of HBL-1 nuclear accumulation. We find that LIN-46 is necessary and sufficient to prevent nuclear accumulation of HBL-1. Our results illuminate that robust progression from L2 to L3 cell fates depends on the combination of two distinct modes of HBL-1 downregulation: decreased synthesis of HBL-1 via *let-7*-family microRNA activity, and decreased nuclear accumulation of HBL-1 via action of the *lin-28-lin-46* pathway.

KEY WORDS: microRNA, Transcription factor, Nuclear-cytoplasmic partitioning, Heterochronic, Cell-fate progression, Developmental robustness

INTRODUCTION

Precise and robust gene regulation is crucial for animal development. Optimal doses of developmental gene products expressed with spatiotemporal precision produce the wild-type body plan, whereas abnormally lower or higher doses or ectopic expression of developmental genes can result in morphological defects that reduce the fitness of the individual. The proper spatiotemporal activity of developmental gene products is ensured by elaborate gene regulatory mechanisms, which often involve collaboration across semi-redundant mechanisms controlling the gene activity at different levels – transcriptional, translational and post-translational.

Caenorhabditis elegans development consists of an invariant set of cell division and differentiation events that produces the stereotyped adult body plan (Sulston and Horvitz, 1977). *C. elegans* developmental regulators are identified by loss-of-function or gain-of-function mutations that cause developmental lethality, or evident morphological defects. One class of developmental defects in *C. elegans* stems from changes in the order and/or timing of larval developmental events, controlled by the heterochronic gene pathway (Ambros and Horvitz, 1984). In this pathway, three transcription factors (LIN-14, HBL-1, LIN-29) control cell-fate transitions from earlier to later stages, and are temporally regulated – directly or indirectly – by certain microRNAs. In particular, LIN-14 is regulated by *lin-4* (Lee et al., 1993), HBL-1 (Hunchback-like-1) is regulated by three members of the *let-7* family (also known as the *let-7* sisters: *mir-48*, *mir-84* and *mir-241*) (Abbott et al., 2005), and LIN-29 is post-transcriptionally regulated by the RNA-binding protein LIN-41 (Slack et al., 2000), which is in turn regulated by *let-7* (Reinhart et al., 2000). These microRNAs are dynamically expressed during larval development and they ensure proper temporal downregulation of their targets, which is crucial for the proper program of stage-appropriate cell-fate transitions.

The conserved RNA-binding protein LIN-28 also plays key roles in the *C. elegans* heterochronic pathway. *lin-28* regulates early cell fates upstream of *lin-46* (Pepper et al., 2004) and in parallel to *mir-48/84/241* (Abbott et al., 2005), and regulates late cell fates upstream of the conserved microRNA *let-7* (Van Wynsberghe et al., 2011; Vadla et al., 2012). In larvae lacking *lin-28*, hypodermal stem cells (called seam cells) skip L2-stage proliferative cell fates and precociously express terminally differentiated adult cell fates (Ambros and Horvitz, 1984; Moss et al., 1997), whereas the rest of the tissues, e.g. the gonad, are still juvenile and developing. *lin-46(lf)* suppresses these *lin-28(lf)* phenotypes (Pepper et al., 2004): *lin-28(lf);lin-46(lf)* double mutants are wild type for the phenotypes observed in *lin-28(lf)* animals. *lin-28(lf)* suppresses the heterochronic phenotypes of *mir-48/84/241* mutants (Abbott et al., 2005), and *lin-46* is required for this suppression (Abbott et al., 2005).

Animals lacking *lin-46* display weak heterochronic phenotypes that are enhanced when the larvae are cultured at low temperatures, such as 15°C, a condition that does not affect wild-type development (Pepper et al., 2004). Although wild-type larval development is similarly robust against other stresses, such as population density pheromones or starvation (Ilbay and Ambros, 2019), *lin-46(lf)* phenotypes are enhanced under these conditions (Ilbay and Ambros, 2019) and when animals develop through a temporary diapause in response to these stresses (Karp and Ambros, 2012). Interestingly, diapause-inducing conditions also repress *let-7* family microRNA expression (Bethke et al., 2009; Hammell et al., 2009); which is thought to be important for an optimal diapause decision (Hammell et al., 2009) and for prolonging HBL-1 expression in coordination with the rate of developmental stage progression (Ilbay and Ambros, 2019). Therefore, LIN-46 activity is important for the proper downregulation of HBL-1 under physiological conditions where *let-7* family microRNA levels are reduced.

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The molecular functions of LIN-46, and how these functions may relate to its role in the heterochronic pathway are not known. However, given the prominent involvement of microRNAs in the heterochronic pathway, in the context of regulating temporal cell fates, LIN-46 has been thought to act possibly by modulating (more precisely 'boosting') the activities of certain microRNAs, e.g. *lin-4* or *let-7* family microRNAs, perhaps by interacting with the miRISC (microRNA-induced silencing complex). By hypothetically boosting the activities of *lin-4* and/or *let-7* family microRNAs in response to environmental stress, LIN-46 could compensate for the reduced levels of these microRNAs. This model is supported by the correlated conservation of LIN-46 with the argonaute family proteins, suggesting a potential function for LIN-46 related to microRNA or small RNA pathways (Tabach et al., 2013). Alternatively, LIN-46 could regulate HBL-1 activity by a mechanism independent of microRNAs.

Here, we report that deleting a genomic region encompassing the *let-7/lin-4* complementary sites (LCSs) in the *hbl-1* 3′UTR results in strong extra seam cell phenotypes, which is consistent with lack of *let-7/lin-4* microRNA regulation and a gain-of-function of HBL-1. Importantly, we find that *lin-28(lf)* suppresses, and *lin-46(lf)* enhances, the extra seam cell phenotype of *hbl-1(gf/ΔLCSs)*, indicating that regulation of HBL-1 by the *lin-28-lin-46* pathway is independent of the LCSs. Moreover, HBL-1, which normally localizes to the nucleus, accumulates in the cytoplasm of hypodermal seam cells in *lin-28(lf)* and *hbl-1(gf/ΔLCSs)* animals, and *lin-46* is required for this cytoplasmic accumulation of HBL-1. This cytoplasmic accumulation is accompanied by reduced nuclear accumulation of HBL-1, which also correlates with reduced HBL-1 activity. Lastly, we found that precocious expression of LIN-46 in L2-stage seam cells is sufficient to localize HBL-1 to the cytoplasm, reducing the nuclear accumulation of HBL-1, and thereby suppressing *hbl-1* gain-of-function phenotypes in *hbl-1(gf/ΔLCSs)* mutants. Our results indicate that the *C. elegans lin-28-lin-46* pathway regulates the temporal dynamics of nuclear accumulation of the HBL-1 transcription factor, acting in parallel with the translational repression exerted by the let-7-family microRNAs, to confer precision and robustness to the temporal downregulation of HBL-1 activity.

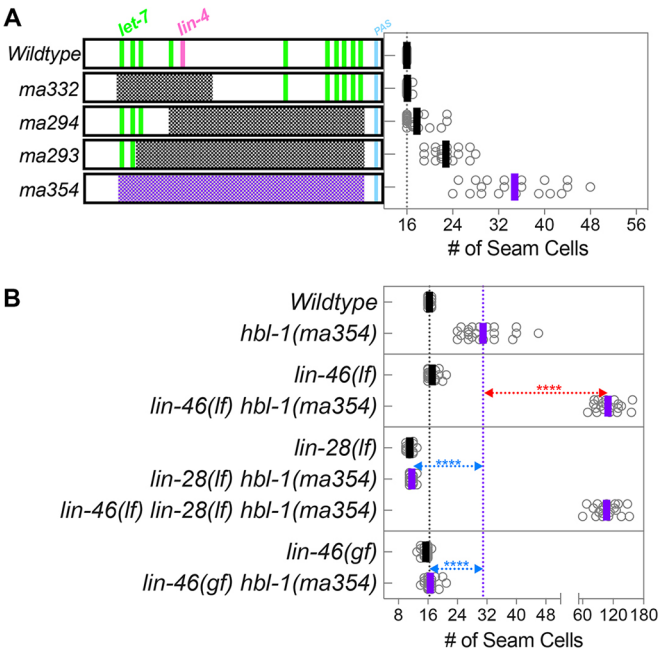


Fig. 1. The *C. elegans lin-28-lin-46* pathway regulates L2-to-L3 cell-fate transitions independently of the *let-7* and *lin-4* complementary sites in the *hbl-1* 3′UTR. (A) Deletion of genomic regions encompassing the *let-7* and *lin-4* complementary sites in the *hbl-1* 3′UTR results in extra seam cell phenotypes. The *hbl-1* 3′UTR contains ten *let-7* (green bars) and one *lin-4* (pink bar) complementary sites. Wild-type *hbl-1* 3′UTR and four deletion alleles are depicted on the *y*-axis and the number of seam cells observed in young adults of animals bearing these alleles are shown on the *x*-axis. Each dot shows the number of seam cells observed in a single animal and the vertical bars show the average seam number in the group of animals observed for each allele. Note that the polyadenylation signal (PAS) in the *hbl-1* 3′UTR is not disrupted in the mutants. (B) Number of seam cells in single and compound mutants containing the *hbl-1(ma354)* allele are plotted. The *lin-46* null allele enhances the extra seam cell phenotype of *hbl-1(ma354)*. *lin-28(lf)* suppresses the extra seam cell phenotype of *hbl-1(ma354)* and this suppression is *lin-46* dependent. The *lin-46* gain-of-function allele similarly suppresses the extra seam cell phenotypes of *hbl-1(ma354)*. In A and B, n=12 for wild type and n=20 for all other strains tested for the number of seam cells. Student's *t*-test was used to calculate statistical significance. ****P<0.0001.

RESULTS

Deletion of genomic regions encompassing the *let-7* and *lin-4* complementary sites in the *hbl-1* 3′UTR results in extra seam cell phenotypes

To explore whether *lin-46* acts downstream of *lin-28* in the heterochronic pathway by modulating the regulation of *hbl-1* by *let-7* family and/or *lin-4* microRNA, we sought to generate *hbl-1(gf)* alleles free from regulation by these microRNAs. The *hbl-1* 3′ UTR contains ten *let-7* complementary sites (LeCSs) and a single *lin-4* complementary site (LiCS), collectively abbreviated as LCSs. In order to abrogate *let-7*- and *lin-4*-mediated regulation of *hbl-1*, we deleted a genomic region encompassing all LCSs in the *hbl-1* 3′ UTR (*ma354*; Fig. 1A; Table S1). We found that, similar to *mir-48/84/241(0)* mutants, *hbl-1(ma354[ΔLCSs])* animals have retarded seam cell defects, wherein L2-stage fates are reiterated at later stages, resulting in extra seam cells in young adult animals (Fig. 1A).

Additionally, in our screens for large deletions in the progeny of CRISPR/Cas9-injected animals, we recovered smaller 3′UTR deletions of various sizes that removed several but not all LeCSs in the *hbl-1* 3′UTR (Fig. 1A; Table S1). We analyzed these smaller deletions along with the largest deletion (*ma354*), and found that most of these mutants also have extra seam cell phenotypes, although weaker than the *ma354* deletion (Fig. 1A). 3′UTR deletions that removed more LeCSs resulted in stronger extra seam cell phenotypes, which is consistent with the idea that these LeCSs are functional and they act partially redundantly.

lin-28(lf) suppresses and *lin-46(lf)* enhances the extra seam cell phenotype of *hbl-1(ma354[ΔLCSs])*

Next, to test if the regulation of *hbl-1* by the *lin-28-lin-46* pathway was dependent on the *let-7* and *lin-4* complementary sites (LCSs) in the *hbl-1* 3′UTR, we generated compound mutants containing the *ma354* deletion, together with null alleles of *lin-28*, and/or *lin-46*. We found that *lin-28(lf)* suppresses and *lin-46(lf)* substantially enhances the extra seam cell phenotype of *hbl-1(ma354)* (Fig. 1B), indicating that the regulation of *hbl-1* by *lin-28* does not require the LCSs in the *hbl-1* 3′UTR. We also found that *lin-46* is required for the suppression of *hbl-1(ma354)* by *lin-28(lf)* (Fig. 1B). These results suggest that the *lin-28-lin-46* pathway regulates HBL-1 amount or activity through a mechanism independent of the *let-7/lin-4* regulation of HBL-1.

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A *lin-46(gf)* mutation can suppress the extra seam cell phenotypes of *hbl-1(ma354[ΔLCSs])*

LIN-46 is expressed precociously in *lin-28(lf)* animals (Ilbay et al., 2019 preprint) and so suppression of *hbl-1(gf)* by *lin-28(lf)* could be solely due to precocious LIN-46, which is sufficient to inhibit L2 cell fates and promote transition to L3 and later cell fates (Ilbay et al., 2019 preprint). To determine whether precocious LIN-46 expression alone is also sufficient to suppress the extra seam cell phenotypes of *hbl-1(ma354)* animals, we employed a *lin-46(gf)* mutation, *lin-46(ma467)*, that consists of a 12 bp deletion of *lin-46* 5′UTR sequences, and that results in precocious expression of LIN-46 and *lin-28(lf)*-like phenotypes (Ilbay et al., 2019 preprint). We generated double mutant animals carrying *hbl-1(ma354)* and *lin-46(ma467)*, and found that the gain-of-function allele of *lin-46* suppresses the extra seam cell phenotypes of *hbl-1(ma354)* (Fig. 1B), suggesting that precocious LIN-46 expression is sufficient to suppress the *hbl-1(gf)* phenotypes. This result supports the interpretation of the suppression of *hbl-1(gf)* by *lin-28(lf)* as resulting from precocious LIN-46 expression.

Endogenously tagged HBL-1 is expressed in the nuclei of L1- and L2-stage hypodermal seam and hyp7 cells

The suppression and enhancement of the extra seam cell phenotypes of *hbl-1(ma354)* by *lin-28(lf)* and *lin-46(lf)*, respectively, could reflect changes in the level of HBL-1 protein. In order to test for changes in the levels of HBL-1 protein in *lin-28(lf)* or *lin-46(lf)* mutants, we tagged *hbl-1* at the endogenous locus with mScarlet-I using CRISPR/Cas9 (Ilbay and Ambros, 2019). We observed that in wild-type L1 and L2 larvae HBL-1::mScarlet-I localizes exclusively to the nucleus (Fig. 2A; Fig. S1A), which is consistent with HBL-1 functioning as a transcription factor (Fay et al., 1999; Niwa et al., 2009). Moreover, consistent with previous reports, endogenously tagged HBL-1 was expressed in the wild type in the hypodermal seam and hyp7 cells of L1- and L2-stage larvae and was not detected in L3- and L4-stage larvae (Lin et al., 2003; Abrahante et al., 2003; Ilbay and Ambros, 2019) (Fig. 2A; Fig. S1A).

HBL-1 is overexpressed and accumulates in the cytoplasm of L3- and L4-stage seam cells in larvae lacking LCSs in the *hbl-1* 3′UTR

To examine the impact of the loss of the LCSs in the *hbl-1* 3′UTR on the expression pattern of HBL-1, we deleted a region in the *hbl-1* 3′ UTR of the mScarlet-I-tagged *hbl-1* allele, generating the *ma430ma475 [hbl-1::mScarlet-I::Δ8LeCSs]* allele. The *ma475* deletion encompasses eight LeCSs and the LiCS, similar to and only three base pairs shorter than the *ma293* deletion (Fig. 1A; Table S1). Consistent with previous reports that utilized GFP reporters fused with wild-type *hbl-1* 3′UTR (Abrahante et al., 2003; Abbott et al., 2005), absence of these LeCSs resulted in HBL-1 expression that persisted in the L3- and L4-stage hypodermal cells (Fig. 2B versus 2A). Interestingly, we observed that at the L3 and L4 stages, HBL-1 accumulates in the cytoplasm of the seam cells, which is accompanied by a reduction in the nuclear accumulation of HBL-1 (Fig. 2B; Fig. S1B). This observation suggested that perhaps the nuclear accumulation of HBL-1 is hindered (or cytoplasmic accumulation of HBL-1 is facilitated) in these L3/L4-stage seam cells.

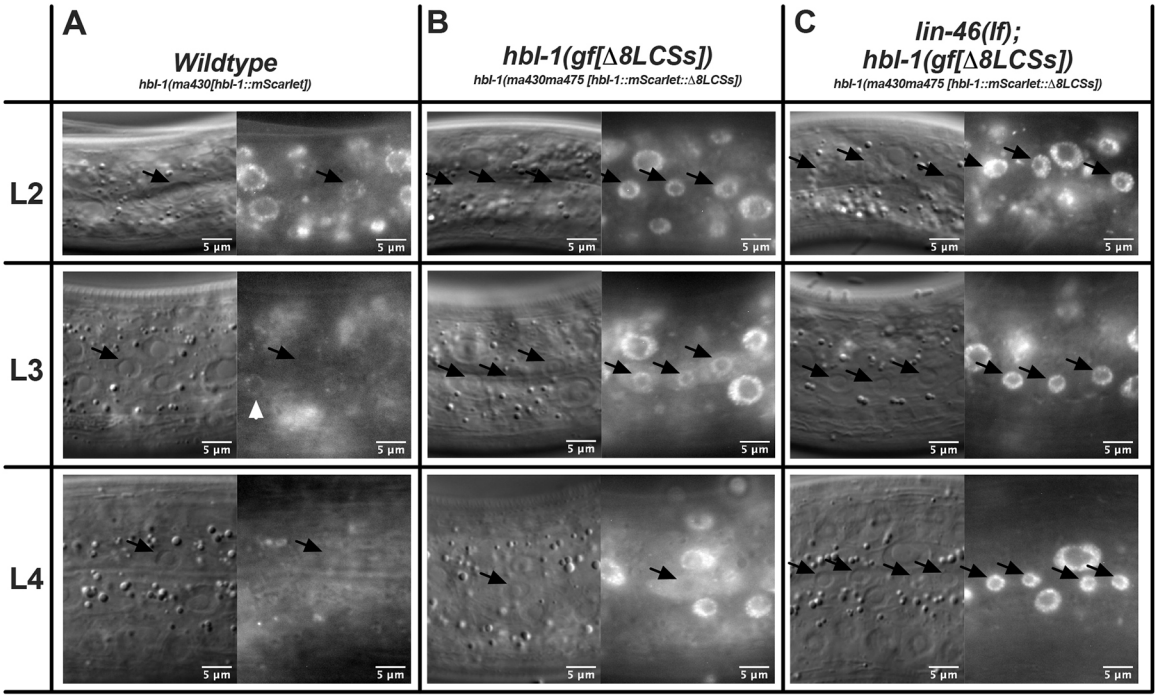


Fig. 2. HBL-1 accumulates in the cytoplasm of L3- and L4-stage seam cells in larvae lacking LCSs in the *hbl-1* 3′UTR, and *lin-46* is required for this cytoplasmic accumulation of HBL-1. All seam cell nuclei are marked with black arrows. (A) HBL-1 is expressed in hypodermal seam (black arrows) and hyp7 cells of L1- and L2-stage larvae but is not detected in L3- and L4-stage larvae. A rare occurrence of an L3-stage hyp7 cell expressing HBL-1 is marked with a white arrowhead. Note that HBL-1 is absent in all other nuclei, including the seam nucleus (black arrow) in the L3 stage panel. (B) In animals that lack a region of the *hbl-1* 3′UTR containing eight *let-7* complementary sites (LCSs), *hbl-1(ma430ma475)*, HBL-1 is present in hypodermal seam (black arrows) and hyp7 cells at all stages (L2-L4 shown). In these animals, HBL-1 accumulates in the cytoplasm of seam cells, at the L3 and L4 stages. L3-stage seam cells still display a marked nuclear HBL-1 accumulation whereas L4-stage seam cells display almost an equal distribution of HBL-1 in both the nucleus and the cytoplasm. (C) In animals lacking *lin-46* in addition to the eight LCSs in the *hbl-1* 3′UTR, HBL-1 does not accumulate in the cytoplasm of seam cells in L3- or L4-stage animals, rather HBL-1 accumulates in the nucleus of seam cells at all stages.

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lin-28 is required for the nuclear accumulation of HBL-1 in the seam cells of L2-stage larvae

lin-28(lf) animals skip L2-stage proliferative seam cell fates, suggesting that *lin-28* is required to support the activity of HBL-1 at the L2 stage. Moreover, loss of *lin-28* can suppress the extra seam cell phenotypes of *hbl-1* gain-of-function (gf) mutants (Fig. 1B), indicating that *lin-28* is also required to support high and/or prolonged expression of HBL-1. We observed that in double mutants containing *hbl-1(ma430ma475)* and *lin-28(lf)*, HBL-1 accumulates primarily in the cytoplasm and is largely absent from the nucleus of the L2-stage seam cells (Fig. 3B versus 3A), which explains the lack of HBL-1 activity and the suppression of *hbl-1(gf)* extra seam cell phenotypes in larvae lacking *lin-28*.

In order to test the possibility of an effect of the linker or the mScarlet-I tag on the localization of HBL-1, we also tagged another transcription factor, *daf-12* (nuclear accumulation of which is not affected by *lin-28(lf)*; Fig. S2A), at its endogenous locus with the same linker and mScarlet-I and determined whether the localization of DAF-12 changed in *lin-28(lf)* animals (Fig. S2). We observed that, unlike HBL-1, linker-mScarlet-I tagged DAF-12 did not accumulate in the cytoplasm of L2-stage seam cells in *lin-28(lf)* animals (Fig. S2B), suggesting that the linker-mScarlet-I tag could not be the cause of cytoplasmic accumulation of HBL-1.

lin-46 activity is required for the cytoplasmic accumulation of HBL-1 in both *hbl-1(ma430ma475)* and *lin-28(lf)* animals

HBL-1 accumulated both in the nucleus and the cytoplasm of L3- and L4-stage seam cells in *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* larvae (Fig. 2B). When we combined *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* with *lin-46(lf)* we did not observe cytoplasmic accumulation of HBL-1 at the L3 and L4 stages, rather HBL-1 accumulated in the nucleus at all stages (Fig. 2C; Fig. S1C), indicating that the L3/L4-stage cytoplasmic accumulation of HBL-1 requires *lin-46* activity.

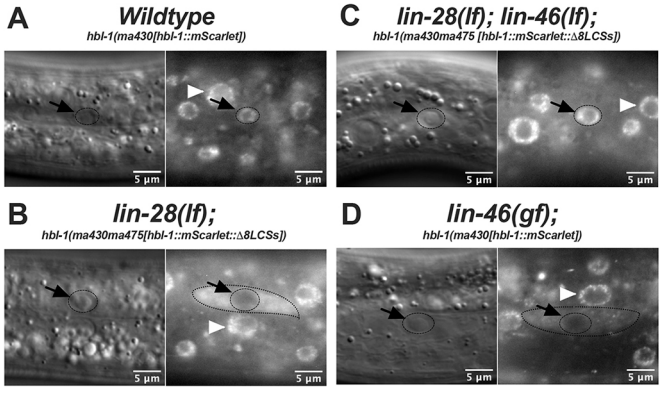


Fig. 3. The *lin-28-lin-46* pathway regulates nuclear accumulation of HBL-1. Differential interference contrast and fluorescent images of hypodermal (seam and hyp7) cells in L2-stage larvae. Black arrows and dashed circles show seam nuclei and white arrowheads show examples of hyp7 nuclei. (A) HBL-1 accumulates in the nucleus in wild-type animals. (B) HBL-1 is dispersed in the cytoplasm (outlined by dotted line) of L2-stage seam cells in *lin-28(lf)* animals, indicating that *lin-28* is required for the nuclear accumulation of HBL-1 in the seam cells of L2-stage larvae. Note that HBL-1 still accumulates in hyp7 nuclei (e.g. white arrowhead). (C) *The lin-28* target *lin-46* is required to prevent the nuclear accumulation of HBL-1 in L2-stage seam cells of *lin-28(lf)* larvae. (D) Precocious/ectopic LIN-46 expression is sufficient to reduce the nuclear accumulation of HBL-1 in the seam cells of L2-stage larvae. HBL-1 is present in the cytoplasm (outlined by dotted line) of the seam cell in the picture. Note that HBL-1 still accumulates in hyp7 nuclei (e.g. white arrowhead).

HBL-1 accumulated primarily in the cytoplasm of L2-stage seam cells in *lin-28(lf)* larvae (Fig. 3B). By contrast, in L2 larvae lacking both *lin-28* and *lin-46* HBL-1 no longer accumulated in the cytoplasm of L2 or later stage seam cells, rather HBL-1 accumulated in the nucleus of the seam cells at all stages (Fig. 3C; Fig. S1F).

In brief, *lin-46(lf)* resulted in the loss of cytoplasmic HBL-1 accumulation at all stages, accompanied by restoration of nuclear accumulation of HBL-1 (Fig. S1).

Precocious LIN-46 expression is sufficient to reduce the nuclear accumulation of HBL-1

Lastly, we found that precocious LIN-46 expression, which is sufficient to suppress the extra seam cell phenotypes of *hbl-1(ma354)* (Fig. 1B), is also sufficient to reduce the nuclear accumulation of HBL-1 (Fig. 3D).

These results, together with those presented above show that the suppression of *hbl-1(ma354)* phenotypes by *lin-28(lf)* or *lin-46(gf)* is accompanied by increased cytoplasmic accumulation of HBL-1 and a reduction in the nuclear accumulation of HBL-1. These findings suggest that LIN-46 negatively regulates HBL-1 activity by hindering its nuclear accumulation.

Spatiotemporal occurrence of cytoplasmic HBL-1 accumulation coincides with LIN-46 expression

Endogenously tagged *lin-46* is expressed in the seam cells of L3- and L4-stage larvae but not in the seam cells of L1- and L2-stage larvae (Ilbay et al., 2019 preprint). Therefore, the onset of LIN-46 expression coincides with the onset of cytoplasmic accumulation of HBL-1 in *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* animals (which are, unlike the wild type, not capable of properly downregulating HBL-1 at the end of the L2 stage owing to the lack of LCSs) (Fig. S1). In *lin-28(lf)* animals, LIN-46 is expressed precociously (Ilbay et al., 2019 preprint), starting in mid L1-stage seam cells. This precocious onset of LIN-46 expression in *lin-28(lf)* animals also coincides with the precocious onset of the cytoplasmic accumulation of HBL-1 in *lin-28(lf)* animals (Fig. S1).

We note that LIN-46 expression is detected in the seam cells but not in the hyp7 cells (Ilbay et al., 2019 preprint), and the presence/absence of LIN-46 expression in these two hypodermal cell types correlates with the presence/absence of cytoplasmic accumulation of HBL-1. For example, whereas HBL-1 accumulates in both the cytoplasm and the nucleus of seam cells L3/L4-stage *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* animals (Fig. 2B), HBL-1 accumulates only in the nuclei of the hyp7 cells (Fig. 2B). Similarly, whereas HBL-1 accumulates primarily in the cytoplasm of L2-stage seam cells of *lin-28(lf)* animals, HBL-1 still accumulates in the nuclei of hyp7 cells in these animals (Fig. 3B, black arrow versus white arrowhead).

In brief, cytoplasmic accumulation of HBL-1 was observed in the hypodermal cells where (seam) and when [by the L3 stage in wild type and by the late L1 stage in *lin-28(lf)*] LIN-46 is expressed (Fig. S1).

DISCUSSION

Our results suggest that the *C. elegans lin-28-lin-46* pathway regulates the nuclear accumulation of HBL-1, a transcription factor that specifies L2-stage proliferative cell fates and opposes the progression to L3-stage self-renewal cell fates during *C. elegans* development. *lin-28* is required for the nuclear accumulation of HBL-1 in hypodermal seam cells at the L2 stage, which is, in turn, necessary for the execution of L2-stage proliferative cell fates. The *lin-28* target *lin-46* is responsible for preventing the nuclear accumulation of HBL-1 in *lin-28(lf)* animals, and in wild-type

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animals *lin-28*-mediated repression of the LIN-46 expression at the L1 and L2 stages (Ilbay et al., 2019 preprint) allows the nuclear accumulation of HBL-1 at those early larval stages. Using a *lin-46* 5′UTR mutation that renders LIN-46 expression poorly repressed by *lin-28*, we show that precocious LIN-46 expression in the seam cells of the L1/L2-stage larvae is sufficient to reduce the nuclear accumulation of HBL-1. Furthermore, using *hbl-1* gain-of-function mutations with *let-7* and *lin-4* sites deleted from the *hbl-1* 3′UTR, we show that the *lin-28-lin-46* pathway acts in parallel with *let-7* family microRNAs. Hence, these two parallel pathways – the microRNA pathway controlling the rate of synthesis of HBL-1 through repression of *hbl-1* mRNA translation, and the *lin-28-lin-46* pathway controlling the nuclear accumulation of HBL-1 – function together to ensure the precision and robustness of stage-specific HBL-1 downregulation (Fig. 4).

In wild-type animals, HBL-1 and LIN-46 are expressed at temporally distinct stages: HBL-1 is expressed at the L1 and L2 stages whereas LIN-46 is expressed at the L3 and L4 stages. In larvae of certain mutants, such as *hbl-1(gf)* L3 and L4 larvae, or *lin-28(0)* L1 and L2 larvae, LIN-46 and HBL-1 expression overlap, and cytoplasmic accumulation of HBL-1 is observed, accompanied by a reduction in the nuclear accumulation of HBL-1. Our data further show that the nucleus-to-cytoplasm displacement of HBL-1 in these contexts depends on *lin-46* activity. Therefore, one might have expected to observe cytoplasmic accumulation of HBL-1 after the L2-to-L3 transition in wild-type larvae, when LIN-46 begins to accumulate. Curiously, in wild-type animals, cytoplasmic accumulation of HBL-1 is not evident at any stage, despite the presence of LIN-46 in L3-adult animals. If LIN-46 causes cytoplasmic accumulation of HBL-1 in the wild type, why is HBL-1 not detected in L3 and L4 larvae? One explanation could be that in wild-type larvae, the post-translational repression of HBL-1 activity by LIN-46 (via cytoplasmic localization) functions semi-redundantly with the translational repression of HBL-1 by *let-7* family microRNAs. In this scenario, the microRNA pathway could exert the lion's share of HBL-1 downregulation at the L2-to-L3 transition, and the upregulation of LIN-46 at the L3 stage could play a secondary role to inhibit the nuclear accumulation of any residual HBL-1 protein. Indeed, in support of this idea, a low level of HBL-1 expression persists in the nuclei of L3-stage seam cells in *lin-46(lf)* animals (Fig. S1). This scenario is also consistent with the differing strengths of the weaker retarded phenotypes of *lin-46(lf)* animals compared with *hbl-1(gf)* animals under standard culture conditions (Fig. 1B).

Interestingly, conditions such as diapause-inducing stress signals that enhance *lin-46(lf)* phenotypes (Ilbay and Ambros, 2019) also result in a reduction in the expression of *let-7* family microRNAs (Hammell et al., 2009), resulting in a shift from primarily microRNA-mediated regulation of HBL-1 to primarily LIN-46-mediated regulation (Ilbay and Ambros, 2019). In this context, where wild-type larvae experience diapause-inducing stress signals, and hence LIN-46-mediated cytoplasmic localization becomes the primary mode of HBL-1 downregulation, we expected to observe cytoplasmic accumulation of HBL-1 after the L2d-to-L3 transition.

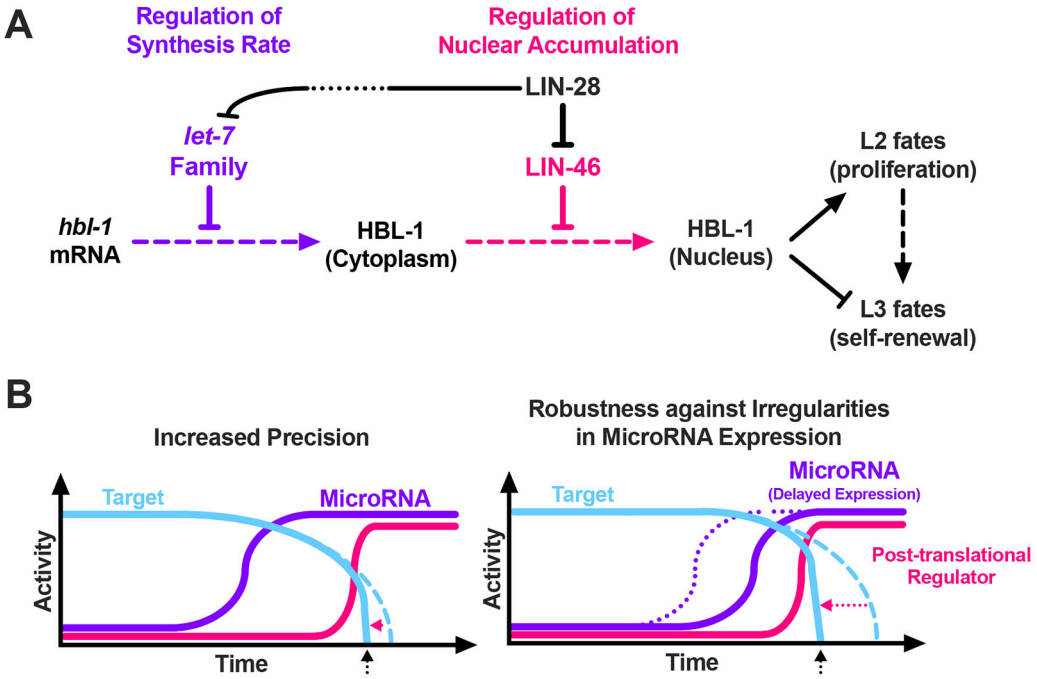


Fig. 4. Regulation of gene activity through microRNA-mediated repression of translation accompanied by post-translational regulation of microRNA targets. (A) The conserved RNA-binding protein LIN-28 indirectly (indicated by the dotted line break) regulates the transcription (Tsialikas et al., 2017) and activities (Nelson and Ambros, 2019) of *let-7*-family (*mir-48*, *mir-84*, *mir-241*) microRNAs, which inhibit the synthesis of HBL-1. LIN-28 also represses the expression of LIN-46 (Ilbay et al., 2019 preprint), which controls the nuclear accumulation of HBL-1. Temporal downregulation of LIN-28 at the end of the L2 stage allows LIN-46 to accumulate, which acts together with the *let-7* family microRNAs to ensure precise and robust temporal downregulation of HBL-1 activity. (B) Hypothetical activity trajectories of a microRNA and its target(s) against time are plotted. Dashed blue lines represent the trajectory in the absence of the hypothetical post-translational regulator. Black arrows indicate a specific critical time when the target must be downregulated to permit normal development. A post-translational regulator of a microRNA target can increase the precision of temporal downregulation of the target (red arrow, left) or confer robustness against irregularities in microRNA expression (red arrow, right). This second scenario is similar to what is thought to happen in *C. elegans* larvae developing in the presence of pheromones or other L2d-inducing conditions: *let-7*-family expression is delayed (Bethke et al., 2009; Hammell et al., 2009) and LIN-46 activity becomes more important for downregulating HBL-1 (Ilbay and Ambros, 2019). Thus, LIN-46 confers robustness against a physiological delay in the expression of *let-7* family microRNAs.

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However, we could not detect any cytoplasmic HBL-1::mScarlet-I fluorescence in wild-type L3 larvae under L2d-promoting conditions. It is possible that the cytoplasmic HBL-1 in L3 larvae is unstable and/or dispersed such that the HBL-1::mScarlet-I signal is below the limit of detection in our fluorescence microscopy assays.

Sequence homology places LIN-46 into a conserved protein family, members of which include bacterial MOEA as well as human GPHN (gephyrin), which are implicated in molybdenum co-factor (MoCo) biosynthesis (Schwarz et al., 2009). GPHN has also been reported to function as a scaffold protein that is required for clustering of neurotransmitter receptors (Feng et al., 1998; Kneussel et al., 1999), and has been shown to physically interact with several other proteins (Fritschy et al., 2008), including tubulin (Kirsch et al., 1991), dynein (Fuhrmann et al., 2002) and mTOR (Sabatini et al., 1999). It is not known whether LIN-46 possesses MOEA-related enzymatic activity and/or has scaffolding functions similar to GPHN, and, if so, how such activities could (directly or indirectly) contribute to inhibition of the nuclear accumulation of a transcription factor such as HBL-1.

Analysis of the HBL-1 amino acid sequence does not reveal a predicted nuclear localization signal (NLS) that could mediate HBL-1 nuclear transport. If HBL-1 has an unconventional or 'weak' NLS, it is possible that other unknown factors may be required to efficiently couple HBL-1 to the nuclear import machinery. LIN-46 might inhibit HBL-1 nuclear accumulation by binding or modifying a factor that is crucial for HBL-1 nuclear transport. Alternatively, LIN-46 could bind or modify HBL-1 directly in order to prevent its association with the nuclear import machinery. It is also possible that LIN-46 could act not by directly preventing nuclear import of HBL-1, but by causing HBL-1 to be trapped in the cytoplasm, for example through the formation of LIN-46–HBL-1 complexes in association with a cytoplasmic compartment.

Regulation of nuclear accumulation in the context of temporal cell-fate specification during *C. elegans* development has not previously been reported. Other transcription factors, including LIN-14, DAF-12 (Antebi et al., 2000) and LIN-29, play key roles in regulating temporal cell fates during *C. elegans* development. These other transcription factors are also regulated by microRNAs, like HBL-1, and regulation of their temporal abundances is important for the proper execution of stage-specific cell fates. Although we have no evidence that LIN-46 may also regulate the nuclear/cytoplasmic partitioning of other heterochronic pathway proteins (except for DAF-12; Fig. S2), our findings suggest that similar post-translational mechanisms might be in place to function in parallel with the microRNA-mediated regulation and hence promote the robust temporal regulation of key developmental regulators.

Many *let-7* targets, as well as many targets of other microRNAs, in worms, flies and mammals are transcription factors (Ambros, 2004; Bartel, 2004; Enright et al., 2004; John et al., 2004). Therefore, similar mechanisms, whereby a transcription factor is regulated both by a microRNA and in parallel by a gene product that controls the nuclear accumulation of the same transcription factor, may be common. Additionally, the regulation of the *let-7* microRNA by LIN-28 is widely conserved. Targets of LIN-28 in other species may have roles, similar to LIN-46, in regulation of *let-7* targets, controlling their nuclear accumulation in particular and their activities by means of post-translational interventions in general. A dual control of a gene product – its synthesis rate by microRNAs and activity by post-translational regulators – would allow more precise and/or more robust transitions between active and inactive states (Fig. 4B).

MATERIALS AND METHODS

C. elegans culture conditions

C. elegans strains used in this study and corresponding figures in the paper are listed in Table S2. *C. elegans* strains were maintained at 20°C on nematode growth media (NGM) and fed with the *Escherichia coli* HB101 strain.

Assaying extra seam cell phenotypes

The worms were scored at the young adult stage (determined by the gonad development) for the number of seam cells using fluorescence microscopy with the help of the *maIs105* [*pCol-19::gfp*] transgene, which marks the lateral hypodermal cell nuclei, or the *wIs51*[*pScm::gfp*] transgene, which marks the seam cell nuclei.

Each circle on the genotype versus number of seam cells plots shows the observed number of seam cells on one side of a single young adult worm. Twenty worms (except for wild type, n=12) for each genotype were analyzed and the average number of seam cells are denoted by vertical bars in the genotype versus number of seam cell plots. Student's t-test was used to calculate statistical significance when comparing different genotypes. GraphPad Prism 8 software was used to plot the graphs and for statistical analysis.

Microscopy

All differential interference contrast and fluorescent images were obtained using a ZEISS Imager Z1 equipped with a ZEISS Axiocam 503 mono camera, and ZEN Blue software. Prior to imaging, worms were anesthetized with 0.2 mM levamisole in M9 buffer and mounted on 2% agarose pads. ImageJ Fiji software was used to adjust the brightness and contrast of the images to enhance the visualization of the fluorescent signal. All images were taken using the same microscopy settings and a standard exposure time for all larval stages and genetic background, but because the brightness and contrast of the individual images were enhanced separately, the signal intensities do not represent the relative expression levels and cannot be used to compare expression levels across different larval stages of genetic backgrounds.

Generation of new alleles using CRISPR/Cas9

CRISPR/Cas9 genome editing tools were used to generate the *hbl-1* 3′UTR deletion alleles, the *lin-46* open-reading frame (ORF) deletion allele, and to tag the *daf-12* gene with GFP and mScarlet-I at its endogenous locus.

For the *hbl-1* 3′UTR deletions and the *lin-46* ORF deletion (for alleles, see Table S2), a mixture of plasmids encoding SpCas9 (pOI90), and a pair of single guide RNAs (sgRNAs, expressed from pOI83; Ilbay and Ambros, 2019) targeting both sites of interest (for primers, see Table S3) and the *unc-22* gene (pOI91) as co-CRISPR marker (Kim et al., 2014), and a rol-6(su1006)-containing plasmid (pOI124) as co-injection marker was injected into the gonads of young adult worms. F1 roller and/or twitcher animals (~50 or more worms until the desired allele was detected) were cloned and screened by PCR amplification (for primers, see Table S3) for the presence of the expected size PCR product consistent with deletion of the genomic region spanning between the sites targeted by the pair of guides.

To tag *daf-12* at the endogenous locus with the same linker and mScarlet-I sequence as the *hbl-1*(ma430) allele, a homologous recombination (HR) donor plasmid (pOI193) and sgRNA plasmid (pOI93) were included in the CRISPR mix, which contained plasmids pOI90 (spCas9), pOI91 (*unc-22* guide) and pOI124 (*rol-6*). The HR plasmid pOI193 contains the C-terminal end of the *daf-12* gene fused in-frame with the linker and mScarlet-I sequence, subcloned from pOI191, which was used to tag *hbl-1* to generate the *ma430* allele. To tag *daf-12* with GFP, instead of pOI193, an HR donor plasmid (pOI122) that contained the GFP sequence flanked by HR sequences was included in the CRISPR mix.

In all new CRISPR alleles, genomic regions spanning the deletion site or the HR arms and the tags introduced were sequenced using Sanger sequencing. For each allele, a single worm with a precise (HR) edited locus was cloned and backcrossed twice before being used in the experiments.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: O.I., V.A.; Methodology: O.I., V.A.; Formal analysis: O.I.; Investigation: O.I.; Resources: V.A.; Writing - original draft: O.I.; Writing - review & editing: V.A.; Visualization: O.I.; Supervision: V.A.; Project administration: V.A.; Funding acquisition: V.A.

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Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.183111.supplemental

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Supplemental Figures

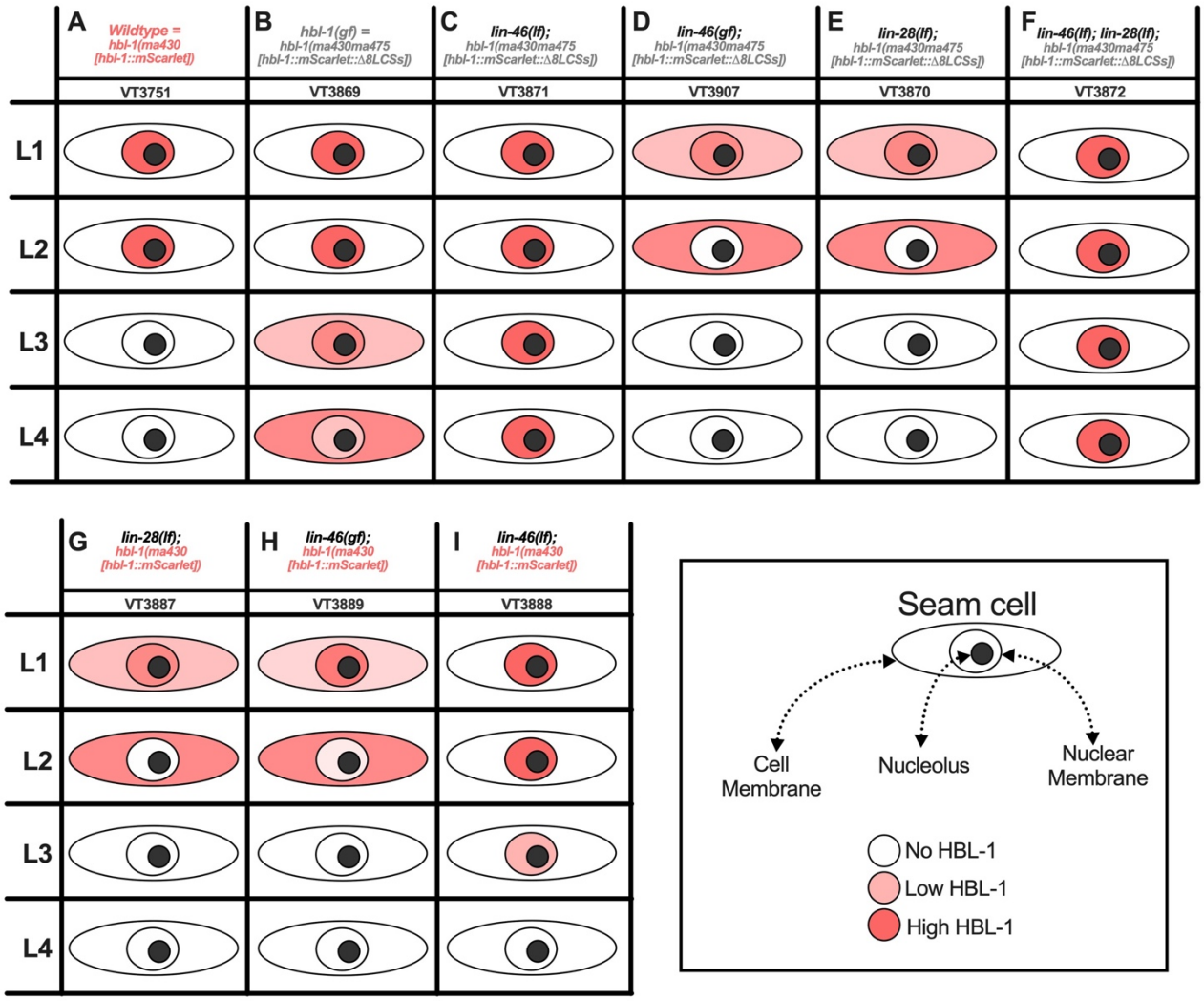


Fig. S1. Schematic representations of nucleo-cytoplasmic localization of HBL-1 in hypodermal seam cells across four larval stages and various genetic backgrounds. HBL-1 expression (pink color) is denoted in nuclear/cytoplasmic compartments of seam cells for each larval stage and genotype. Ten animals for each larval stage and genotype were analyzed. Genotypes and corresponding strain names (VT#) are denoted at the top of each column.

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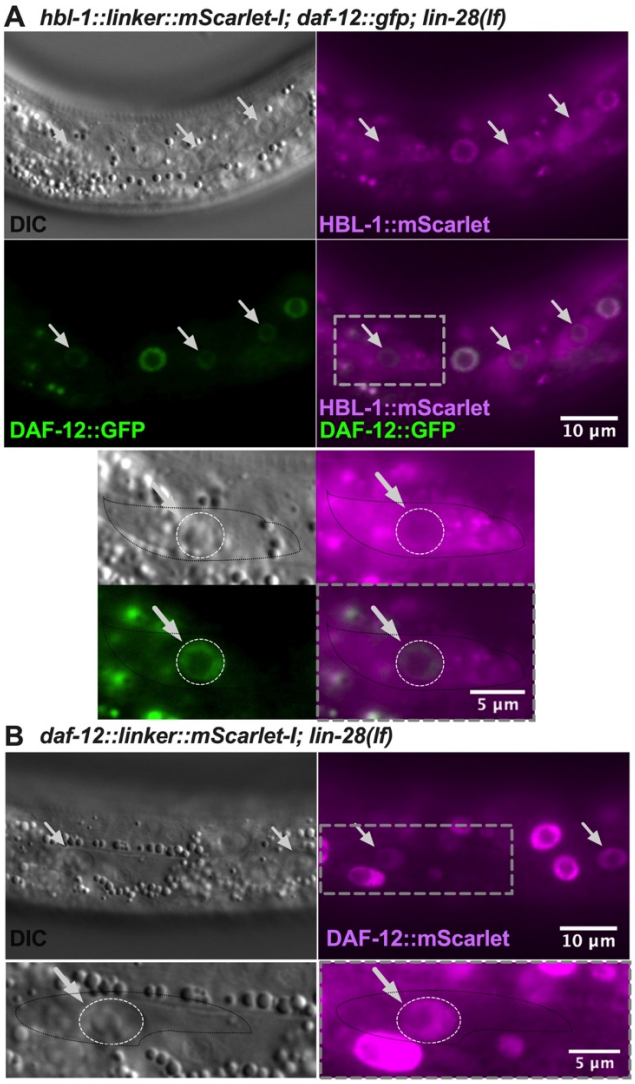


Fig. S2. Nuclear localization of GFP or mScarlet-I tagged DAF-12 is not regulated by *lin-28*. DIC and fluorescent images showing hypodermal seam and hyp7 nuclei of L2 stage *lin-28(lf)* larvae. White arrows indicate the seam cell nuclei. (A) In the absence of *lin-28*, HBL-1 is majorly excluded from the nuclei of L2 stage seam cells whereas nuclear localization of DAF-12 (GFP tagged) is not affected. (B) The linker and mScarlet-I tag that was used to tag *hbl-1* [*hbl-1(ma430)*] do not have an effect on nuclear localization of DAF-12 in the *lin-28(lf)* background.

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Supplemental Tables

Gene	Allele Name	Description	FP tagged	25 bp sequences flanking the deletion site and the size of the deletion
hbl-1	*ma322*	Deletes 4 LCSs in the 3'UTR	No	TCGTTAAGGAAACACTTCCCATAGC <-545 bp deleted-> ATTGTTTAACTATGCACACATTTGT
	ma294	Deletes 7 LCSs in the 3'UTR	No	ATCTAGAAGCAATTGTATACTGTTC <-892 bp deleted-> AAACTTCAGTGCGTTCTTCTGTCAT
	ma293	Deletes 8 LCSs in the 3'UTR	No	ACTTGTTACTGTTTTCTTTTACCTC <-1051 bp deleted-> AAACTTCAGTGCGTTCTTCTGTCAT
	ma430 *ma475*	Deletes 8 LCSs in the 3'UTR	Yes (mScarlet-I)	TGTTACTGTTTTCTTTTACCTCTGA <-1048 bp deleted-> AAACTTCAGTGCGTTCTTCTGTCAT
	ma354	Deletes all LCSs in the 3'UTR	No	TTCTAATCATGGCCAGTTTCTTGCA <-1120 bp deleted-> GTGCGTTCTTCTGTCATCATGTACA
lin-46	*ma385*	Deletes all *lin-46* exons = *lin-46* null	No	AAACCAAGAATTGTATCAGTGGGAG <-1681 bp replaced with "AATTGT"> TACGCTTTGCATGAAAATTCACCAG
daf-12	*ma497*	Introduces a GFP tag at the C-terminal end of *daf-12*	Yes (GFP)	GCCAGGAGAATTTTTCAAAATCAAA <-agtaaaggagaa…864 bp of GFP …gaactatacaaa-> TAGACCTACTAGAAATCATCTAC(C/g)A Stop codon of daf-12, C/g = PAM mutation (Note: GFP tag contains a sense mutation = [TGATTTTAAAGAAGATGGAA[A/g]CATTCTTGGACACAAATTGG])
	ma498	Introduces a linker::mScarlet-I tag (same as ma430) at the C-terminal end of *daf-12*	Yes (mScarlet-I)	GCCAGGAGAATTTTTCAAAATCAAA <- tctggaggtggatctggaggtggatctggaggtggatct:: GTCAGCAAGGGAGAGGCAGTTATCA --rest of the 643 bp mScarlet-- CGGAGGAATGGACGAGCTCTACAAG-> TAGACCTACTAGAAATCATCTAC(C/g)A Linker, Stop codon of daf-12, C/g = PAM mutation

Table S1. New *hbl-1, lin-46, daf-12* alleles generated for this study

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Strain name	Genotype	Related figures
VT1367	*maIs105 V*	Fig. 1A&B
VT3307	*maIs105 V; hbl-1(ma294) X.*	Fig. 1A
VT3336	*maIs105 V; hbl-1(ma293) X.*	Fig. 1A
VT3399	*maIs105 V; hbl-1(ma332) X*	Fig. 1A
VT3500	*wIs51 V; hbl-1(ma354) X*	Fig. 1A&B
VT3593	*lin-46(ma385) I; maIs105 V.*	Fig. 1B
VT3696	*lin-46(ma385) wIs51 V; hbl-1(ma354) X*	Fig. 1B
VT790	*lin-28(n719) I; maIs105 V*	Fig. 1B
VT3571	*lin-28(n719) I; maIs105 V; hbl-1(ma354) X*	Fig. 1B
VT3698	*lin-28(n719) I; lin-46(ma385) wIs51 V; hbl-1(ma354) X*	Fig. 1B
VT3855	*lin-46(ma467) maIs105 V*	Fig. 1B
VT3891	*lin-46(ma467) maIs105 V; hbl-1(ma354) X*	Fig. 1B
VT3751	*maIs105 V; hbl-1(ma430[hbl-1::mScarlet-I]) X*	Fig. 2A&3A, Fig. S1A
VT3869	*wIs51 V; hbl-1(ma430ma475[hbl-1::mScarlet-I::UTRdel]) X*	Fig. 2B, Fig. S1B
VT3871	*wIs51 lin-46(ma385) V; hbl-1(ma430ma475[hbl-1::mScarlet-I::UTRdel]) X*	Fig. 2C, Fig. S1C
VT3870	*lin-28(n719) I; maIs105 V; hbl-1(ma430ma475) X*	Fig. 3B, Fig. S1E
VT3872	*lin-28(n719) I; wIs51 lin-46(ma385) V; hbl-1(ma430ma475) X*	Fig. 3C, Fig. S1F
VT3889	*lin-46(ma467) maIs105 V; hbl-1(ma430) X*	Fig. 3D, Fig. S1H
VT3907	*lin-46(ma467) maIs105 V; hbl-1(ma430ma475) X*	Fig. S1D
VT3887	*lin-28(n719) I; maIs105 V; hbl-1(ma430) X; syIs(ajm-1::gfp)*	Fig. S1G
VT3888	*lin-46(ma385) maIs105 V; hbl-1(ma430) X; syIs(ajm-1::gfp)*	Fig. S1I
VT3922	*lin-28(n719) I; daf-12(ma497[daf-12::gfp] hbl-1(ma430[hbl-1::mscarlet-I]) X*	Fig. S2A
VT3924	*lin-28(n719) I; daf-12(ma498[daf-12::mScarlet-I] X*	Fig. S2B

Table S2. *C. elegans* strains used in this study.

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Cloning/ PCR	Primer Name	Primer Sequence	Plasmid name and/or purpose
Cloning: Annealed primer-pairs that are cloned into pOI83 to express sgRNAs	priOI250F	tcttggaacgcactgaagtttgagg	pOI104& pOI138 are injected together to delete a region of *hbl-1* 3'UTR encompassing all *let-7*-complementary sites.
	priOI251R	aaaccctcaaacttcagtgcgttcc	
	priOI421F	tcttgaggtgtacgtgcaagaaac	
	priOI422R	aaacgtttcttgcacgtacacctc	
	priOI362F	tcttgcgtagatcaaccacgtctc	pOI113&pOI160 are injected together to delete the *lin-46* coding sequences (all exons).
	priOI363R	aaacgagacgtggttgatctacgc	
	priOI517F	tcttgtcaatccaatgagttcttc	
	priOI518R	aaacgaagaactcattggattgac	
	priOI175F	tcttgattgaaggcatggcatcgtt	pOI93 is used to tag *daf-12* with gfp or mScarlet-I.
	priOI176R	aaacaacgatgccatgccttcaatc	
Cloning: HR Template to tag daf-12 with gfp (pOI122)	priOI346F	cgggaattctttaggttcttcggtt tcttcgac	5-prime HR arm, PCR amplified using N2 DNA as template. pOI346F contains an EcoRI cut site (underlined).
	priOI349R	attctcctggcagctcttcg	
	priOI232F	ggtgaagccgaagagctgccaggag aatttttcaaaatcaaaagtaaagg agaagaacttttc	To amplify GFP from pCM1.53. Primers contain tails complementary to *daf-12* 5-prime and 3-prime HR arms to allow PCR stitching of HR arms with GFP.
	priOI239R	ggaggcaatatagaatcaagttgtg aggtattgaaggcatggcatcgttt cgtagatgatttctagtaggtctat ttgtatagttcgtccatgccatg	
	priOI348F	aaacgatgccatgccttcaatac	3-prime HR arm, PCR amplified using N2 DNA as template. pOI346F contains a HindIII cut site (underlined).
	priOI347R	gccaagcttggctaggctgcatgaa tcac	
Cloning: HR Template to tag daf-12 with linker::mScarlet-I (pOI193)	priOI670	gaaggtctcatctggaggtggatct ggaggtggatctggaggtggatctg tcagcaagggagaggcagttatc	To amplify the linker+mScarlet sequences from pOI191, which is the plasmid that was used as the HR template to tag *hbl-1* with mScarlet. Primers contain Golden Gate (NEB) cloning tails (underlined).
	priOI671	gaaggtctcacttgtagagctcgtc cattcc	
	priOI741	ggctacggtctcccagatttgattt tgaaaaattctcctggc	To amplify 5-, 3-primer HR arms and the backbone of pOI122 to fuse with the linker+mScarlet sequence. Primers contain Golden Gate (NEB) cloning tails (underlined).
	priOI742	ggctacggtctcccaagtagaccta ctagaaatcatctac	
PCR Primers	priOI147F	tgcaaaccgacctagtgcat	These primers flank the *hbl-1* 3'UTR. They are used to detect large deletions in the F1/F2 progeny of pOI104& pOI138 (in a CRISPR mix) injected animals.
	priOI148R	aaagtagccagtcccctcgt	
	priOI515F	accatactgctgaaatcccaa	These primers flank the *lin-46* exons. They are used to detect the ORF deletion in the F1/F2 progeny of pOI113&pOI160 (in a CRISPR mix) injected animals.
	priOI516R	taagtacgcaaacacgctgc	
	priOI3	gaggcgtttcgtcaaagttg	These primers flank the HR arms in pOI122&pOI193. They are used to detect GPF or mScarlet integration in F1/F2 progeny of animals injected with CRISPR mixes containing these plasmids together with pOI93.
	priOI114	cccttatgggttggctgaga	

Table S3. Cloning and PCR Primers used in this study.

Development • Supplementary information