The Caenorhabditis elegans hunchback-like Gene
lin-57/hbl-1 Controls Developmental Time
and Is Regulated by MicroRNAs

Juan E. Abrahante, Aric L. Daul, Ming Li,
Mandy L. Volk, Jason M. Tennessen, Eric A. Miller,
and Ann E. Rougvie*
Department of Genetics, Cell Biology
and Development
University of Minnesota
6-160 Jackson Hall
321 Church Street SE
Minneapolis, Minnesota 55455

Summary

Temporal control of development is an important aspect of pattern formation that awaits complete molecular analysis. We identified lin-57 as a member of the C. elegans heterochronic gene pathway, which ensures that postembryonic developmental events are appropriately timed. Loss of lin-57 function causes the hypodermis to terminally differentiate and acquire adult character prematurely. lin-57 is hbl-1, revealing a role for the worm hunchback homolog in control of developmental time. Significantly, fly hunchback (hb) temporally specifies cell fates in the nervous system. The hbl-1/lin-57 3'UTR is required for postembryonic downregulation in the hypodermis and nervous system and contains multiple putative binding sites for temporally regulated microRNAs, including let-7. Indeed, we find that hbl-1/lin-57 is regulated by let-7, at least in the nervous system. Examination of the hb 3'UTR reveals potential binding sites for known fly miRNAs. Thus, evolutionary conservation of hunchback genes may include temporal control of cell fate specification and microRNA-mediated regulation.

Introduction

The heterochronic genes of C. elegans provide a framework for dissecting the molecular mechanisms that time development in animals (reviewed in Rougvie, 2001). Mutations in these genes cause temporal transformations in cell fate; certain cells express developmental programs normally restricted to either earlier or later stages within the same cell lineage. For example, heterochronic gene mutations alter the temporal identity of a set of stem cell-like hypodermal cells, called seam cells, such that certain stage-specific division patterns are omitted or reiterated, resulting in premature or delayed terminal differentiation of the hypodermis.

A remarkable feature of the heterochronic gene pathway is that it includes among its members the first two microRNAs (miRNAs) identified, encoded by lin-4 and let-7 (Lee et al., 1993; Reinhart et al., 2000). These small, noncoding RNAs are key to programming temporal progression of cell fate in C. elegans, and their phylogenetic conservation and expression patterns suggest that they may also function in temporal control mechanisms in other organisms (Lagos-Quintana et al., 2002; Pasquinelli et al., 2000). The 21 nt lin-4 miRNA is a component of an “early timer” (Ambros, 2000), which specifies seam cell temporal identity from embryo hatching through the L3 stage. lin-4 transcription begins in the mid L1 stage and progressively downregulates protein accumulation from lin-14 and lin-28 mRNAs by binding to antisense sites in their 3'UTRs (Feinbaum and Ambros, 1999; Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993). The graded decrease in LIN-14 and LIN-28 levels is critical for the temporal progression to the L3 stage seam cell fate. During the L3 stage, let-7 is transcriptionally activated and initiates a “late timer” by downregulating target genes, including lin-41, ultimately allowing temporal seam cell identity to progress to the terminally differentiated adult state (Reinhart et al., 2000; Slack et al., 2000).

The ultimate target of the heterochronic genes is the LIN-29 zinc finger transcription factor whose presence in the L4 stage hypodermis is required for seam cell terminal differentiation (Bettinger et al., 1996; Rougvie and Ambros, 1995). Hypodermal LIN-29 accumulation occurs abnormally early in precocious mutants, while, in retarded mutants, it is delayed or absent. In lin-29 mutants, the larval program of seam cell division replaces terminal differentiation at the fourth molt and is reiterated through supernumerary molting cycles.

Genetic evidence suggests that key heterochronic genes that act to connect lin-4 activation in the L1 stage to lin-29 activation in the L4 stage remain to be identified. For example, the weak heterochronic phenotype of lin-41 null mutants predicts the existence of additional, partially redundant genes in the late timer. Similarly, additional control inputs to lin-29 are suggested by the observation that let-7 null mutations produce a retarded phenotype with the execution of a single extra “larval” molt, which is less severe than that caused by lin-29 mutations. Moreover, not all of the let-7 targets have been found, and there may be additional microRNAs that act with let-7 to regulate lin-41 and these other targets.

To identify additional players, we carried out a genetic screen for heterochronic mutants and identified lin-57; loss of lin-57 activity causes precocious seam cell terminal differentiation during the L3 molt. Molecular cloning revealed that lin-57 is hbl-1, a gene previously identified on the basis of its sequence relationship to Drosophila hunchback (Fay et al., 1999). For simplicity and consistency with published literature, we will henceforth refer to lin-57 as hbl-1. hbl-1 is partially redundant with lin-41 in the late timer and is regulated by the let-7 miRNA. We discuss the role of Drosophila hunchback in the temporal specification of cell fate and speculate that microRNA regulation may be a conserved feature of hunchback genes.

*Correspondence: rougvie@cbs.umn.edu
Results

Locomotion-Based Screen for Heterochronic Mutants
To identify additional members of the heterochronic gene pathway, we screened for mutants with a temporally altered locomotion behavior. Loss-of-function (lf) mutations in rol-1 cause a stage-specific locomotion defect: larvae swim in the normal sinusoidal pattern, but adults “roll” in circles (Brenner, 1974; Higgins and Hirsh, 1977). This adult-specific Rol phenotype depends upon the synthesis of adult cuticle and is temporally altered by mutations in previously identified heterochronic genes. For example, lin-4(lf) mutants never synthesize adult cuticle, and, consequently, lin-4 rol-1 double mutants never roll. Conversely, lin-14(lf) mutants synthesize adult cuticle early, and rol-1; lin-14 double mutants roll as larvae.

rol-1(e91) animals were mutagenized, and 8000 haploid genomes were screened for animals that either roll precociously, during larval stages, or fail to roll as adults. A precocious roller that identified hbl-1 as a member of the heterochronic gene pathway was among the mutants recovered. rol-1(e91); hbl-1(ve18) mutant animals begin rolling as L4 stage larvae.

Precocious Hypodermal Cell Terminal Differentiation in hbl-1(ve18) Mutants
A larval Rol phenotype can also be generated by mutations in genes that do not regulate the time of adult cuticle synthesis (Cox et al., 1980). To test hbl-1(ve18) mutants for a timing defect, we monitored developmentally staged animals for the execution of the seam cell terminal differentiation program. Seam cells divide in a characteristic pattern until the L4 molt, when they instead terminally differentiate, fuse, and secrete an adult-type cuticle containing alae, cuticular ridges that are a hallmark of terminal differentiation.

We observed a precocious hypodermal phenotype in hbl-1(ve18) mutants: seam cell fusion and adult alae synthesis occurred one stage early, during the L3 to L4 molt (referred to as the L3 molt; Table 1; Figures 1A and 1B). Seam cell lineages appear essentially wild-type in hbl-1(ve18) mutants until the L3 molt, when, in contrast to wild-type, they do not divide (Figure 1C). These results indicate that the precocious phenotype of hbl-1 mutants includes many aspects of seam cell terminal differentiation. However, the precocious phenotype is incomplete in one respect. The seam cell nuclei do not exit permanently from the division cycle during the third molt as expected for full execution of the terminal differentiation program; they usually divide again during the L4 stage intermolt, resulting in seam syncytia containing as many as 28 nuclei, rather than 16 as in wild-type animals (Figures 1D and 1E; 2). The L4 stage nuclear division phenotype is not unique to the ve18 allele; it is also seen when hbl-1 activity is depleted by RNA interference (RNAi) (Figure 2). One explanation for this phenotype is that LIN-29 levels in hbl-1 mutants may not rise to sufficient levels by the L3 molt to completely inhibit the nuclear division.

hbl-1(ve18) mutants remain in the molting cycle following the third molt, executing a fourth molt during which adult-type cuticle is again synthesized (Table 1). hbl-1(ve18) mutants often have trouble executing this molt and remain stuck in the L4 stage cuticle.

Precocious Vulval Cell Divisions in hbl-1(ve18) Mutants
hbl-1(ve18) mutants also have a protruding vulva (Pvul), which appears after the L3 molt, causing an egg-laying defect (Table 1). Examination of vulval cell divisions in hbl-1(ve18) animals revealed precocious divisions of vulval precursor cells (VPCs) (Figures 1F and 1G). In wild-type animals, three VPCs, P5.p–P7.p (Pn.p), undergo three stereotypic rounds of cell division during the mid L3 stage to generate the 22 cells that comprise the vulva (Sulston and Horvitz, 1977). In hbl-1(ve18) mutants, one or more of these cells initiates its division pattern by the L2 molt. This phenotype is also observed in hbl-1(RNAi) animals and with higher penetrance; more Pn.p divisions are completed by the L2 molt, and, often, morphogenesis has begun.

The seam cell division, precocious fusion, and vulval phenotypes are all suppressed in ve18 mutants when the animals develop through the alternative dauer larva stage (Table 1; Figure 2). Phenotypic suppression by...
predicted wild-type open reading frame is disrupted by or secrete alae, and the animals continue to molt. lin-29 downstream ATG could produce a truncated protein through/H11032. In one, the exon 2/3 splice junction was shifted 1 nt 5
nine zinc fingers (Figure 3A). The hypothesis that ve18 mutants produce a partially functional HBL-1 protein is supported by the observation that a genomic PCR product containing the hbl-1(ve18) lesion rescues the hbl-1(ve18) phenotype (data not shown).

Animals bearing hbl-1(ve18) in trans to lwDf12, a deficiency that removes the locus, are viable and appear similar to ve18 homozygotes (Table 1). However, our molecular analyses indicate that ve18 is a hypomorphic allele, and depletion of hbl-1 activity by RNAi suggests that the null phenotype is embryonic lethality (Fay et al., 1999). Because RNAi administered postembryonically (see below) results in a phenotype similar to ve18 animals, we interpret the hbl-1(ve18) phenotype as deficient in postembryonic function.

**hbl-1 Acts Upstream of lin-29 in the Heterochronic Pathway**

To position hbl-1 in the heterochronic gene pathway, we analyzed double mutants between hbl-1 and the retarded heterochronic genes lin-29 and lin-4. In contrast to the precocious phenotype observed in hbl-1 mutant animals, hbl-1; lin-29 double mutants resemble lin-29 mutants and fail to execute the seam cell terminal differentiation program (Table 1). Seam cells in these double mutants remain in the cell cycle and fail to fuse or secrete alae, and the animals continue to molt.

Molecular epistasis analysis confirms that hbl-1 acts through lin-29. In hbl-1(ve18) mutants, LIN-29 accumulates precociously in the L3 stage hypodermis, rather than the L4 as in wild-type (Figure 1H; Bettinger et al., 1996). Together, these data indicate that hbl-1 requires...
lin-29(+) activity to exert its function, and HBL-1 times seam cell differentiation by preventing hypodermal LIN-29 accumulation before the L4 stage.

Of the four previously described heterochronic genes whose loss-of-function causes precocious phenotypes, mutations in three, lin-14, lin-28, and lin-42, suppress the retarded hypodermal phenotype of lin-4 mutations (Ambros, 1989; Jeon et al., 1999). In contrast, mutations in lin-41 do not, even though lin-41 acts downstream of lin-4 by other criteria (Slack et al., 2000). Examination of lin-4; hbl-1 double mutants reveals a failure of seam cell terminal differentiation at the fourth molt (Table 1), and, therefore, as was found for lin-41, lin-4 is epistatic to hbl-1 in this test.

We next asked whether lin-41 and hbl-1 activities must both be reduced to allow adult cuticle synthesis in lin-4 mutants. Seam cell terminal differentiation was examined in a lin-4(e912) background where hbl-1 and/or lin-41 activities were depleted by RNAi (see Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/4/5/625/DC1). To provide a sensitive measure of adult cuticle synthesis, we also assayed for expression of col-19::gfp, a reporter gene whose expression is restricted to adult hypodermis in wild-type animals (Abrahante et al., 1998). Depletion of neither hbl-1 or lin-41 alone restores alae synthesis to lin-4 mutants, although loss of lin-41 activity allows weak col-19::gfp expression. In contrast, simultaneous depletion of lin-41 and hbl-1 partially restores alae synthesis (52% of animals have detectable patches of alae) and increases the intensity of col-19::gfp expression to nearly wild-type levels. This partial restoration of the adult seam cell program to lin-4 mutants indicates that hbl-1 acts downstream of lin-4 and is partially redundant with lin-41.

hbl-1 RNA Interference Produces Multiple Phenotypes

Previous RNAi experiments showed that depletion of HBL-1 causes a variety of phenotypes, including embryonic lethality, malformed larvae, and egg laying-defective adults, but did not reveal a heterochronic defect (Fay et al., 1999). To reconcile these results with the hbl-1(ve18) phenotype, we performed a series of RNAi experiments (see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/4/5/625/DC1). hbl-1(RNAi) resulted in animals that were semi-Dumpy and Pvul. Examination of the hypodermis of these animals during the L3 molt revealed an hbl-1(ve18)-like phenotype; the seam cells terminally differentiate precociously. Abnormalities were also observed

Figure 2. hbl-1 Loss-of-Function Alters Seam Cell Lineages

Seam nuclei were counted on one side of animals of the indicated genotype and stage by scm::gfp expression. Six seam cell lineages undergo a proliferative division during the L1 molt (Figure 1C) to increase the wild-type cell number from 10 to 16. RG733 is wIs78 (containing scm::gfp). RG734 is wIs78; hbl-1(ve18). RNAi was administered by feeding. RG734 dauer animals were selected by 30 min incubation in 1% SDS, recovered on seeded plates, and examined at post-dauer stages (L3, L4, and Adult).
at earlier stages, especially with higher dsRNA concentrations. The most severe phenotype observed was embryonic lethality near the start of morphogenesis, as described by Fay et al. (1999).

cDNA and Northern blot analyses indicate that a single species of hbl-1 message is produced (Fay et al., 1999). Together with the observed concentration dependence of RNAi experiments, this suggests that the distinct phenotypes seen result from different levels of HBL-1 protein remaining in the RNAi-treated animals. Embryonic lethality likely results from complete depletion of HBL-1, while a partial reduction reveals the postembryonic requirement, indicating that postembryonic development is more sensitive to perturbations of HBL-1 levels. Consistent with this view, hbl-1 expression levels are highest during embryogenesis and decrease thereafter (Fay et al., 1999), and, thus, small alterations in HBL-1 levels may have a more dramatic effect during later development.

Depletion of hbl-1 activity by RNAi administered postembryonically results in a slight decrease in seam cell number in L2 stage animals (Figure 2). This observation suggests that the proliferative cell division at the L1 molt (see Figure 1C) is skipped in some seam cells and that hbl-1 also plays a role in programming this stage-specific event.

The hbl-1 3' Untranslated Region Is Required for Temporal Downregulation

Because temporal regulation through 3' UTR sequences is a common theme in the heterochronic gene pathway and fly hunchback spatial expression is translationally regulated via its 3' UTR (Wharton and Struhl, 1991), it is of interest to test whether the hbl-1 3' UTR plays a key regulatory role. Fay et al. (1999) used hbl-1::gfp fusion constructs that differed in their 3' UTRs to show that the hbl-1 3' UTR does not regulate the spatial pattern of HBL-1 accumulation. In these constructs, the hbl-1 promoter and sequences encoding the first 133 amino acids of HBL-1 are fused in-frame to gfp. One construct then contains the native hbl-1 3' UTR, while the other contains the unc-54 3' UTR (Fire et al., 1999), which does not confer appreciable developmental regulation to linked genes.

We examined transgenic animals containing these constructs to test whether the 3' UTR contributes to the postembryonic temporal pattern of hbl-1 expression. hbl-1::gfp::hbl-1 expression is observed at high levels in the main body hypodermis (hyp7) of newly hatched L1 larvae and decreases as development proceeds to the early L3 stage, becoming undetectable thereafter (Figures 4A, 4C, and 4E). In contrast, animals bearing hbl-1::gfp::unc-54 accumulate GFP in hyp7 at all stages of development, suggesting that hbl-1 expression is regulated posttranslationally through its 3' UTR (Figures 4A–4F). hbl-1::gfp::hbl-1 expression is not detected in the seam after the mid L1 stage, and this expression pattern appears unaffected by 3' UTR sequences (Figures 4A–4F).

Expression of the hbl-1::gfp::hbl-1 reporter is also temporally downregulated in neuronal cells. At hatching, hbl-1::gfp::hbl-1 expression is most intense in P cells (Fay et al., 1999; Figure 4A), which migrate into the ventral cord and undergo several rounds of division in the late L1 stage to give rise to ventral cord neurons and Pn.p’s, including the six vulval precursor cells. Expression is maintained at moderate levels in the P-derived neurons of the ventral nerve cord through the mid L3 stage, and then it lessens as development proceeds to the adult, with weak expression sometimes remaining detectable (Figures 4E and 4G). A similar decrease in expression was observed in neurons of the anterior nerve ring. Finally, hemaphrodite-specific neuron (HSN) expression was strong in the L1 and L2 stages and decreased during the L3 stage, becoming undetectable in adults (Figures 4M and 4N). In contrast to the temporal downregulation of hbl-1::gfp::hbl-1, hbl-1::gfp::unc-54 expression remained high in each of these neuronal cell types at later developmental stages (Figures 4G–4P). Thus, although the hbl-1 3' UTR is dispensable for spatial regulation, it is required for temporal downregulation of hbl-1::gfp during postembryonic development of the hypodermis and nervous system.

To test whether the hbl-1 3' UTR is sufficient to program temporal downregulation, we substituted the hbl-1 3' UTR into a col-10::lacZ::unc-54 reporter, which is otherwise expressed in the hypodermis at all developmental stages (Wightman et al., 1993). Similar to the hbl-1::gfp::hbl-1 fusion, the col-10::lacZ::hbl-1 construct was temporally downregulated (Figures 4Q–4T). col-10::lacZ::hbl-1 expression was detected at later stages than...
Figure 4. *hbl-1* Is Temporally Regulated via Its 3' UTR

(A–P) Fluorescent images of animals bearing *hbl-1::gfp* reporter fusions. Developmental stage and 3' UTR identity are indicated.

(A) GFP is detected in hyp7 and P cells and weakly in the seam.
the corresponding gfp construct, extending into the L4 stage and occasionally including adult head or tail hypodermal cells, likely reflecting a difference in sensitivity of detection or protein perdurance. Together, these experiments indicate that the hbl-1 3 'UTR plays a temporal regulatory role.

The hbl-1 3' UTR Contains Multiple Putative let-7 Binding Sites

Several lines of evidence implicate the heterochronic gene let-7 as a candidate negative regulator of hbl-1. The let-7 microRNA negates regulate target genes through antisense binding sites in their 3' UTRs (Reinhart et al., 2000; Slack et al., 2000), and the onset of let-7 expression during the L3 stage coincides with downregulation of hbl-1::gfp fusions, at least in neuronal cells. In addition, whereas loss of hbl-1 function causes a precocious phenotype, loss-of-function mutations in let-7 cause a retarded phenotype, in which seam cells terminally differentiate one stage late, during a supernumerary molt (Reinhart et al., 2000). Notably, animals that presumably overexpress HBL-1 because of multiple copies of hbl-1(+) on extrachromosomal arrays can have let-7-like phenotypes, including weak or incomplete alae synthesis and bursting (21% of adults examined, n = 66).

The hbl-1 3' UTR contains multiple evolutionarily conserved regions predicted to form heteroduplexes with the let-7 miRNA (Figures 5A and 5B). The overall sequence identity between the C. elegans hbl-1 3' UTR and that of C. remanei and C. briggsae is 76% and 71%, respectively, and contains eight 12–30 nt blocks of perfect conservation. The putative let-7 binding sites vary in the degree of conservation observed. The most highly conserved site resides near the 3' end of the UTR (nt 1266–1287). It is 100% conserved in C. briggsae and 91% conserved in C. remanei. Moreover, there is a 23 out of 25 bp match encompassing this sequence in the region of the lin-41 3' UTR that confers its let-7 responsiveness (Slack et al., 2000).

Two conserved, putative lin-4 binding sites are also present in the hbl-1 3' UTR (Figure 5C), raising the possibility that the lin-4 miRNA may also participate in the downregulation of hbl-1.

Loss of hbl-1 Activity Suppresses let-7 Defects

If hbl-1 acts downstream of the let-7 miRNA, then mutations in hbl-1 should suppress the heterochronic defects associated with let-7 mutations. To test this idea, we hatched eggs homozygous for the let-7 null allele, mn112, on bacteria expressing hbl-1 dsRNA. In contrast to the retarded defect of let-7(mn112) animals grown on control bacteria, adult-type cuticle was synthesized as early as the L3 molt in animals fed bacteria producing hbl-1 dsRNA, indicating that let-7 acts through hbl-1 to time seam cell differentiation (Table 2). In addition, the let-7 larval lethality was also suppressed. Analysis of hbl-1(ve18) let-7(mn112) double mutants confirmed these results (Table 2).

Experiments presented above showed that lin-41 and hbl-1 are partially redundant with respect to lin-4 suppression. If the steps controlled by these two genes reside downstream of let-7, a similar redundancy should be uncovered in analysis of let-7 suppression. Indeed, simultaneous reduction of hbl-1 and lin-41 in a let-7(null) background dramatically improves the penetrance and expressivity of the precocious (L3 molt) alae phenotype relative to single depletion of either gene activity (Table 2). Depletion of hbl-1 in a let-7 background results in patches of weak alae on 70% of L3 molt animals, and depletion of lin-41 results in small patches of well-formed alae. In contrast, simultaneous loss of these gene activities yields L3 molt animals with generally full-length alae that are wild-type in appearance. Moreover, rare patches of well-formed alae can form two stages early in these animals, at the L2 molt. This enhanced precocious phenotype in animals lacking both hbl-1 and lin-41 activities is also observed in a let-7(+/-) background; 35% of these animals synthesize patches of alae at the L2 molt (Table 2), and the inappropriate L4 stage nuclear division observed in hbl-1 single mutants is suppressed (Figure 2). These results indicate that let-7 acts through hbl-1 and lin-41, which together prevent seam cell terminal differentiation until the final molt.

If let-7 plays a predominant role as an hbl-1 repressor, we would expect misregulation of the hbl-1::gfp::hbl-1 construct in a let-7 mutant background. However, hypodermal expression of hbl-1::gfp::hbl-1 in let-7(n2853) mutants was largely unchanged from the wild-type pattern. Only occasional weak hbl-1::gfp::hbl-1 expression

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**References**

1. Slack, J. and Slack, J. (2000). Overexpression of HBL-1 because of multiple copies of hbl-1(+) on extrachromosomal arrays can have let-7-like phenotypes, including weak or incomplete alae synthesis and bursting.
2. Reinhart, J. and Slack, J. (2000). HBL-1 is required for the expression of let-7 miRNA.
could be discerned in the hypodermis of let-7 mutants after the early L3 stage, suggesting that factors other than let-7 are required for efficient posttranscriptional downregulation of hbl-1::gfp::hbl-1 in hyp7. Consistent with this view, the drop in hypodermal hbl-1 expression begins prior to activation of let-7 during the L3 stage. In the nervous system, hbl-1::gfp::hbl-1 deregulation was more pronounced, with 35% of animals exhibiting increased expression in the VNC of let-7 mutant adults during blind scoring tests (Figures 4G and 4H). Similar deregulation was also observed in the anterior nerve ring (Figures 4J and 4K). These results suggest that let-7 plays a role in controlling hbl-1::gfp::hbl-1 expression in the nervous system.

![Diagram](image)

**Figure 5. miRNA Binding Sites in the 3' UTRs of Worm and Fly hunchback Genes**

(A) Stick diagram comparing sequence identities among C. elegans, C. briggsae, and C. remanei hbl-1 orthologs. The hbl-1 3' UTR is represented by the red bar. stretches of ≥10 nt that are identical among all three species are indicated by black boxes. The positions of potential let-7 and lin-4 binding sites shown in (B) and (C) are indicated.

(B) Nucleotides conserved in hbl-1 in all three Caenorhabditidae species, blue; nucleotides that are not conserved but that could maintain base pairing in each species, purple. D. virilis sequence was unavailable for the putative mir-3 binding sites.

(E) Sequence alignment of the let-7, mir-84, mir-48, and mir-241 miRNAs (Lau et al., 2001). Nucleotides identical to let-7, red; those shared by at least two other miRNAs, blue.

**Discussion**

**hbl-1 Is a Developmental Timing Gene**

The C. elegans hunchback-like gene hbl-1 regulates postembryonic developmental time. In ve18 mutants, vulval cell divisions occur abnormally early during the L2 stage, seam cell fusion and adult cuticle synthesis occur precociously during the L3 molt, and seam cell nuclei divide inappropriately during the L4 stage. Removal of hbl-1 activity postembryonically through RNAi causes similar defects, and, in addition, proliferative seam cell divisions are sometimes skipped during the L1 molt. These observations indicate that the worm hunchback ortholog functions at multiple times during postembryonic development and plays key roles in specifying temporal cell fates in the vulva and hypodermis.

Because the L1 molt seam cell division phenotype correlates with seam cell expression of hbl-1::gfp during the L1 stage, hbl-1 may function cell autonomously in the seam to control this early division pattern. However, hbl-1::gfp is not detected in the seam after the L1 stage, nor is it detected in the vulval precursor cells (Fay et al., 1999), suggesting that hbl-1 may function non-cell autonomously to time cell divisions at later stages in these tissues. One candidate tissue for producing an hbl-1-dependent signal is the hyp7 syncytial hypodermis that surrounds the seam, abuts the VPCs, and is a major contributor to cuticle synthesis. The roller phenotype used in our screens is likely to result from hyp7-mediated ultrastructural defects in the cuticle (Peixoto...
Table 2. Loss of hbl-1 Activity Suppresses a let-7(null) Mutation

<table>
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<tr>
<th>Strain</th>
<th>dsRNA construct</th>
<th>Larval Lethality (%)</th>
<th>Sterile (%)</th>
<th>Brood Size</th>
<th>Number of Animals with Alae (n = 20)</th>
<th>L3 Molt Alae Extent (% with gaps)</th>
<th>L4 Molt Alae Extent (% with gaps)</th>
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**Notes:**
- All strains also contained unc-3(e151), except for hbl-1 alone (bottom line). unc-3 was present in the let-7(mn112) strain, necessitating its use as a control. RNAi experiments comparing N2 and unc-3 gave essentially identical results in these assays (data not shown), so unc-3 has been omitted for simplicity.
- RNAi was performed by ingestion of dsRNA-expressing bacteria, as described in Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/4/5/625/DC1.
- For these RNAi experiments, n = 25 except for let-7 on control vector (n = 50) and dsbhl-1 (n = 28). Larval lethality is a measure of vulval bursting at the L4/A molt, a phenotype associated with let-7 mutations. For the genetic analyses on the bottom three lines, n = 50, 54, and 49, respectively. Brood size is averaged over the 25 animals scored.
- Animals with alae were counted as the number of sides scored, typically, one side per animal. Alae varied in quality and extent. Alae quality ranges from weak and indistinct to robust, as observed in wild-type L4 molt animals. Alae extent was graded as +++++ (86%–100% of full-length), ++++ (66%–85%), ++ (26%–55%), and + (6%–25%). The percentage of animals with alae that had gaps is given in parentheses.
- Animals contained small patches (1–2 seam cells) of robust alae.
- One animal had three progeny and 1 had two progeny; all 5 exploded as adults and gave no offspring.
et al., 1998), and, therefore, our identification of ve18 as a precocious roller suggests that temporal identity of hyp7 is also affected in hbl-1 mutants. Thus, similar to the seam, hyp7 initiates its adult program, possibly including signaling to surrounding tissues, precociously. There is precedent for signaling from hyp7: lin-15(+) is required in hyp7 for repression of cell divisions in VPCs that do not normally contribute to vulva formation (Herman and Hedgecock, 1990). Thus, one possibility is that hbl-1 function in hyp7 is required for correct timing of seam and vulval cell fates.

**hunchback and Control of Developmental Time**

In flies, *hunchback* (*hb*) is best known for its crucial role in spatial patterning of the embryo, as a member of the gap class of segmentation genes (Lehmann and Nusslein-Volhard, 1987; Struhl et al., 1992; Tautz et al., 1987). Null mutations in *hb* cause deletion of specific sets of segments along the anterior-posterior axis.

Similar to the regulation of *hbl-1* during temporal patterning of the nematode, the *hb* 3' UTR plays a key role during spatial patterning in the early fly embryo. Pumilio, Nanos, and Brat form a quaternary complex on a pair of conserved 32 nt elements in the *hb* 3' UTR, called Nanos response elements (NREs), and repress *hb* translation in the posterior of the embryo, contributing to the formation of an *hb* protein gradient emanating from the anterior (Sonoda and Wharton, 1999, 2001; Wharton and Struhl, 1991). The *hbl-1* 3' UTR does not contain canonical fly NREs, although there are three “B box” sequences (AUUGUA), two of which are conserved in *C. briggsae* and *C. remanei*. The *C. elegans* genome does contain candidate homologs of the key fly *hb* regulatory proteins (Frank et al., 2002; Subramaniam and Seydoux, 1999; Wickens et al., 2002), and the evolutionary maintenance of these components raises the possibility that additional aspects of *hb* regulation are conserved between these species, perhaps acting through divergent 3' UTR sequences.

*hb* also plays a role in patterning the fly central nervous system where, intriguingly, relative to our studies, *hb* programs temporal specification of cell fate (Ishikii et al., 2001). Neuroblasts divide in invariant stem cell-like patterns, giving rise to specific lineages, depending on the position and timing of divisions. These neuroblasts sequentially express a set of transcription factors, and the fate of progeny cells produced depends on the transcription factor present at their birth (Cui and Doe, 1992; Ishikii et al., 2001; Kambadur et al., 1998; Mellerick et al., 1992). The first transcription factor expressed is Hb, and cell division gives rise to neurons that maintain *hb* expression and adopt a characteristic “first-born” cell fate. *Krüppel* expression appears next in the neuroblast and is then maintained in the cells generated at the subsequent division, which adopt a “second-born” fate. In *hb* mutant animals, the first-born identity is skipped, and the progeny cells execute the second-born fate prematurely (Ishikii et al., 2001). In contrast, continued expression of *hb* at later developmental times results in a reiteration of the first-born fate.

These Hb-mediated temporal phenotypes are reminiscent of those reported here for *hbl-1* loss-of-function. Loss of *hbl-1* activity causes the adult seam cell fate to be executed one stage too early, whereas overexpression of *hbl-1* can cause failure of seam cell terminal differentiation, likely as a result of reiteration of L4 fates in the adult. Together, these observations suggest that the general involvement of *hunchback* proteins in programming temporal identity within specific cell lineages has been evolutionarily conserved.

There is a dramatic difference in seam cell versus neuroblast cell cycle time. Seam cells are divided once per molt, at approximately 7 hr intervals, whereas fly neuroblasts undergo rapid cell divisions in the embryo, on the order of 40 min. How the rapid transitions in transcription factor expression are controlled in fly neuroblasts is unknown, but it is tempting to speculate that miRNAs could play a role in facilitating expeditious transnational downregulation of *hb* and other transcription factors in this developmental context.

Indeed, the *hb* 3' UTR contains potential fly microRNA binding sites in evolutionarily conserved regions (Figure 5D). Of particular note, *mir-184*, *mir-4*, and *mir-13a* are predicted to duplex with sequences overlapping the two NREs (NRE1 at nt 46–78 of the 3' UTR and NRE2 at nt 98–129), suggesting involvement of these miRNAs in fundamental aspects of *hb* regulation. Whether the predicted *mir-3* binding sites are conserved in *D. virilis* is unknown, but the two *D. melanogaster* sites share high identity (13 out of 18 nt). Moreover, *mir-3* and *mir-4* are expressed during embryogenesis, but not thereafter (Lagos-Quintana et al., 2001), consistent with an early regulatory role. In summary, our work extends the similarities between the worm and fly *hunchback* genes beyond the level of sequence conservation. Both genes are deployed to temporally specify cell fates, and their expression patterns rely on 3' UTR sequences likely to be modulated through the action of microRNAs.

**The hbl-1 Message Is an miRNA Target**

Postembryonic temporal downregulation of *hbl-1* in the worm nervous system and hypodermis is programmed, at least in part, through its 3' UTR, which contains multiple putative *let-7* binding sites that are evolutionarily conserved. In the nervous system, an *hbl-1:gfp::hbl-1* reporter construct is temporally deregulated in a *let-7* mutant background; enhanced expression is observed in the central nerve cord and anterior nerve ring of adults. Together, these results imply that the *hbl-1* 3' UTR is a direct target of the *let-7* miRNA.

The extent of *hbl-1:gfp::hbl-1* misexpression in *let-7* mutants is less than might be expected if *let-7* acts alone to downregulate neuronal expression and suggests that additional factors, perhaps other microRNAs, act together with *let-7*. Indeed, a large and diverse family of miRNAs has been discovered in *C. elegans* and other organisms (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Reinhart et al., 2002). Among the worm miRNAs reported are three, *mir-84*, *mir-48*, and *mir-241*, that share sequence identity with *let-7* RNA and are expressed with the same temporal specificity as is *let-7* (Figure 5E; Lim et al., 2003). The sequence conservation among these miRNAs, particularly between *mir-84* and *let-7* (81% identical), suggests that they may have target sites in common. Thus, complete temporal deregulation of the *hbl-1* reporter may require simultaneous inactivation of multiple miRNAs.

The role of *let-7* in control of *hbl-1* in the hypodermis is less clear. The simplest way to interpret *let-7* suppression by *hbl-1*, together with *let-7* binding sites in the
hbl-1 3' UTR, is that hbl-1 is a direct target of the let-7 miRNA. However, hypodermal hbl-1::gfp expression begins to subside in the L2 and disappears in the early L3, prior to let-7 accumulation in the mid to late L3 stage (Reinhart et al., 2000). Assuming that the hbl-1::gfp construct (which contains a 6.4 kb 5' flanking sequence through the first three introns) contains all relevant enhancer regions, this implies that 3' UTR-mediated down-regulation of hbl-1 in hyp7 is controlled by other factors, perhaps including earlier-acting miRNAs.

let-7 could add to the repression of hbl-1 mRNA from the mid L3 stage onward, ensuring its silence at late developmental stages. However, consistent hbl-1::gfp::hbl-1 misexpression was not detected in the hypodermis of let-7 mutants, suggesting only a minor role for let-7 or redundant action by let-7-related genes. Alternatively, a low threshold level of the HBL-1 presumed transcription factor (not detectable by gfp assay) may be required for hypodermal function. Thus, small changes in HBL-1 level could lead to major developmental consequences through deregulation of target genes.

Temporal regulation of hbl-1 differs from that of lin-41, the other known let-7 target. lin-41::gfp is expressed in both neurons and hypodermis but is temporally down-regulated only in the hypodermis (Slack et al., 2000), and this downregulation is mediated by let-7 control of the lin-41 3' UTR during the L4 stage (Reinhart et al., 2000). The discordant patterns of regulation suggest inherent differences between the hbl-1 and lin-41 3' UTRs and the assembled factors that orchestrate their function.

*Hbl-1 Functions with lin-41 in the Late Timer*

Reduction of hbl-1 activity by mutation or RNAi does not fully suppress let-7 null mutations. Explanations for this partial epistasis include incomplete loss of hbl-1 function, misexpression of let-7 targets, or redundancy at the hbl-1 step in the pathway. Our work suggests that the let-7 target, lin-41, is at least part of the answer. Simultaneous removal of hbl-1 and lin-41 activities produces stronger suppression of the let-7 phenotype than does single depletion of either gene (Table 2). In let-7(+/-) animals, depletion of hbl-1 and lin-41 activities produces a fully penetrant L3 molt phenotype and can cause terminal differentiation at the L2 molt, one stage earlier than in either single mutant. Together, these results indicate that let-7 acts through both hbl-1 and lin-41 and that these genes function with partial redundancy to inhibit premature activation of the adult hypodermal program at the L2 and L3 molts in wild-type animals.

These findings extend the intriguing parallels between the early and late timers of the heterochronic gene pathway, which together mediate stage-specific temporal identities (Figure 6). Each timer is initiated by a microRNA that has two known targets; in the early timer, lin-4 downregulates lin-14 and lin-28, and, in the late timer, let-7 acts through both hbl-1 and lin-41. In each case, one target encodes a transcription factor (LIN-14 and HBL-1), and the other encodes a protein with hallmarks of a translational regulator (LIN-28 and LIN-41) (Hong et al., 2000; Moss et al., 1997; Slack et al., 2000). Since loss-of-function for each pair of targets causes enhanced precocious phenotypes (Ambros, 1989; this work), it appears that both transcriptional and translational controls are necessarily integrated into both timers to ensure proper timing of cell fate specification.

*Evidence for a Branched Heterochronic Gene Pathway*

Previous studies have generally supported a linear pathway of heterochronic genes, with lin-4 acting as the most upstream and global regulator. Our analyses suggest that the pathway is branched. Concomitant loss of hbl-1 and lin-41 activities suppresses the let-7 mutant phenotype more completely than that of lin-4. Loss of hbl-1 and lin-41 activities only weakly restores alae synthesis at the L4 molt in lin-4 mutants, whereas it leads to essentially complete execution of the adult seam cell program at the L3 molt in a let-7 mutant background. These observations indicate that lin-4 or the genes it regulates have additional targets, which time the adult hypodermal program independently of hbl-1 and lin-41. Thus, multiple temporal inputs converge upon the transcription factor LIN-29, indicating that a branched pathway functions to ensure proper timing of seam cell terminal differentiation. Elaboration of these proposed branches will require searches for additional components of the heterochronic gene pathway.

*Experimental Procedures*

**Nematode Strains and Methods**

Worm strains were grown and maintained as in Sulston and Hodgkin (1988). Experiments were performed at 20°C, unless otherwise noted. The following strains were used: BW1891 ctsi37 (hbl-1::gfp::unc-54 3' UTR), BW1932 ctsi39 (hbl-1::gfp::hbl-1 3' UTR),
Molecular Analysis of hbl-1

ve18 was mapped by standard techniques. Clones were tested for transformation rescue of the ve18 phenotype with rol-6(sv1006sd) as a coinjection marker (Mello and Fire, 1995). YACs and cosmids were obtained from The C. elegans Genome Consortium at the Sanger Center, Cambridge. YACs were prepared as described (Davies et al., 1999). The ve18 allele was sequenced by PCR amplification of predicted exons and intron/exon junctions. C. briggsae and C. remanei hbl-1 clones were identified from a gridded fosmid library (Incyte Pharmaceuticals) and a cDNA library (gift of the Ambros lab), respectively. The col-10::lacZ::unc-54 construct used was pC10U54 (Wightman et al., 1993). The hbl-1 3' UTR was substituted into this construct to yield col-10::lacZ::hbl-1. Additional methodological detail is available in Supplemental Data at http://www.developmentalcell.com/cgi/content/full/4/5/625/DC1.

RNA Interference Assays

RNAi was performed by feeding as in protocol 1 of Kamath et al. (2001), except that 12.5 μg/ml tetracycline was included in the plates. Eggs were dissected from gravid adults in M9 buffer and transferred to new RNAi plates and analyzed at the indicated stage. For analysis of let-7 unc-3 adults were dissected, and unc hatchings were transferred to new RNAi plates for analysis. When two bacteria were used together, they were grown in separate cultures for 8–12 hr, and equal volumes were mixed prior to seeding plates. The following plasmids were used: control vector, pPD129.36; dsh-1, pJA44; and dsl-1 (gift of F. Slack). pJA44 contains the 1.1 kb Ncol hbl-1 cDNA fragment cloned into pPD129.36.

Immunofluorescence and Microscopy

Immunolocalization of LIN-29 and AJM-1 (the MH27 antigen) was as described (Bettinger et al., 1996). Microscopy was performed with a Zeiss AxioPlan 2 equipped with differential interference contrast and fluorescence optics. Images were captured with a Zeiss AxioCam and analyzed with Axiovision 3.0.6 and Adobe Photoshop 5.0 software.

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