DOI: 10.1534/genetics.110.123992

# Note

## The Developmental Timing Regulator *hbl-1* Modulates the Dauer Formation Decision in *Caenorhabditis elegans*

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Manuscript received May 17, 2010
Accepted for publication October 12, 2010

ABSTRACT

Animals developing in the wild encounter a range of environmental conditions, and so developmental mechanisms have evolved that can accommodate different environmental contingencies. Harsh environmental conditions cause *Caenorhabditis elegans* larvae to arrest as stress-resistant "dauer" larvae after the second larval stage (L2), thereby indefinitely postponing L3 cell fates. HBL-1 is a key transcriptional regulator of L2 *vs.* L3 cell fate. Through the analysis of genetic interactions between mutations of *hbl-1* and of genes encoding regulators of dauer larva formation, we find that *hbl-1* can also modulate the dauer formation decision in a complex manner. We propose that dynamic interactions between genes that regulate stage-specific cell fate decisions and those that regulate dauer formation promote the robustness of developmental outcomes to changing environmental conditions.

ACHIEVING the correct outcome of developmental processes is essential for survival and fitness in animal species. Therefore, developmental outcomes must be robust to the range of environmental conditions experienced by animals developing in the wild. While much is understood about genetic pathways that regulate temporal and spatial cell fate specification, less is known about how these pathways are integrated with pathways that regulate the response to environmental cues. A dramatic example of a response to environmental cues is dauer diapause in *Caenorhabditis elegans*. In favorable environmental conditions, *C. elegans* larvae progress rapidly and continuously through four larval stages prior to adulthood (Sulston and Horvitz 1977). However, unfavorable environmental cues sensed in the first larval stage (L1) cause larvae to enter the predauer L2d stage, which is >50% longer than the rapid L2 stage (Golden and Riddle 1984). During the extended L2d stage, L3 cell fates are temporarily postponed while larvae continue to sense their environment and prepare for the possibility of continued harsh conditions. At the end of L2d, larvae make a choice between one of two distinct life histories: (1) to continue with developmental progression, molt to the L3 stage, and express L3 cell fates or (2) to interrupt development by entry to the stress-resistant dauer diapause, thereby indefinitely postponing L3 cell fates (Cassada and Russell 1975; Golden and Riddle 1984). Thus, the timing of expression of L3 cell fates is inextricably linked to the animal's decision of whether or not to enter the dauer diapause. This suggests that pathways regulating stage-specific cell fate decisions are coordinated with pathways that regulate dauer diapause.

The dauer formation decision occurs in response to environmental cues, including temperature, food supply, and population density. These cues are perceived by sensory neurons, resulting in up- or downregulation of two partially parallel signaling pathways, TGFβ and insulin-/insulin-growth-factor-1-like (IIS). These two pathways in turn regulate the activity of the third major dauer formation pathway, nuclear hormone receptor signaling (for review see Fielenbach and Antebi 2008). Favorable environmental cues lead to upregulation of the TGFβ encoded by *daf-7* (Ren *et al.* 1996; Schackwitz *et al.* 1996). High DAF-7/TGFβ signaling opposes the activity of the downstream transcription factor DAF-3/SMAD that complexes with DAF-5/Sno-Ski to regulate target gene expression (Patterson *et al.*1997; Da Graca *et al.* 2004). Also in favorable environmental conditions, the insulin-like receptor (InsR) DAF-2 is activated by one or more of ~40 insulin-related proteins (Kimura *et al.* 1997; Pierce *et al.* 2001). High DAF-2/InsR activity opposes the activity of the downstream transcription factor DAF-16/FOXO (Lin *et al.* 1997; Ogg *et al.* 1997). When active, both DAF-16 and DAF-3-DAF-5 pro-

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.123992/DC1.

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mote dauer formation by affecting expression of downstream genes (Thatcher *et al.* 1999; Jensen *et al.* 2006), including *daf-9* (Gerisch *et al.* 2001; Gerisch and Antebi 2004; Mak and Ruvkun 2004; Motola *et al.* 2006). *daf-9* encodes a homolog of cytochrome P450 (Gerisch *et al.* 2001) and is required to produce dafachronic acid (DA), the ligand for the DAF-12 nuclear hormone receptor (Motola *et al.* 2006). Liganded DAF-12 promotes reproductive growth, whereas unliganded DAF-12 is absolutely required for dauer formation (Antebi *et al.* 1998; Antebi *et al.* 2000; Gerisch *et al.* 2001; Motola *et al.* 2006).

***hbl-1* promotes dauer formation downstream or in parallel to *daf-9*:** The study of *daf-12* provided the first indication of coordination between the regulation of L2 *vs.* L3 cell fates and the regulation of the dauer formation decision. This was based on the finding that certain *daf-12* alleles cause the inappropriate reiteration of L2 cell fates in L3-staged larvae (Antebi *et al.* 1998). More recently, additional genes that regulate the L2 *vs.* L3 cell fate decision have been shown to influence the dauer formation decision (Hammell *et al.* 2009; Tennessen *et al.* 2010). *hbl-1* promotes L2 cell fates and inhibits L3 cell fates, downstream from other known L2 *vs.* L3 cell fate regulators (Abrahante *et al.* 2003; Lin *et al.* 2003; Abbott *et al.* 2005; Bethke *et al.* 2009; Hammell *et al.* 2009). We therefore tested whether *hbl-1* could similarly modulate the dauer formation decision, perhaps by interacting with *daf-12*. The *daf-12(rh273)* allele encodes a DAF-12 protein with a defective ligand binding domain (Antebi *et al.* 2000). Because this mutant protein does not bind ligand well, it causes a constitutive dauer formation phenotype ("Daf-c") similar to the loss of DA in *daf-9* mutants (Antebi *et al.* 1998; Gerisch *et al.* 2001; Motola *et al.* 2006). To test the effect of lowering *hbl-1* activity in a *daf-12* mutant background we constructed double mutant strains between *daf-12(rh273)* and either of two hypomorphic *hbl-1* alleles: *hbl-1(ve18)*, and *hbl-1(mg285)*. [Null alleles of *hbl-1* are not available. Furthermore, RNAi experiments indicate that loss of *hbl-1* is likely to result in embryonic lethality (Fay *et al.* 1999)]. Both *hbl-1(ve18) daf-12(rh273)* and *hbl-1(mg285) daf-12(rh273)* strains showed moderate but statistically significant suppression of the *daf-12 (rh273)* Daf-c phenotype (Figure 1A). That the same effect was observed in both *hbl-1(ve18)*- and *hbl-1(mg285)*-containing strains indicates that the suppression is likely to be due to reducing *hbl-1* activity rather than genetic background (Harvey *et al.* 2008). The suppression of a Daf-c phenotype by reduction of *hbl-1* activity suggests that *hbl-1* has a dauer-promoting activity.

The Daf-c phenotype of *daf-12(rh273)* is caused by an excess of the unliganded (dauer promoting) form of DAF-12, since the mutant DAF-12 protein encoded by *daf-12(rh273)* binds its ligand poorly (Motola *et al.* 2006). However, because ligand binding is not completely abrogated in this mutant, *hbl-1* could potentially

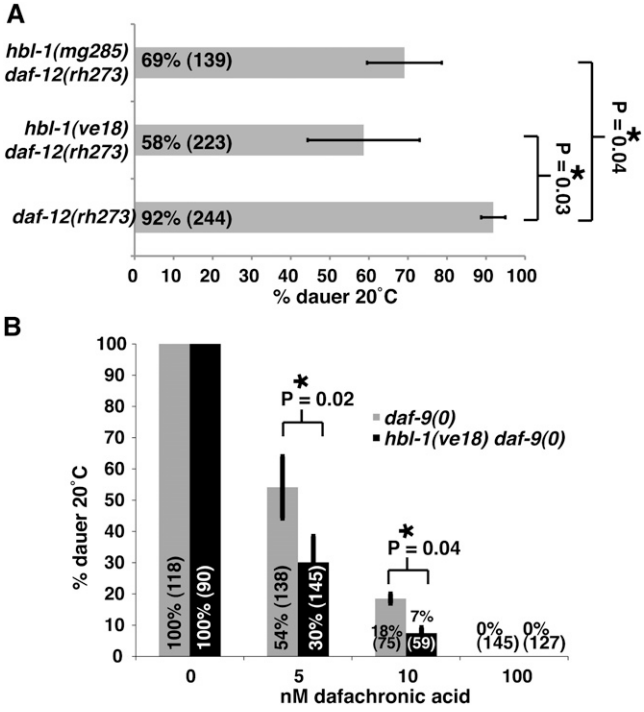


Figure 1.—*hbl-1* promotes dauer formation downstream or in parallel to *Daf-9*. Bar graphs indicate the mean percentage of dauer formation ± SEM. At least three independent trials were carried out for each experiment. For each trial, gravid adults of control and test strains were allowed to lay eggs for several hours on 60-mm NGM plates with the OP50 strain of *Escherichia coli* as a food source. (While *hbl-1* mutant hermaphrodites are egg-laying defective if they have developed continuously, postdauer animals do lay eggs; Abrahante *et al.* 2003). The adults were then removed and plates were incubated side by side at 20° until nondauer animals were L4 or early adult stage (generally 3–4 days). All animals on each plate were scored as dauer or nondauer. As *daf-12(rh273)* and *daf-9* mutant larvae form partial and/or transient dauers (Antebi *et al.* 1998; Gerisch *et al.* 2001), dauer larvae were identified by the presence of dauer alae on the cuticle. Since we were interested in the decision to form dauer, and not in the ability to fully execute or maintain dauer arrest, we counted animals with dauer alae anywhere along the length of the body as dauers. The total number of animals scored across all trials is indicated in parentheses. Asterisks indicate statistically significant differences ($P < 0.05$ by a paired student's *t*-test, where the pairs consisted of control and test strains grown side by side). (A) Reduction of *hbl-1* by either of two hypomorphic alleles can suppress the Daf-c phenotype of the *daf-12(rh273)* mutation. Strains: AA87 *daf-12(rh273)*, VT2010 *hbl-1(ve18) daf-12(rh273)*, and VT2280 *hbl-1(mg285) daf-12(rh273)*. (B) Endogenous dafachronic acid (DA) production was eliminated by the *daf-9(dh6)* mutation, resulting in 100% dauer formation. Exogenous DA [(25S),26–3-keto-4-cholestenoic acid] added to the medium at the indicated concentration can rescue this Daf-c phenotype. This rescue is more efficient if *hbl-1* activity is reduced. Dafachronic acid was provided by David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). Experiments were performed as described (Hammell *et al.* 2009). Strains: AA199 *daf-9(dh6); dhEx24[daf-9+, sur-5::GFP]* and VT1996 *hbl-1(ve18) daf-9(dh6); dhEx24[daf-9+, sur-5::GFP]*. Only $GFP^-$ animals were scored.

promote dauer formation either upstream or downstream of ligand production by DAF-9. To test whether *hbl-1* could promote dauer formation independently of DA ligand production, we used a *daf-9(0)* mutation to eliminate endogenous DA (Motola *et al.* 2006). The *daf-9(dh6)* mutant forms dauers constitutively since all DAF-12 is unliganded in these animals (Gerisch *et al.* 2001; Motola *et al.* 2006). This Daf-c phenotype can be rescued by addition of exogenous DA (Motola *et al.* 2006). At low levels of DA, populations of *daf-9(dh6)* larvae contain some dauers and some nondauers. At these limiting DA levels, reduction of *hbl-1* caused a moderate but statistically significant suppression of the Daf-c phenotype (Figure 1B). Since *hbl-1* cannot be affecting *daf-9* expression or activity in a *daf-9(0)* background, this suggests that the dauer-promoting activity of *hbl-1* is downstream of or in parallel to *daf-9* activity and ligand production. *hbl-1* may promote the activity of unliganded DAF-12, oppose the activity of liganded DAF-12, and/or regulate the transcription of common dauer-promoting targets.

In the absence of DA, *hbl-1(ve18) daf-9(dh6)* double mutants form dauers constitutively, indicating that reduction of *hbl-1* cannot bypass the need for *daf-9* to produce DA ligand (Figure 1B). By contrast, 100 nM DA was sufficient to completely rescue the *daf-9(dh6)* Daf-c phenotype. At this high concentration of DA, reduction of *hbl-1* had no effect (Figure 1B). The inability of reduction of *hbl-1* to affect dauer formation at very low or very high DA concentrations suggests that the dauer-promoting activity of *hbl-1* is modulatory and is most important in moderately stressful situations where environmental cues do not strongly drive the larvae to a particular life history (*i.e.*, a percentage of larvae choose the dauer life history, whereas others choose the continuous life history). We also find that the suppression of *daf-9(dh6)* by reduction of *hbl-1* is specific for the dauer formation phenotype because reducing *hbl-1* activity in a *daf-9(dh6)* background has no effect on the gonad migration phenotype observed in these animals at limiting concentrations of DA (supporting information, Figure S1) (Motola *et al.* 2006).

***hbl-1* opposes dauer-promoting signals from both the TGFβ and insulin-like pathways:** Having detected a modulatory activity for *hbl-1* in promoting dauer formation in response to exogenous DA, we tested whether *hbl-1* would influence dauer formation in genetic backgrounds impaired in signals upstream of DA production. Specifically, we tested whether reduction of *hbl-1* could suppress the incompletely penetrant Daf-c phenotypes of components of the two major signaling pathways that operate upstream of *daf-9*: *daf-7/TGFβ* and *daf-2/INSR* (reviewed in Fielenbach and Antebi 2008). Again, we used two different hypomorphic alleles of *hbl-1* to ensure that any interactions we saw between *hbl-1* and *daf-7* or *daf-2* were likely due to reducing *hbl-1* activity and not to background mutations that affect dauer formation (Harvey *et al.* 2008). Surprisingly, we found that instead of suppressing the Daf-c phenotype, reduction of *hbl-1* enhanced the Daf-c phenotype of both *daf-7(e1372)* and *daf-2(e1370)* at semipermissive temperatures. Again, the effects were moderate but statistically significant (Figure 2, A and B). This suggests that *hbl-1* has reciprocal roles—acting upstream of *daf-9* to negatively modulate dauer-promoting signals and also acting downstream of *daf-9* to positively modulate the dauer formation decision.

Neither *daf-7(e1372)* nor *daf-2(e1370)* are null alleles. Therefore, the enhancement of the Daf-c phenotype of these alleles by reduction of *hbl-1* does not distinguish between whether *hbl-1* acts in the *daf-7* pathway, the *daf-2* pathway, or in both pathways. DAF-7/TGFβ signaling antagonizes the activity of the DAF-3/SMAD + DAF-5/Sno-Ski transcription complex, whereas signaling through DAF-2/InsR antagonizes the activity of the DAF-16/FoxO transcription factor (for review see Fielenbach and Antebi 2008). Loss of either *daf-3* or *daf-5* completely suppresses the Daf-c phenotype of *daf-7* alleles, but has no effect on the Daf-c phenotype of *daf-2* alleles. Conversely, loss of *daf-16* completely suppresses the Daf-c phenotype of *daf-2* alleles, but has no effect on the Daf-c phenotype of *daf-7* alleles (Vowels and Thomas 1992; Larsen *et al.* 1995). We took advantage of these genetic relationships to test whether *hbl-1* can oppose dauer formation via TGFβ or IIS pathways. First, we constructed a *daf-5(0); daf-2(e1370)* double mutant strain. At the semipermissive temperature, the Daf-c phenotype of this strain is as penetrant as a *daf-5(+); daf-2(e1370)* strain (Figure 2A). If the enhancement of the Daf-c phenotype of *daf-2(e1370)* by reduction of *hbl-1* were due to an activity of *hbl-1* in the TGFβ pathway, that activity should be abrogated in the *daf-5(0); daf-2(e1370)* strain. However, we found that reduction of *hbl-1* in this background still resulted in a significant enhancement of the Daf-c phenotype (Figure 2A). Therefore, the enhancement of the Daf-c phenotype of *daf-2(e1370)* by reduction of *hbl-1* activity is not due solely to a role for *hbl-1* in the TGFβ pathway upstream of *daf-5*, because *daf-5* is not required to obtain the enhancement.

We next performed the reciprocal experiment: we asked whether *daf-16* was required to observe the enhancement of the Daf-c phenotype of *daf-7(e1372)*. If *hbl-1* opposes dauer formation in the IIS pathway upstream of the DAF-16/FoxO transcription factor, removing *daf-16* activity should abrogate the dauer opposing effect of *hbl-1*. First, we examined the control *daf-16(0); daf-7(e1372)* strain. Surprisingly, the mean percentage of dauer formation is higher in the *daf-16(0); daf-7(e1372)* strain than the *daf-7(e1372)* strain grown in parallel, although this difference is slightly outside our cutoff for statistical significance (Figure 2B). We next tested the effect of reducing *hbl-1* activity using the *hbl-1(ve18)* allele in the *daf-16(0); daf-7(e1372)* background. We found that 90% of *daf-16(0); daf-7(e1372); hbl-1(ve18)* triple mutant

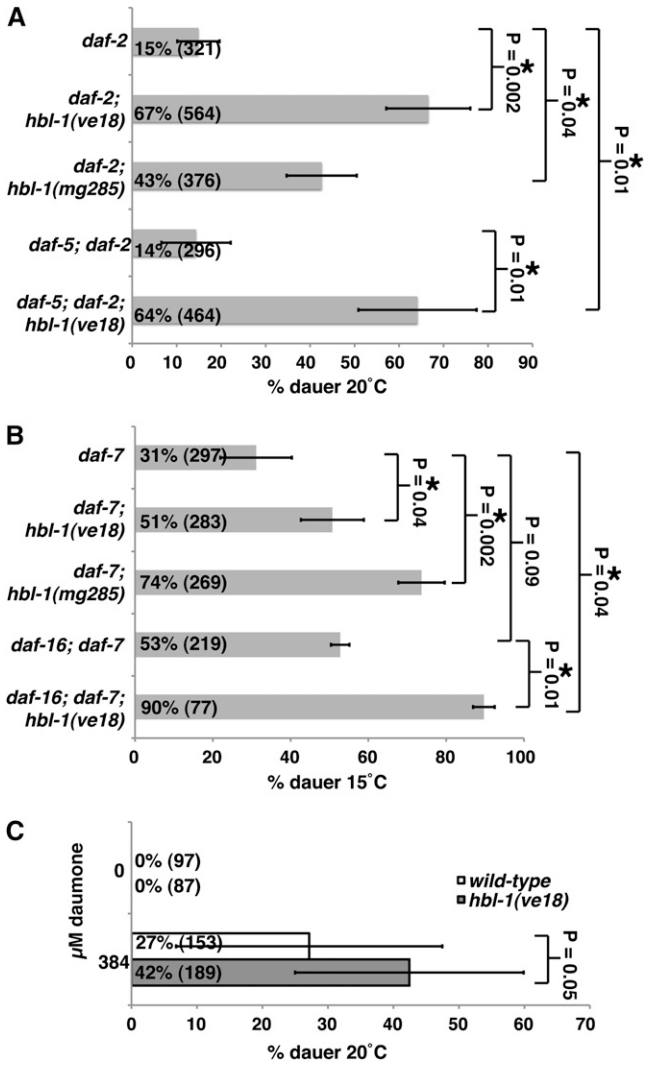


FIGURE 2.—*hbl-1* opposes dauer formation in parallel to insulin and TGFβ signaling. Bar graphs indicate the mean percentage of dauer formation ± SEM. At least three independent trials were carried out for each experiment. For each trial, gravid adults of control and test strains were allowed to lay eggs on 60-mm NGM plates for several hours. (While *hbl-1* mutant hermaphrodites are egg-laying defective if they have developed continuously, postdauer animals do lay eggs; ABRAHANTE *et al.* 2003). The adults were then removed and plates were incubated side by side at the indicated temperature until nondauer animals were L4 or early adult stage (generally 3–4 days at 20° or 5 days at 15°). All animals on each plate were scored as dauer, or nondauer. Any remaining L1 or L2 animals were not included in the percentage of dauer calculation (see Figure S2). In most strains, dauers and nondauers could be easily distinguished by morphology under the dissecting microscope. However, because *daf-16* mutant larvae can form partial and/or transient dauers (VOWELS and THOMAS 1992), we did not rely on the dissecting microscope to score dauer formation in strains carrying these mutations. Instead, we examined the animals by Nomarski optics for the presence of dauer alae. Since we were interested in the decision to form dauer, and not in the ability to fully execute or maintain dauer arrest, we counted animals with dauer alae anywhere along the length of the body as dauers. The total number of animals scored across all trials is indicated in parentheses. Asterisks indicate statistically significant differences ($P < 0.05$ by a paired student's *t*-test, where the pairs consisted of control and test strains grown side by side). (A) Reduction of *hbl-1* enhances the Daf-c phenotype of *daf-2(e1370)*. This enhancement remains even when TGFβ signaling is abrogated by a *daf-5* mutation. Strains (grown at 20°): CB1370 *daf-2(e1370)*, VT1773 *daf-2(e1370); hbl-1(ve18)*, VT2200 *daf-2(e1370); hbl-1(mg285)*, VT2311 *daf-5(m512); daf-2(e1370)*, and VT2312 *daf-5(m512); daf-2(e1370); hbl-1(ve18)*. (B) Reduction of *hbl-1* enhances the Daf-c phenotype of *daf-7(e1372)*. This enhancement remains even when IIS signaling is abrogated by a *daf-16* mutation. Strains (grown at 15°): CB1372 *daf-7(e1372)*, VT1796 *daf-7(e1372); hbl-1(ve18)*, VT2316 *daf-7(e1372); hbl-1(mg285)*, VT2317 *daf-16(mgDf50); daf-7(e1372)*, and VT2318 *daf-16(mgDf50); daf-7(e1372), hbl-1(ve18)*. (C) In a wild-type background, reduction of *hbl-1* fails to display a convincing effect on dauer formation, as the *P*-value was just outside our threshold for significance, and the error bars overlap substantially. A synthetic component of dauer pheromone called daumone [(−)-(6R)-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid; JEONG *et al.* 2005] was added to a final concentration of either 0 μM or 384 μM to ~55° NG agar lacking peptone and with 30 μg/ml streptomycin. We poured ~750 μl of this modified NGM into each well of 12-well culture plates. When cool, 8.3 μl 6× concentrated OP50 bacteria were added to each well. Plates were used within 1–2 days of pouring. As above, gravid adults were added to each well and allowed to lay ~50 eggs and then removed. Plates were incubated at 20° for 3 days and then scored. Strains: N2 *wild-type*, VT1874 *hbl-1(ve18)*.

larvae formed dauers, significantly more than either the *daf-16(+); daf-7(e1372); hbl-1(+)* or *daf-16(0); daf-7 (e1372); hbl-1(+)* strains above (Figure 2B). Therefore, the enhancement of the Daf-c phenotype of *daf-7(e1372)* by reduction of *hbl-1* activity is not due solely to a role for *hbl-1* in the IIS pathway upstream of *daf-16*, because *daf-16* is not required to obtain the enhancement.

These results indicate that *hbl-1* opposes dauer formation in a fashion that does not involve a role only in the DAF-7/TGFβ pathway or only in the DAF-2/IIS pathway. Therefore, *hbl-1* could modulate critical factors in both pathways and/or could act in a parallel pathway. If *hbl-1* opposes dauer formation in a parallel pathway, then a Daf-c phenotype should still be evident when both TGFβ and IIS pathways are inactivated through simultaneous removal of *daf-5* and *daf-16*. However, because *daf-7* and *daf-2* mutations do not produce a Daf-c phenotype in a *daf-16(0); daf-5(0)* background, we are unable to test for enhancement of their Daf-c phenotype by reduction of *hbl-1*. Therefore, the question of whether *hbl-1* can oppose dauer formation in the absence of both TGFβ and IIS signaling can be addressed only if *hbl-1* mutations cause a Daf-c phenotype independently of mutations in *daf-7* and *daf-2*. However, we failed to observe a convincing *hbl-1* Daf-c phenotype in the absence of *daf-7* and *daf-2* alleles, even in animals sensitized for dauer formation by the addition of daumone (JEONG *et al.* 2005) to the growth medium (Figure 2C). In an independent study, no Daf-c phenotype was observed in *hbl-1* mutant animals grown at 27°

(Tennessen *et al.* 2010). We interpret this result to indicate that, under the conditions that we culture our developing larvae, the opposing roles for *hbl-1* in modulating dauer formation effectively cancel each other. We propose that the dauer-modulatory activities of *hbl-1* are more critical under particular environmental or physiological conditions that are not well replicated in our laboratory conditions. The natural habitat for *C. elegans* is complex, including soil, decomposing organic material, and even in association with various invertebrate species (Barriere and Felix 2005). Additionally, one or the other of these opposing roles for *hbl-1* could become critical when larvae develop in environmental conditions that change over time (see model in the next section).

Although we are unable to unambiguously determine whether *hbl-1* acts through both TGFβ and IIS pathways or in parallel to these pathways, we favor the latter possibility for two reasons. First, it is the simplest model. Second, consistent with *hbl-1* acting in parallel to *daf-16*, we observed that when *hbl-1* activity is reduced in the absence of *daf-16*, one-third of larvae arrest in the L1 stage (Figure S2). This synthetic phenotype is not observed in the control strains, although it is possible that it is related to the larval lethality observed after reduction of *hbl-1* activity by RNAi (Figure S2 and legend) (Fay *et al.* 1999).

There is evidence for the existence of dauer formation pathways in addition to TGFβ, IIS, and DAF-12/nuclear hormone pathways. One example is the *daf-11* pathway that is thought to function upstream of TGFβ and IIS pathways. (*daf-11* encodes a transmembrane guanylyl cylclase) (reviewed in Fielenbach and Antebi 2008). *hbl-1* could act in the *daf-11* pathway to oppose dauer formation. However we do not favor this possibility because both *daf-5* and *daf-16* mutations can partially suppress the Daf-c phenotype of *daf-11(-)* (Vowels and Thomas 1992; Thomas *et al.* 1993), whereas we see no evidence of such suppression of the *hbl-1* Daf-c phenotype in our assay (Figure 2, A and B).

There is also some evidence for unknown pathways regulating dauer formation, because dauer pheromone can induce larvae to enter dauer diapause even in the absence of both *daf-3/SMAD* and *daf-16/FOXO* (Ogg *et al.* 1997). Therefore, even when TGFβ and IIS pathways are removed, unfavorable environmental cues are transduced into the decision to enter dauer diapause. Since unliganded DAF-12 is absolutely required for dauer formation (Thomas *et al.* 1993; Antebi *et al.* 1998), one interpretation is that this unknown dauer formation pathway is also upstream of *daf-9* expression or activity. Since *hbl-1* encodes a transcription factor, we hypothesize that the dauer opposing role for *hbl-1* could reflect direct transcriptional regulation by HBL-1 of some of the transcriptional targets of the TGFβ and IIS pathways.

Many genes that influence dauer formation also influence lifespan, including *daf-2*, *daf-7*, *daf-9*, and *daf-12* (Kenyon *et al.* 1993; Larsen *et al.* 1995; Gerisch *et al.* 2007; Shaw *et al.* 2007). In particular, the longevity observed in strains carrying *daf-2* mutations has been long studied, and the role of the IIS pathway in aging is conserved to mammals (for review see Broughton and Partridge 2009). Since reduction of *hbl-1* can enhance the dauer formation phenotype of *daf-2(e1370)* (Figure 2A), we asked whether reduction of *hbl-1* activity might similarly enhance the longevity phenotype of the *daf-2(e1370)* allele. We found no effect on the longevity of *daf-2(e1370)* animals when *hbl-1* was reduced using either the *hbl-1(ve18)* or the *hbl-1(mg285)* alleles (Figure 3). Furthermore, we found no effect on lifespan by either *hbl-1* allele in a wild-type background (Figure 3). Since these are not null alleles of *hbl-1*, this does not rule out a role for *hbl-1* in aging. However, this observation suggests that the ability of *hbl-1(−)* to enhance the phenotype of *daf-2(e1370)* is specific for the dauer formation decision. This is consistent with *hbl-1* affecting the dauer formation decision independently from *daf-16*, since negative regulation of *daf-16* by *daf-2* (and *daf-7*) is the cause of the longevity phenotype observed in these mutant strains (Kenyon *et al.* 1993; Shaw *et al.* 2007).

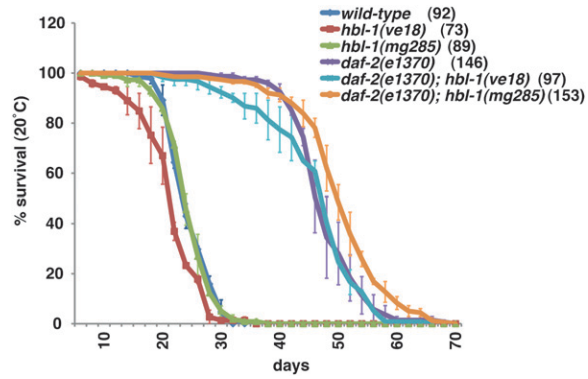


Figure 3.—*hbl-1* does not affect lifespan. Survival curves show the mean percentage of survival over two independent trials. Error bars indicate maximum and minimum percentage of survival in the two trials. Neither hypomorphic allele of *hbl-1* affected lifespan either in wild-type or *daf-2(e1370)* backgrounds. Approximately 100 synchronized L4 larvae that had grown on standard NGM plates with plenty of food at 15° were transferred to two NGM plates + 50 μg/ml FUDR (5-fluorodeoxyuridine, Sigma). Plates were incubated at 20° and examined every 2 days for living or dead animals. Animals that failed to respond to stimulation by touch were counted as dead and removed. Animals that crawled off the plate or exploded through the vulva were censored. All strains were grown in parallel. Strains: N2 *wild-type*, VT1874 *hbl-1(ve18)*, CT11 *hbl-1(mg285)*, CB1370 *daf-2(e1370)*, VT1773 *daf-2(e1370); hbl-1(ve18)*, and VT2200 *daf-2(e1370); hbl-1(mg285)*.

**Model for the integration of *hbl-1* into dauer formation pathways:** *hbl-1* expression during continuous development is negatively regulated by let-7-family microRNAs, such that *hbl-1* levels are high in the L1 stage, but low in the L3 stage (Abrahante *et al.* 2003; Lin *et al.* 2003; Abbott *et al.* 2005). We monitored *hbl-1* expression during dauer-interrupted development us-

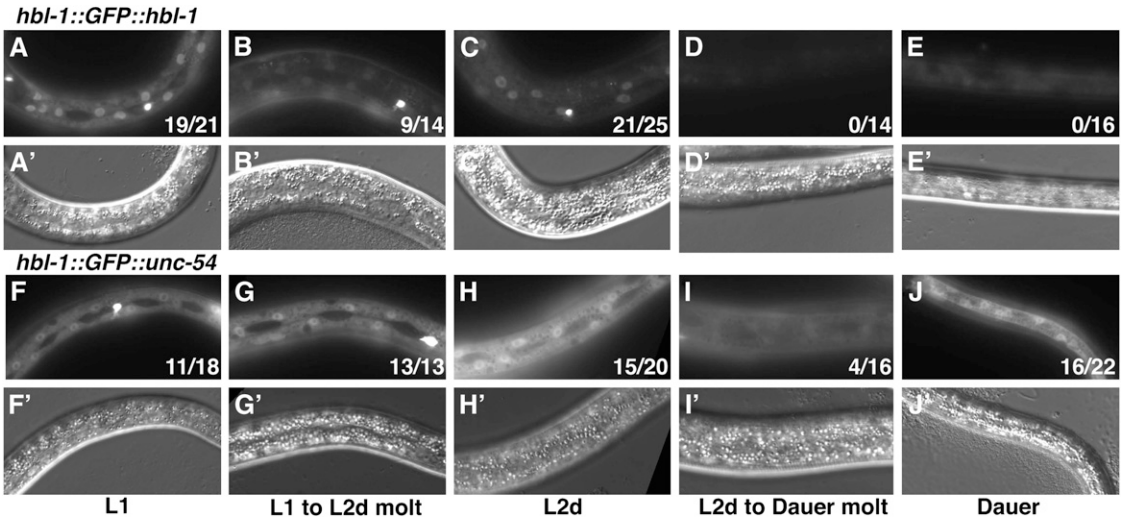


FIGURE 4.—*hbl-1* expression in the hypodermis of dauer and predauer larvae. Gravid adults carrying *hbl-1* reporter transgenes were allowed to lay eggs on 35-mm NG agar plates containing 50 μl crude dauer pheromone and seeded with 20 μl 6× concentrated OP50 as a food source (VOWELS and THOMAS 1994). Mothers were removed after several hours, and the progeny were incubated at 20° and monitored for GFP expression throughout predauer developmental stages. Developmental stages were determined by the extent of gonad development, and molting larvae were identified by the presence of an extra cuticle. Dauer larvae were harvested from crowded and starved plates incubated at 20°. Dauer larvae and larvae at the L2d to Dauer molt were identified by the presence of dauer alae on the cuticle. Developmental stages are indicated underneath the images. GFP images appear in the top panels, and the corresponding DIC images appear beneath them. Images were obtained on a Zeiss Axio Imager D1 with an AxioCam MRm camera, and a X-Cite 120Q light source (EXFO Photonic Solutions) using a ×63 objective. Anterior is to the left. (A–E) Larvae of the strain BW1932 *ctIs39[hbl-1::GFP::hbl-1, rol-6(su1006)]* (FAY *et al.* 1999). (F–J) Larvae of the strain BW1891 *ctIs37[hbl-1::GFP::unc-54, rol-6(su1006)]* (FAY *et al.* 1999). GFP images of BW1932 were taken using a 100-ms exposure time, and GFP images of BW1891 were taken using a 40-ms exposure time. Note that *ctIs39* and *ctIs37* contain the identical regulatory sequence, apart from the 3′-UTR. BW1932 contains the *hbl-1* 3′-UTR, whereas *ctIs37* contains the unregulated *unc-54* 3′-UTR. Numbers indicate the number of larvae at that developmental stage that display unambiguous GFP expression in hypodermal nuclei at the indicated exposure time, out of the total number examined.

ing the same reporter transgenes as in the above studies and found a similar expression pattern: *hbl-1::GFP* levels are high in L1 and L2d stages, but downregulated at the L2d to dauer molt (Figure 4, A–D). *hbl-1::GFP* remains off during dauer diapause (Figure 4E). The downregulation at the L2d to dauer molt appears to be partially 3′-UTR independent, because a *hbl-1::GFP* transgene that lacks the *hbl-1* 3′-UTR is largely downregulated at that stage (Figure 4I). However, expression of this transgene returns during dauer diapause, indicating that the continued lack of *hbl-1* expression during dauer diapause requires the *hbl-1* 3′-UTR (Figure 4J). While there are many possible models for how *hbl-1* could modulate the dauer formation decision, below and in Figure 5 we discuss a model in which the temporal regulation of *hbl-1* expression is important for its effect on dauer formation.

In the L1 stage, larvae assess their environment and decide whether to enter L2 or L2d, a decision involving DAF-7/TGFβ signaling (GOLDEN and RIDDLE 1984; VOWELS and THOMAS 1992). Both DAF-7/TGFβ and DAF-28/insulin-like are expressed in sensory neurons in L1 (and later) stage larvae experiencing favorable environmental conditions, but downregulated in L1 (and later) stage larvae experiencing unfavorable environmental conditions (REN *et al.* 1996; SCHACKWITZ *et al.* 1996; LI *et al.* 2003). Indeed, the critical periods for the assessment of environmental stimuli through *daf-7* and *daf-2* occur within the L1 or early L2 stage (SWANSON and RIDDLE 1981; GOLDEN and RIDDLE 1984). We propose that the early, high expression of HBL-1 at this stage has an activity distinct from the later, low expression of HBL-1. For example, high levels of HBL-1 could be required to bind to the promoter of certain target genes, whereas low HBL-1 levels could be sufficient for others. It is also possible that HBL-1 could regulate the same targets early and late in development, but that the regulation could be in opposite directions. The related Drosophila Hunchback transcription factor has been shown to repress a target gene at high concentrations, but activate it at low concentrations (SCHULZ and TAUTZ 1994; SCHULZ and TAUTZ 1995; PAPATSENKO and LEVINE 2008). We hypothesize that during the L1 and early L2d stage, high HBL-1 levels promote L2 cell fates and prevent the precocious expression of late-stage programs, including not just L3 cell fates (ABRAHANTE *et al.* 2003; LIN *et al.* 2003; ABBOTT *et al.* 2005) but also dauer formation (Figure 2). The novel dauer-opposing activity described here may occur at the level of integration between DAF-7/TGFβ and DAF-2/IIS signaling in response to environmental cues (blue arrow in Figure 5). If environmental conditions are mildly stressful at this time, *hbl-1* activity may modulate the sensitivity of the larva to dauer-

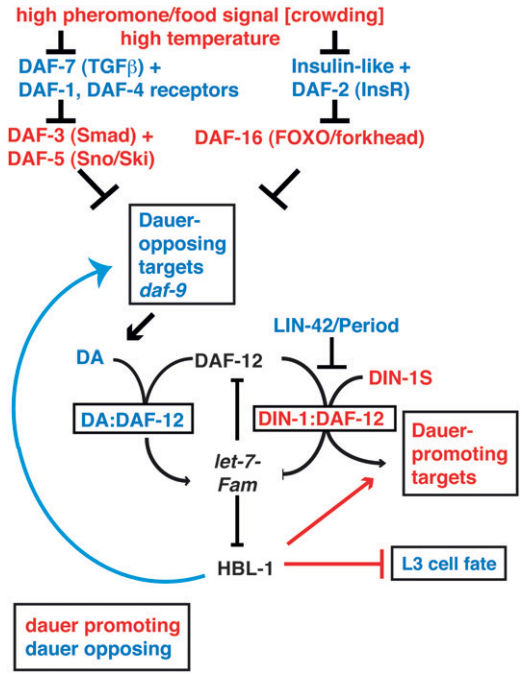


Figure 5.—A model for integration of *hbl-1* activity with the regulation of dauer formation. Early in larval development, environmental cues signal through TGFβ and IIS pathways to direct progression to either the rapid L2 that leads to continuous development or the extended L2d that can lead to dauer diapause. Downstream transcription factors DAF-3 (complexed with DAF-5) and DAF-16 regulate target genes that in turn regulate dauer formation. One consequence of this regulation is *daf-9* expression in favorable environmental conditions. High levels of HBL-1 at this stage promote L2 cell fates and also oppose dauer formation, perhaps by regulating common targets with DAF-3-DAF-5 and DAF-16 (blue arrow). By the time of the L2d-to-dauer molt, HBL-1 levels have been reduced (Figure 4). Lower levels of HBL-1 oppose L3 cell fates and also promote dauer formation, perhaps by regulating common targets with DAF-12. At the same time, a feedback loop between DAF-12 and let-7-family microRNAs is operating (Hammell *et al.* 2009), and LIN-42/Period modulates the dauer formation decision by opposing the activity of unliganded DAF-12 (Tennessen *et al.* 2010). This complex set of interactions may coordinate stage-specific cell fates with the dauer formation decision.

promoting signals to delay expression of the dauer program and help to keep development progressing rapidly.

Following the L1 stage, larvae either commit to continuous development by entering the rapid L2 stage or leave open the possibility for either continuous or dauer interrupted development by entering L2d (Golden and Riddle 1984). Although the critical period for *daf-12* activity in the dauer formation decision is not known, one possibility is that *daf-12* acts after the L2 *vs.* L2d decision, to regulate the L3 *vs.* dauer decision within the L2d stage. This possibility is suggested by the observation that, in contrast to *daf-3* and *daf-5* mutants that are defective in entry to L2d, dauer-defective *daf-12* mutants enter L2d normally (Vowels and Thomas 1992). This idea is consistent with the model that TGFβ and IIS pathways affect *daf-9* expression, which eventually translates into altered levels of DA and an effect on DAF-12 activity (Gerisch *et al.* 2001; Motola *et al.* 2006). During the L2d stage, the let-7-family microRNAs and LIN-42/Period fine-tune DAF-12 expression and activity, respectively, permitting high sensitivity to environmental conditions (Hammell *et al.* 2009; Tennessen *et al.* 2010). In wild-type larvae, the final decision to commit to dauer diapause is made during the early L2d molt (Golden and Riddle 1984). We hypothesize that at the late L2d or L2d molt stage, the lower levels of HBL-1 that remain act to oppose L3 cell fates, thereby postponing their expression. Along with opposing L3 cell fates during L2d, we suggest that HBL-1 would simultaneously promote dauer formation, perhaps by assisting with DAF-12–dependent dauer gene expression (red arrow, Figure 5). If this dauer-promoting activity of HBL-1 indeed occurs only when HBL-1 levels have been sufficiently lowered, that could help to link dauer commitment to a particular time in the L2d stage. Furthermore, the potential ability of HBL-1 activity to change from dauer opposing to dauer promoting over time could be important in the context of the rapidly changing environmental conditions that may be experienced in the wild.

The discovery that *hbl-1* can modulate the dauer formation decision adds an important dimension to our understanding of the coordination between the regulation of dauer formation and the regulation of stage-specific cell fate decisions. As mentioned above, several other regulators of the L2 *vs.* L3 cell fate decision can also influence the dauer formation decision, including *lin-42*, let-7-family microRNAs, and the core dauer pathway component *daf-12* (Antebi *et al.* 1998; Hammell *et al.* 2009; Tennessen *et al.* 2010). Interestingly, the effects of these genes on dauer formation are not necessarily predictable from their roles in regulating stage-specific cell fates. For example, while *hbl-1* and *lin-42* both promote L2 cell fates and oppose L3 cell fates, they appear to act in opposition with respect to modulation of *daf-12* activity. Specifically, *hbl-1* promotes dauer formation downstream or in parallel to *daf-9* (Figure 1), whereas *lin-42* opposes dauer formation downstream of *daf-9* (Tennessen *et al.* 2010).

Additionally, *lin-4*, *lin-14*, and *lin-28* influence different aspects of dauer formation (Liu and Ambros 1989). *lin-14* encodes a transcription factor that regulates L1 *vs.* L2 and L2 *vs.* L3 cell fate decisions, *lin-28* encodes an RNA binding protein that regulates the L2 *vs.* L3 cell fate decision, and *lin-4* encodes a microRNA that targets both *lin-14* and *lin-28* (Ambros and Horvitz 1984, 1987; Lee *et al.* 1993; Moss *et al.* 1997; Hristova *et al.* 2005). *lin-4(0)* larvae are unable to enter dauer diapause, a phenotype that is due to persistent *lin-14* expression. By contrast, no Daf-c or Daf-d phenotypes have been reported to result from loss of *lin-14* or *lin-28*. However, *lin-14* acts to restrict the entry into dauer diapause to the proper stage (following the L2 molt), and

both *lin-14* and *lin-28* are required for proper dauer morphogenesis (Liu and Ambros 1989). The genetic relationships between *lin-4*, *lin-14*, and *lin-28* are different for the dauer formation phenotypes than they are for stage-specific cell fate decisions (Ambros 1989; Liu and Ambros 1989). Therefore, while the modulation of the dauer formation decision by regulators of stage-specific cell fates is a common theme that is emerging, there are unique effects on dauer formation by specific genes. The complexity of the interactions described here may underscore the need for coordination between pathways that regulate developmental timing and pathways that respond to environmental conditions for development to occur robustly in changing environments.

We are grateful to Jason Tennessen and Ann Rougvie at the University of Minnesota for discussing their data prior to publication. We also thank Molly Hammell for statistical advice and Iva Greenwald for the use of reagents and equipment. This work was supported by National Institutes of Health (NIH) grants GM30428 (to V.A.) and F32 GM73307 (to X.K.). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. V.A. is a member of the UMass DERC (DK32520), which provided Core Resources.

## LITERATURE CITED

Abbott, A. L., E. Alvarez-Saavedra, E. A. Miska, N. C. Lau, D. P. Bartel *et al.*, 2005 The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev. Cell **9:** 403–414.

Abrahante, J. E., A. L. Daul, M. Li, M. L. Volk, J. M. Tennessen *et al.*, 2003 The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Dev. Cell **4:** 625–637.

Ambros, V., 1989 A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell **57:** 49–57.

Ambros, V., and H. R. Horvitz, 1984 Heterochronic mutants of the nematode Caenorhabditis elegans. Science **226:** 409–416.

Ambros, V., and H. R. Horvitz, 1987 The lin-14 locus of Caenorhabditis elegans controls the time of expression of specific postembryonic developmental events. Genes Dev. **1:** 398–414.

Antebi, A., J. G. Culotti and E. M. Hedgecock, 1998 daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans. Development **125:** 1191–1205.

Antebi, A., W. H. Yeh, D. Tait, E. M. Hedgecock and D. L. Riddle, 2000 daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes Dev. **14:** 1512–1527.

Barriere, A., and M. A. Felix, 2005 High local genetic diversity and low outcrossing rate in Caenorhabditis elegans natural populations. Curr. Biol. **15:** 1176–1184.

Bethke, A., N. Fielenbach, Z. Wang, D. J. Mangelsdorf and A. Antebi, 2009 Nuclear hormone receptor regulation of microRNAs controls developmental progression. Science **324:** 95–98.

Broughton, S., and L. Partridge, 2009 Insulin/IGF-like signalling, the central nervous system and aging. Biochem. J. **418:** 1–12.

Cassada, R. C., and R. L. Russell, 1975 The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev. Biol. **46:** 326–342.

da Graca, L. S., K. K. Zimmerman, M. C. Mitchell, M. Kozhan-Gorodetska, K. Sekiewicz *et al.*, 2004 DAF-5 is a Ski oncoprotein homolog that functions in a neuronal TGF beta pathway to regulate C. elegans dauer development. Development **131:** 435–446.

Fay, D. S., H. M. Stanley, M. Han and W. B. Wood, 1999 A Caenorhabditis elegans homologue of hunchback is required for late stages of development but not early embryonic patterning. Dev. Biol. **205:** 240–253.

Fielenbach, N., and A. Antebi, 2008 C. elegans dauer formation and the molecular basis of plasticity. Genes Dev. **22:** 2149–2165.

Gerisch, B., and A. Antebi, 2004 Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. Development **131:** 1765–1776.

Gerisch, B., C. Weitzel, C. Kober-Eisermann, V. Rottiers and A. Antebi, 2001 A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Dev. Cell **1:** 841–851.

Gerisch, B., V. Rottiers, D. Li, D. L. Motola, C. L. Cummins *et al.*, 2007 A bile acid-like steroid modulates *Caenorhabditis elegans* lifespan through nuclear receptor signaling. Proc. Natl. Acad. Sci. USA **104:** 5014–5019.

Golden, J. W., and D. L. Riddle, 1984 The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Dev. Biol. **102:** 368–378.

Hammell, C. M., X. Karp and V. Ambros, 2009 A feedback circuit involving let-7-family miRNAs and DAF-12 integrates environmental signals and developmental timing in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **106:** 18668–18673.

Harvey, S. C., A. Shorto and M. E. Viney, 2008 Quantitative genetic analysis of life-history traits of Caenorhabditis elegans in stressful environments. BMC Evol. Biol. **8:** 15.

Hristova, M., D. Birse, Y. Hong and V. Ambros, 2005 The Caenorhabditis elegans heterochronic regulator LIN-14 Is a novel transcription factor that controls the developmental timing of transcription from the insulin/insulin-like growth factor gene ins-33 by direct DNA binding. Mol. Cell. Biol. **25:** 11059–11072.

Jensen, V. L., M. Gallo and D. L. Riddle, 2006 Targets of DAF-16 involved in Caenorhabditis elegans adult longevity and dauer formation. Exp. Gerontol. **41:** 922–927.

Jeong, P. Y., M. Jung, Y. H. Yim, H. Kim, M. Park *et al.*, 2005 Chemical structure and biological activity of the Caenorhabditis elegans dauer-inducing pheromone. Nature **433:** 541–545.

Kenyon, C., J. Chang, E. Gensch, A. Rudner and R. Tabtiang, 1993 A C. elegans mutant that lives twice as long as wild type. Nature **366:** 461–464.

Kimura, K. D., H. A. Tissenbaum, Y. Liu and G. Ruvkun, 1997 daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science **277:** 942–946.

Larsen, P. L., P. S. Albert and D. L. Riddle, 1995 Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics **139:** 1567–1583.

Lee, R. C., R. L. Feinbaum and V. Ambros, 1993 The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell **75:** 843–854.

Li, W., S. G. Kennedy and G. Ruvkun, 2003 daf-28 encodes a C. elegans insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev. **17:** 844–858.

Lin, K., J. B. Dorman, A. Rodan and C. Kenyon, 1997 daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science **278:** 1319–1322.

Lin, S. Y., S. M. Johnson, M. Abraham, M. C. Vella, A. Pasquinelli *et al.*, 2003 The C. elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Dev. Cell **4:** 639–650.

Liu, Z. C., and V. Ambros, 1989 Heterochronic genes control the stage-specific initiation and expression of the dauer larva developmental program in Caenorhabditis elegans. Genes Dev. **3:** 2039–2049.

Mak, H. Y., and G. Ruvkun, 2004 Intercellular signaling of reproductive development by the C. elegans DAF-9 cytochrome P450. Development **131:** 1777–1786.

Moss, E. G., R. C. Lee and V. Ambros, 1997 The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell **88:** 637–646.

Motola, D. L., C. L. Cummins, V. Rottiers, K. K. Sharma, T. Li *et al.*, 2006 Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell **124:** 1209–1223.

Ogg, S., S. Paradis, S. Gottlieb, G. I. Patterson, L. Lee *et al.*, 1997 The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature **389:** 994–999.

Papatsenko, D., and M. S. Levine, 2008 Dual regulation by the Hunchback gradient in the Drosophila embryo. Proc. Natl. Acad. Sci. USA **105:** 2901–2906.

Patterson, G. I., A. Koweek, A. Wong, Y. Liu and G. Ruvkun, 1997 The DAF-3 Smad protein antagonizes TGF-beta-related receptor signaling in the Caenorhabditis elegans dauer pathway. Genes Dev. **11:** 2679–2690.

Pierce, S. B., M. Costa, R. Wisotzkey, S. Devadhar, S. A. Homburger *et al.*, 2001 Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes Dev. **15:** 672–686.

Ren, P., C. S. Lim, R. Johnsen, P. S. Albert, D. Pilgrim *et al.*, 1996 Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. Science **274:** 1389–1391.

Schackwitz, W. S., T. Inoue and J. H. Thomas, 1996 Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron **17:** 719–728.

Schulz, C., and D. Tautz, 1994 Autonomous concentration-dependent activation and repression of Kruppel by hunchback in the Drosophila embryo. Development **120:** 3043–3049.

Schulz, C., and D. Tautz, 1995 Zygotic caudal regulation by hunchback and its role in abdominal segment formation of the Drosophila embryo. Development **121:** 1023–1028.

Shaw, W. M., S. Luo, J. Landis, J. Ashraf and C. T. Murphy, 2007 The C. elegans TGF-beta Dauer pathway regulates longevity via insulin signaling. Curr. Biol. **17:** 1635–1645.

Sulston, J. E., and H. R. Horvitz, 1977 Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. **56:** 110–156.

Swanson, M. M., and D. L. Riddle, 1981 Critical periods in the development of the Caenorhabditis elegans dauer larva. Dev. Biol. **84:** 27–40.

Tennessen, J. M., K. J. Opperman and A. E. Rougvie, 2010 The C. elegans developmental timing protein LIN-42 regulates diapause in response to environmental cues. Development **137:** 3501–3511.

Thatcher, J. D., C. Haun and P. G. Okkema, 1999 The DAF-3 Smad binds DNA and represses gene expression in the Caenorhabditis elegans pharynx. Development **126:** 97–107.

Thomas, J. H., D. A. Birnby and J. J. Vowels, 1993 Evidence for parallel processing of sensory information controlling dauer formation in *Caenorhabditis elegans*. Genetics **134:** 1105–1117.

Vowels, J. J., and J. H. Thomas, 1992 Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. Genetics **130:** 105–123.

Vowels, J. J., and J. H. Thomas, 1994 Multiple chemosensory defects in daf-11 and daf-21 mutants of *Caenorhabditis elegans*. Genetics **138:** 303–316.

Communicating editor: B. J. Meyer

GENETICS

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# The Developmental Timing Regulator *hbl-1* Modulates the Dauer Formation Decision in *Caenorhabditis elegans*

**Xantha Karp and Victor Ambros**


DOI: 10.1534/genetics.110.123992

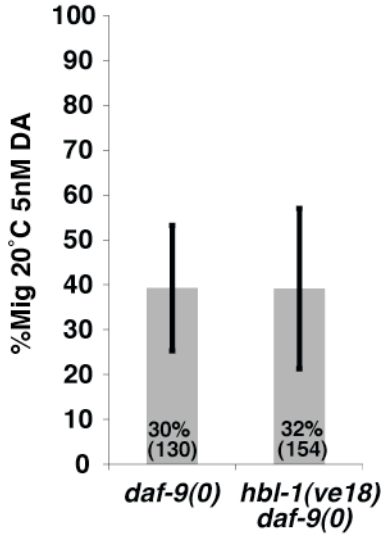


FIGURE S1.—*hbl-1* does not affect gonad migration. During larval development, the distal tip cells of the somatic gonad migrate in a particular pattern in order to extend the gonad and accommodate the increased number of somatic gonad cells and germ cells produced during this period (KIMBLE and WHITE 1981). Unliganded DAF-12 can block gonad migration at a particular step in the late L3 stage. *daf-9(0)* animals form dauers constitutively and cannot be scored for a Mig phenotype. However, if limiting amounts of exogenous DA ligand are added to the culture medium, some animals bypass dauer and/or recover from dauer and can be assessed for the Mig phenotype. At these limiting concentrations of DA, some animals display a Mig phenotype whereas others do not (MOTOLA *et al.* 2006). %Mig indicates the average number of animals that display a Mig phenotype in either or both gonad arms across three independent trials. Error bars indicate SEM. The total number of animals scored across all trials is indicated in parentheses.

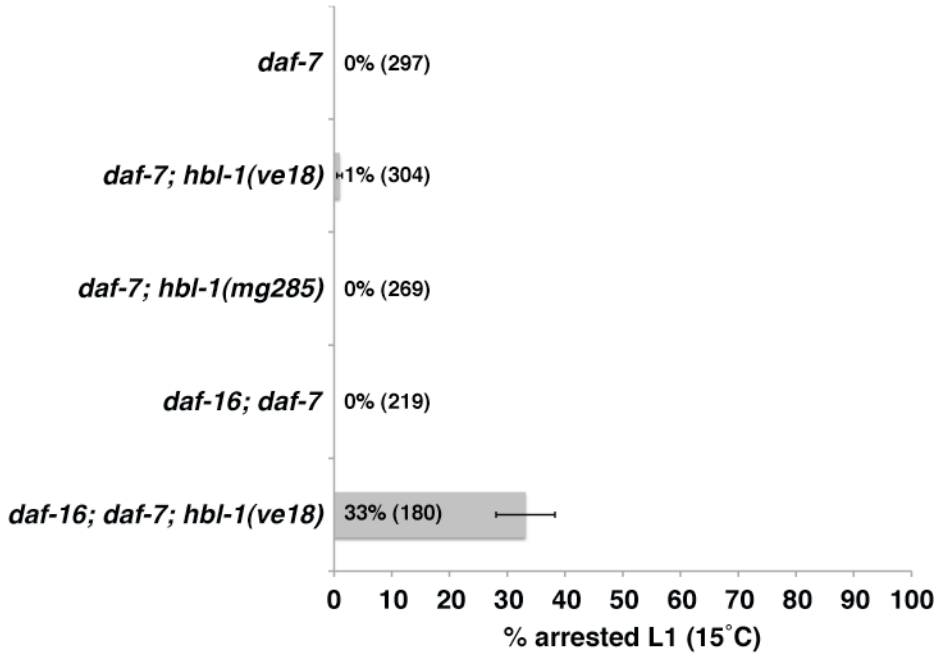


FIGURE S2.—Lowering *hbl-1* and *daf-16* simultaneously causes an L1-arrest phenotype. Bar-graphs indicate the mean percent L1 arrested larvae ± SEM. At least 3 independent trials were carried out for each experiment. The total number of animals scored across all trials is indicated in parentheses. Strains (grown at 15˚C): CB1372 *daf-7(e1372)*, VT1796 *daf-7(e1372); hbl-1(ve18)*, VT2316 *daf-7(e1372); hbl-1(mg285)*, VT2317 *daf-16(mgDf50); daf-7(e1372)*, VT2318 *daf-16(mgDf50); daf-7(e1372), hbl-1(ve18)*. Arrested L1s were noted simultaneously with % dauer as shown in Figure 2B. The L1 stage was verified by the extent of gonad development visible by Nomarski optics. Arrested L1 larvae were not included in the % dauer calculation in Figure 2B, however both dauer and non-dauer animals were included in the % L1 arrested larvae calculation. One possibility is that the arrested L1 phenotype observed here is related to the abnormalities reported in *hbl-1(RNAi)* larvae: Of the 62% of *hbl-1(RNAi)* animals that survive past embryogenesis, most were observed to have abnormal morphology, and many did not survive to adulthood (FAY *et al.* 1999). These phenotypes may be due to the defects in hypodermal cell fate specification that were described (FAY *et al.* 1999). Therefore, if *hbl-1* and *daf-16* function in the same pathway, loss of *daf-16* could enhance the reduction of function mutation *hbl-1(ve18)* and produce a phenotype similar to what may be observed in a *hbl-1(0)* allele. Alternatively, the L1-arrest phenotype could be a new, synthetic phenotype that is not due to defects in hypodermal cell fate and requires the loss of *daf-16* in addition to reduction of *hbl-1* activity.

**SUPPORTING REFERENCES**:

FAY, D. S., H. M. STANLEY, M. HAN and W. B. WOOD, 1999 A Caenorhabditis elegans homologue of hunchback is required for late stages of development but not early embryonic patterning. Dev Biol **205:** 240-253.

KIMBLE, J. E., and J. G. WHITE, 1981 On the control of germ cell development in Caenorhabditis elegans. Dev Biol **81:** 208-219.

MOTOLA, D. L., C. L. CUMMINS, V. ROTTIERS, K. K. SHARMA, T. LI *et al.*, 2006 Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell **124:** 1209-1223.