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The *C. elegans tailless/Tlx* homolog *nhr-67* regulates a stage-specific program of linker cell migration in male gonadogenesis

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Cell migration is a common event during organogenesis, yet little is known about how migration is temporally coordinated with organ development. We are investigating stage-specific programs of cell migration using the linker cell (LC), a migratory cell crucial for male gonadogenesis of *C. elegans*. During the L3 and L4 larval stages of wild-type males, the LC undergoes changes in its position along the migratory route, in transcriptional regulation of the *unc-5* netrin receptor and *zmp-1* zinc matrix metalloprotease, and in cell morphology. We have identified the *tailless* homolog *nhr-67* as a cell-autonomous, stage-specific regulator of timing in LC migration programs. In *nhr-67*-deficient animals, each of the L3 and L4 stage changes is either severely delayed or never occurs, yet LC development before the early L3 stage or after the mid-L4 stage occurs with normal timing. We propose that there is a basal migration program utilized throughout LC migration that is modified by stage-specific regulators such as *nhr-67*.

KEY WORDS: *C. elegans* males, Gonadogenesis, Cell migration, *nhr-67/tailless*, Timing

INTRODUCTION

The importance of spatial cues in the development of multicellular organisms is well established, and much is known about the signaling pathways that provide spatial information within developing tissues (Towers and Tickle, 2009). Less understood, but probably equally important, are temporal cues, which organize cells into organs and coordinate the development of each organ with the developmental program of the whole organism. At least some organs use transcriptional regulation as a mechanism to respond to timing cues. For example, hormones, such as ecdysone in insects or those produced by the pituitary gland in mammals, serve as global regulators of timing by activating organ-specific transcription factors that respond to these cues (Randall et al., 1997). In another example, neuroblast differentiation in mouse and *Drosophila* depends on a series of transcription factors that are expressed in a defined order within a cell lineage (Pearson and Doe, 2004). The importance of proper timing is underscored by the fact that the same transcription factors required during development can lead to disease when expressed at an inappropriate stage (Robson et al., 2006).

Cell migration, which is integral to many types of organogenesis, clearly depends on spatial cues (Cardoso and Lu, 2006; Ghysen and Dambly-Chaudiere, 2007; Montell, 2003; Sauka-Spengler and Bronner-Fraser, 2006). Because cell migrations occur at specific times during organogenesis and often involve stage-specific changes in migratory behavior, they are likely to also be temporally regulated; however, there are few systems tractable for studying temporal regulation in migration. *C. elegans* is particularly conducive for studying developmental timing because it develops with both invariant timing of cell divisions and invariant cell positions, making aberrations easy to identify (Kimble and Hirsh, 1979; Sulston et al., 1980; Sulston and Horvitz, 1977). This feature has allowed the identification of heterochronic mutants, in which the developmental timing of some tissues is altered relative to the rest of the organism (Ambros and Horvitz, 1984). Some of these genes have since been shown to control vertebrate development, but because the effect is more subtle in vertebrates, the studies in *C. elegans* have been pivotal in identifying the role of heterochronic genes (Moss, 2007). The precise timing of *C. elegans* development has also been used to identify the logic of transcriptional regulation of temporal information during the development of organs such as the pharynx (Gaudet and Mango, 2002; Gaudet et al., 2004).

We are studying the stage-specific regulation of migration in the linker cell (LC), an individual leader cell whose migration defines the shape of the developing male gonad and ultimately connects the gonad to the cloaca, enabling sperm release (Kimble and Hirsh, 1979; Klass et al., 1976). The LC clearly requires regulation at different stages as it navigates a complex trajectory during three larval stages (L2-L4) of development. The LC migration route consists of several linear segments and two turns (see Fig. 1). The first turn occurs in L2 larvae, when the LC travels from the ventral to the dorsal bodywall, while changing direction from anterior to posterior. The second turn occurs in mid-L3 larvae, when the LC travels from the dorsal bodywall back down to the ventral bodywall as it migrates posteriorly. The LC finishes migrating in mid-L4 larvae and is engulfed by the U.lp/U.rp cell near the cloaca, undergoing cell death. Some data suggest that timing cues, rather than physical landmarks along the migration route, induce specific LC behaviors at different points of migration. For example, the second turn executed by the LC is regulated by *daf-12*, a nuclear hormone receptor that regulates developmental timing and dauer diapause in response to a hormonal ligand, dafachronic acid (Antebi et al., 1998; Motola et al., 2006; Su et al., 2000). In addition, LC death occurs in the L4 molt, regardless of engulfment by the U.lp/U.rp cell or its position in the worm (Abraham et al., 2007).

Our characterization of the migrating LC has revealed that it changes gene expression and cell shape, in addition to the position along the migratory route. We show that changes in LC gene

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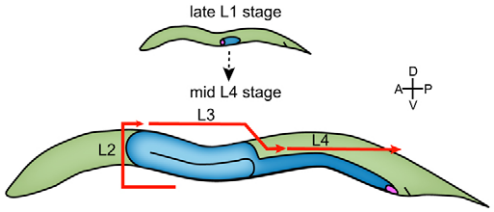


Fig. 1. Linker cell migration. By the late L1 stage, the linker cell (LC; pink) has been specified and is positioned to become the leader cell in the male gonad (blue). The LC migrates from the early L2 through the mid-L4 stage. The red arrows indicate the LC migratory path during each of these larval stages. It begins by migrating anteriorly on the ventral bodywall, then turning from the ventral to dorsal side during the L2 molt. It migrates posteriorly during the L3 and L4 stages. In the mid-L3 stage, it performs a second turn from the dorsal back down to the ventral bodywall. In all figures, anterior (A) is left; posterior (P) is right; dorsal (D) is top; ventral (V) is bottom.

expression and cell shape in L4 larvae are primarily controlled temporally. We have identified the *tailless*/*Tlx* homolog *nhr-67* (Gissendanner et al., 2004), a nuclear hormone receptor, as a stage-specific regulator of LC migration during the L3 and L4 stages, including the negative regulation of the *unc-5* netrin receptor. In particular, *nhr-67* is required for executing LC developmental changes at their proper time during the L3 and L4 stages. We propose that *nhr-67* is a stage-specific regulator that modifies a basal LC migratory program to execute L3 and L4 stage changes with normal timing.

MATERIALS AND METHODS

Worm strains

C. elegans strains were cultured at 20°C using standard protocols (Brenner, 1974) unless indicated otherwise. All strains used carry the *him-5*(*e1490*) mutation, except *nuIs9* [*unc-5::GFP*], which carries the *him-8*(*e1489*) mutation. The alleles and transgenes used in this study are: *zmp-1*(*cg115*)III; *unc-119*(*ed4*)III (Maduro and Pilgrim, 1995); *him-8*(*e1489*)IV (Hodgkin et al., 1979); *him-5*(*e1490*)V (Hodgkin et al., 1979); *him-4(e1267)*X (Hodgkin et al., 1979); *syIs128* [*lag-2::YFP*]II; *syIs49* [*zmp-1::GFP*]IV (Wang and Sternberg, 2000); *nuIs9* [*unc-5b::GFP*] (Su et al., 2000); *muIs27* [*mig-2::GFP*] (Zipkin et al., 1997); *qEx454* [*gon-1::GFP*] (Blelloch and Kimble, 1999); *syEx925* [*nhr-67::GFP*] (Fernandes and Sternberg, 2007); *him-4::YFP*; *nhr-67::unc-5*; *rde-1(ne215)*; *znex338[lag-2::rde-1*, *lag-2::mRFP]* (Lucanic and Cheng, 2008).

RNAi feeding assays

An RNAi screen of 508 known and putative transcription factors was conducted on *him-5* animals. Adult males were scored for incomplete gonad migration under the dissecting microscope. A complete list of transcription factors tested can be found in Fernandes and Sternberg (Fernandes and Sternberg, 2007). A previously described RNAi protocol was used (Kamath et al., 2001) with a few modifications. Eggs were harvested from gravid adults by bleaching and incubated on plates containing RNAi bacteria at 22°C. The RNAi bacteria were obtained from the Ahringer Library (Geneservice).

nhr-67 expression levels were modulated during gonad migration by first growing animals on *nhr-67* RNAi bacterial plates from egg to the late L1/early L2 stage over ~41 hours. The animals were then washed with M9 solution and placed on plates with OP50 bacteria and scored at the late L4 stage. This experiment was repeated by switching plates in the mid-L1 and mid-L2 stages.

Staging males

The early L3 stage was identified under Nomarski optics by the '10-cell stage' configuration of the B lineage (Chamberlin and Sternberg, 1993) and by the yet undivided P10.p and P11.p cells. Late L3 stage animals were identified by there being at least ten P10.p and P11.p progeny cells. The L4 stage was divided into four time categories based on hook and tail retractions. A small hook retraction just anterior to the cloaca was defined as the early L4 stage, and a large retraction characterized by jagged, receding edges, was defined as the early/mid-L4 stage. The beginning of the tail retraction was defined as the mid/late L4 stage, and a large tail retraction to the base of the tail taper was defined as late L4 stage.

Generation of plasmids and transgenic lines

lag-2::YFP plasmid was generated by PCR amplification of a 3.3 kb sequence of the *lag-2* 5′ region using primers GAAACTGCAGTGCCACTCATTATTTTGGACG and GAAGGATCCCTAGCAAAGCTCAAGGTCGAC, and then cloning into the *Pst*I and *Bam*HI sites of vector pPD136.64 (a gift from Andy Fire), modified from Siegfried and Kimble (Siegfried and Kimble, 2002). *him-4::YFP* was generated by a similar strategy using primers TTTAACTGCAGCTGTATGTCCGCTAGGGTCGATT and TTCCGGATCCGATATTGAAGGATTGATGTGCTG to amplify 4 kb of 5′ region, and restriction sites *Pst*I and *Bam*HI. *nhr-67::unc-5* plasmid was generated by first PCR amplifying a 5.3 kb sequence of 5′ *nhr-67* using primers CCCAAGCTTCAATAACTTCCGTTTTGCCAGATC and CCCAAGCTTTCTTGGCGCCAGATTCATTAATTC, and the UNC-5a coding region from cDNA using primers CGGGGTACCATGGACGAAATCACAATCACAACA and CGGGGTACCTTATGGGGACACAATTTGTGGAAA; then, the two fragments were sequentially inserted into plasmid pPD49.26 (a gift from Andy Fire) using *Hin*dIII and *Kpn*I restriction sites, respectively.

The plasmids were injected into *unc-119(ed4); him-5(e1490)* hermaphrodites at 60 ng/μl for *lag-2::YFP* and *him-4::YFP* and at 15 ng/μl for *nhr-67::unc-5*, along with plasmids *unc-119(+)* (Maduro and Pilgrim, 1995) and pBSK+ (Stratagene).

Fluorescence intensity measurements

Fluorescence images of *unc-5::GFP* animals were captured on a Hamamatsu ORCA ER camera and the mean pixel intensity of the LC was calculated using Openlab software (Improvision). DIC and fluorescence images were overlaid using Adobe Photoshop 7.0.

Sequence homology searches

The known *nhr-67* binding site, AAGTCA, was searched for in the regulatory regions of *unc-5* and *zmp-1*. In the 4.6 kb promoter region of *unc-5* used for the reporter strain (Su et al., 2000), four potential binding sites (underlined) were found: AAGCACGGATA<u>AAGTCA</u>CTTTTTCTGTGATTA; AAAAAATTTTA<u>AAGTCA</u>TTTTTGAGCTCTGTAG; TTGAAAAGAAA<u>AAGTCA</u>CAACTAATTGTAAGTTAT; and ACAAATTGAAA<u>AAGTCA</u>AAATACTTTTCAAATTTAT. In the 2.8 kb promoter region of *zmp-1*, four potential binding sites were also found: AAATCAGATTTTTAAAA<u>AAGTCA</u>ATTTTGTAATTACTCATGT; ACGTAATAATTTTTGGA<u>AAGTCA</u>TGTTTTGACGTGTTTCAAA; TTAAAAATGCTGTAAA<u>AAGTCA</u>TTATGCATAGAATATGAA; and TTCTTCAATTGTTTTAA<u>AAGTCA</u>TTACTCATCTTCTTTTTAT.

Because a particular hexamer can occur randomly in the sequence at a high rate (1 in 4000 bases), the non-gap alignment program MUSSA (Kuntz et al., 2008) was used to computationally determine whether these hexamers are in conserved regulatory regions. The *C. elegans unc-5* promoter was aligned with sequences from *C. briggsae* and *C. brenneri* (5′ region plus introns). A similar analysis was performed of the 5′ region of *zmp-1* in the three species. We used 15/15 and 18/20 conserved bases as thresholds for the MUSSA alignment. In addition, we searched by eye for any conserved sequences adjacent to each of the hexamers in the three species.

RESULTS

To find genes that stage-specifically affect LC migration, we performed an RNAi screen of 508 known and putative *C. elegans* transcription factors. Because gonad shape is a trace of the LC migratory path, we searched for genes, the reduction-of-function of which caused abnormal adult male gonad morphology. We identified a number of transcription factor genes, including *lin-29*, *nhr-25* and *egl-5*, already known to have roles in gonadogenesis

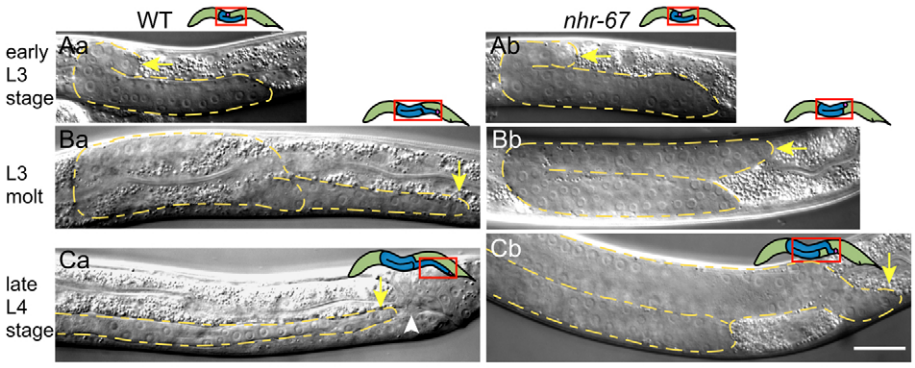
(Abraham et al., 2007; Asahina et al., 2000; Chisholm, 1991; Euling et al., 1999; Ferreira et al., 1999), as well as others with yet unknown gonadal functions, including the *tailless* homolog *nhr-67*. *tailless* has been shown to be required for patterning the anterior and posterior poles of the *Drosophila* embryo (Pignoni et al., 1990), for development of the brain and eye in *Drosophila* and vertebrates (Hollemann et al., 1998; Kitambi and Hauptmann, 2007; Monaghan et al., 1995; Roy et al., 2004; Younossi-Hartenstein et al., 1997; Yu et al., 1994), and for proper vulval morphogenesis and neuronal development in *C. elegans* (Fernandes and Sternberg, 2007; Sarin et al., 2009). We were interested in *nhr-67* because the male gonad shape in *nhr-67*(RNAi) animals suggested a defect specific to the later stages of gonadal development. We thus investigated whether *nhr-67* is a regulator of stage-specific LC development.

nhr-67-deficient animals have L3 and L4 stage defects in gonad morphology

We compared the gonadal morphology of wild-type and *nhr-67*-deficient animals at different stages (Fig. 2A-D). Because *nhr-67* deletion mutants have an embryonic lethal phenotype, we used RNAi feeding on newly hatched worms to study the function of *nhr-67*. *nhr-67*(RNAi) males have a fully penetrant gonadal defect, with none of the LCs completing the migration by the L4 molt (*n*=87). At the early L3 stage, no gonadal defects in *nhr-67*(RNAi) males were observed: 85% of LCs in both *nhr-67*(RNAi) (*n*=31) and wild-type (*n*=22) males executed the turn from the ventral to the dorsal side and migrated posteriorly approximately one-third the length of the ventral gonadal arm (Fig. 2Aa,Ab,D). By the L3-to-L4 molt, however, several gonadal defects had become apparent in *nhr-67*(RNAi) males. First, the LC did not migrate as far in *nhr-67*(RNAi) males as in the wild type, with only 17% (*n*=23) of LCs in *nhr-67*(RNAi) males reaching the P7.p hypodermal cell, compared with 93% (*n*=14) in wild-type males (Fig. 2Ba,Bb,D). Second, by the L3-to-L4 molt, 100% (*n*=14) of LCs in wild-type males turned back down from the dorsal to the ventral bodywall, whereas only 4% (*n*=24) of LCs in *nhr-67*(RNAi) males turned ventrally. Most LCs of *nhr-67*(RNAi) males remained on the dorsal bodywall despite having migrated past the position at which they normally turn. Later during the L4 stage, however, most LCs in *nhr-67*(RNAi) males turned ventrally (95%, *n*=87; Fig. 2Ca,Cb,D). Although LCs in *nhr-67*(RNAi) males continued to migrate during the L4 stage, they were even farther behind their normal position than at the L3-to-L4 molt. As a result, by the late L4 stage, when 100% (*n*=28) of the LCs in wild-type males had finished migrating and been engulfed by the U.lp/U.rp cell, none (*n*=87) of the LCs in *nhr-67*(RNAi) males had completed their migration. LCs in *nhr-67*(RNAi) males might therefore have a defect in executing the correct developmental programs at the appropriate stage.

We found that *nhr-67* functions cell-autonomously in the LC to regulate gonadal migration. We used *rde-1* mutants, which are refractory to RNAi effects, rescued for *rde-1* function cell-specifically in the LC by a *lag-2::rde-1* transgene (Lucanic and Cheng, 2008). When these animals were treated with *nhr-67*(RNAi), 18 of 21 L4 stage males had the same gonadal defects as males receiving systemic *nhr-67*(RNAi). Most *rde-1* mutant males without the rescuing construct did not have gonad migration defects (29/34 were as wild type).

When we examined the expression of a transcriptional *nhr-67::GFP* reporter in the male gonad, we found that it was expressed only in the LC. Furthermore, the duration of *nhr-67::GFP* expression in the LC, from the early L3 stage until LC death (Fig.



D

	early L3 stage	L3 molt		late L4 stage	
	% LC reflexed back 1/3 length of bottom gonad	% LC reaching P7.p	% LC turned ventrally	% LC completed migration	% LC turned ventrally
wild type	86% (19/22)	93% (13/14)	100% (14/14)	100% (28/28)	100% (28/28)
nhr-67 (RNAi)	84% (26/31)	17% (4/23)	4% (1/24)	0% (0/87)	95% (83/87)

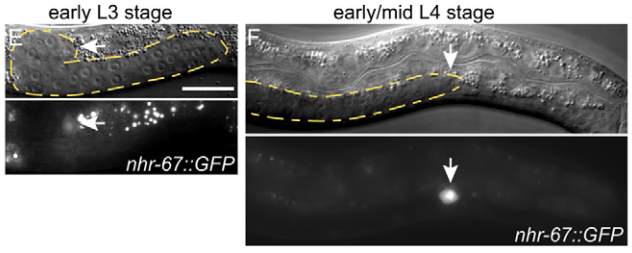


Fig. 2. *nhr-67* is required for gonad migration during the L3 and L4 stages. (**A-C**) Comparison of gonad migration in wild-type and *nhr-67*(RNAi) animals. The gonads of wild-type (Aa,Ba,Ca) and *nhr-67*(RNAi) (Ab,Bb,Cb) animals are shown at the early L3 stage (A), the L3 molt (B), and the late L4 stage (C). (A) The gonads appear identical in the early L3 stage. The LCs are indicated by arrows and the gonads are outlined in yellow. (B) By the L3 molt, the gonad has turned from the dorsal to the ventral side in the wild type (Ba) but not in the *nhr-67*(RNAi) animal (Bb). Also, the gonad migration is slower in the *nhr-67*(RNAi) animal. (C) By the late L4 stage, in the wild-type animal the gonad has completed its migration, having reached the cloaca (Ca, arrowhead). In the *nhr-67*(RNAi) animal, the gonad does not complete its migration, but does finally turn ventrally (Cb). (**D**) Summary of the wild-type versus *nhr-67* gonadal characteristics for the early L3 stage, L3 molt, and late L4 stage. (**E**,**F**) Nomarski images (top) and fluorescence images (bottom) of gonads in an early L3 stage (E) and an early/mid-L4 stage (F) wild-type animal. *nhr-67::GFP* is expressed in the LC (arrow). Scale bars: 20 μm.

2E,F), is consistent with the timing of the gonadal defects observed during the L3 and L4 stages. *nhr-67* is also expressed in the intestine, Pn.p cells, and in a few head and tail cells.

We also used *nhr-67::GFP*-expressing animals to test the efficacy of our *nhr-67*(RNAi) feeding method. Feeding *GFP*(RNAi) to *nhr-67::GFP*-expressing animals abolished GFP expression in all tissues except for a few refractory head neurons (*n*=43/44 no LC GFP).

nhr-67 regulates gonad migration sex-specifically in males

Most genes utilized for proper gonad migration by the male LC are also used by the hermaphrodite for gonad migration (Lehmann, 2001). The best-studied hermaphrodite gonad has two leader cells: the distal tip cells (DTCs) that start migrating from the midbody in opposite directions to create a two-armed gonad. Although *nhr-67* is important for male gonad migration, hermaphrodite DTC migration is not regulated by *nhr-67*. Twenty-nine of thirty hermaphrodites treated with *nhr-67*(RNAi) had normal DTC migrations, compared with 36/37 wild-type hermaphrodites. Consistently, we did not observe the expression of *nhr-67::GFP* in the hermaphrodite DTCs. *nhr-67* is thus a rare instance of a gene used male-specifically in gonadal leader cell migration.

nhr-67 negatively regulates the expression of the netrin receptor UNC-5

We examined whether the failure of the LC in *nhr-67*(RNAi) animals to execute the second turn during the L3 stage involves the regulation of netrin receptors by *nhr-67*. Netrin receptors are known to be necessary for specific *C. elegans* gonadal turns (Su et al., 2000). Two netrin receptors, UNC-5 and UNC-40, are required by the LC for the first turn in the L2 stage, and for a similar, dorsal turn by the hermaphrodite DTCs at the L3 stage. In both migratory cell types, the UNC-40 netrin receptor alone is expressed by the gonadal leader cells in the early stages of migration, causing them to be attracted to netrin molecules on the ventral bodywall and to migrate along it. At the time of their ventral-to-dorsal turn, the leader cells begin to coexpress the UNC-5 netrin receptor, which then changes their response to netrin from attraction to repulsion and guides their migration away from the ventral bodywall.

We examined *unc-5::GFP* expression in wild-type and *nhr-67*(RNAi) males at the L3 and L4 stages. Although UNC-5 expression is visible in the LC by antibody staining at the start of the ventral-to-dorsal turn (Su et al., 2000), the *unc-5::GFP* reporter only becomes visible during the turn in the L2-to-L3 molt. Levels of LC UNC-5 expression remained constant during the L3 and L4 stages (Fig. 3A,B). By contrast, in *nhr-67*(RNAi) animals, LC expression of *unc-5::GFP* became progressively brighter during the L3 and L4 stages, indicating elevated levels of *unc-5* expression (Fig. 3A,B). NHR-67 is therefore required to negatively regulate *unc-5* expression during the L3 and L4 stages.

Suppression of UNC-5 expression is required for the mid-L3 stage turn

We further investigated whether the failure of the LC to turn ventrally at the mid-L3 stage in *nhr-67*(RNAi) animals was due to overexpression of UNC-5 past the mid-L3 stage. We

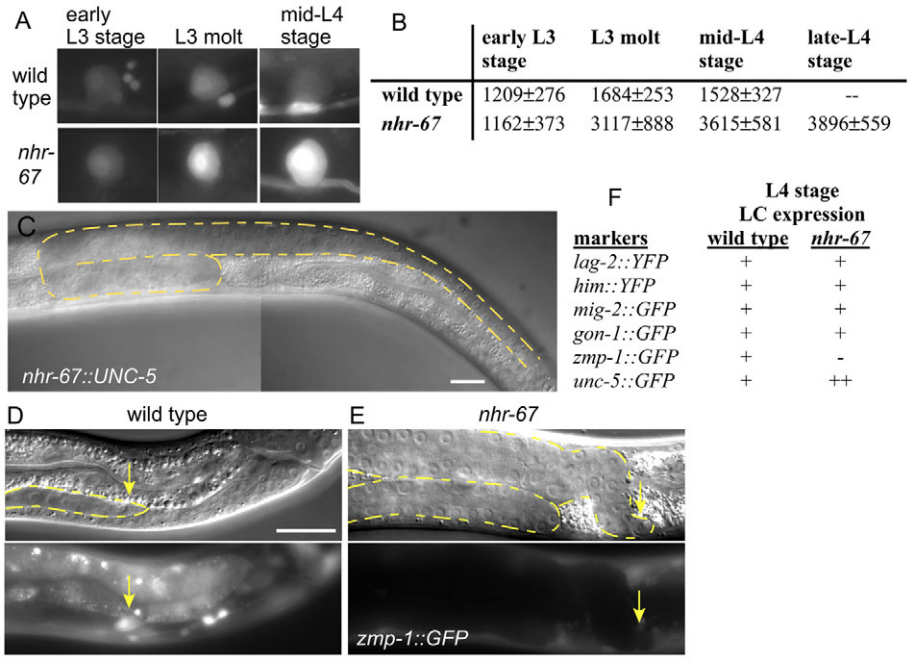


Fig. 3. *nhr-67* negatively regulates *unc-5* in the L3 stage and positively regulates *zmp-1* in the L4 stage. (**A**) *unc-5::GFP* expression in the LC during the L3 and L4 stages remains constant in wild-type animals (top row), but becomes progressively stronger in *nhr-67*(RNAi) animals (bottom row). (**B**) Summary of the levels of *unc-5::GFP* fluorescence in the LC of wild-type and *nhr-67*(RNAi) animals. (**C**) Nomarski image of an animal overexpressing UNC-5 in the LC during the L3 and L4 stages. In this *nhr-67::unc-5*-expressing animal, the LC continues to migrate on the dorsal bodywall instead of turning ventrally in the mid-L3 stage. (**D**) The LC expresses *zmp-1::GFP* during the L4 stage in wild-type animals. The gonad is outlined in yellow and the LC is indicated by an arrow. Nomarski, top; fluorescence, bottom. (**E**) In *nhr-67*(RNAi) animals, *zmp-1::GFP* is not expressed in the L4 stage LC. (**F**) Summary of the expression of fluorescent markers in the LC of wild-type versus *nhr-67*(RNAi) animals in the L4 stage. Genes that are expressed throughout LC migration, such as *lag-2*, *him-4*, *mig-2* and *gon-1*, are not regulated by *nhr-67*. By contrast, *zmp-1* and *unc-5*, which have specific L3 and L4 stage expression, are regulated by *nhr-67*. Scale bars: 20 μm.

ectopically expressed *unc-5* in the LC during both the L3 and L4 stages in wild-type animals to see whether this prevented the LC from turning ventrally in the mid-L3 stage. We used an *nhr-67::UNC-5* construct because the *nhr-67* promoter drives gene expression in the LC during the L3 and L4 stages. Fifty-seven percent (n=30) of *nhr-67::unc-5*-expressing animals showed continued dorsal LC migration throughout the L3 stage, and often into the L4 stage (Fig. 3C). In these animals, unlike in *nhr-67*(RNAi) animals, the LC reached the posterior region of the body before the L4-to-adult molt, indicating that the defects that we observed were due to *unc-5* overexpression and not to an *nhr-67* deficiency resulting from the sequestering of factors that bind the *nhr-67* promoter. Ectopic expression of *unc-5* is therefore sufficient to prevent the LC from turning ventrally in the mid-L3 stage. We conclude that the negative regulation of *unc-5* by NHR-67 is required in the LC for it to execute the mid-L3 stage turn from the dorsal to ventral side.

nhr-67 regulates L4 stage *zmp-1* expression in the LC

We then investigated the effect of *nhr-67* on the LC in L4 larvae. Because little is known about LC development during its migration, we began by identifying changes in LC gene expression, shape and migration during the L4 stage. One change we found is that *zmp-1*, which encodes a zinc matrix metalloprotease, is expressed by the LC from the early L4 stage until LC death in the L4-to-adult molt (n=40/41; Fig. 3D). When we examined *zmp-1::GFP* expression in *nhr-67*(RNAi) animals, we found that it is never expressed in the LC (n=0/35; Fig. 3E). *zmp-1* mutants, however, had a wild-type gonad migration (22/22 animals with complete LC migration by mid/late L4).

We could not find any evidence of *nhr-67* directly regulating either *unc-5* or *zmp-1* based on sequence conservation. Although the promoter regions of *unc-5* and *zmp-1* used in the reporter strains contain the hexameric *nhr-67* binding sequence (DeMeo et al., 2008), the sequences adjacent to the hexamers are not conserved in *C. briggsae* and *C. brenneri* (see Materials and methods).

nhr-67 specifically regulates genes that change in expression during the L3 and L4 stages

nhr-67 must regulate other genes besides *unc-5* and *zmp-1* because animals that are both mutant for *zmp-1* and overexpress *unc-5* resemble the *unc-5*-overexpression phenotype, rather than the *nhr-67* phenotype. We examined 85 animals with both *zmp-1* and *unc-5* perturbations at various times during the L4 stage and found that most LCs reached the posterior region of the animal by the mid/late L4 stage (n=60/61) and became polarized (n=31/36).

Having found that NHR-67 negatively regulates *unc-5* and positively regulates *zmp-1*, we examined whether NHR-67 specifically regulates genes that become expressed during the L3 and L4 stages, or whether it regulates gene expression in the LC more broadly. We tested the effect of *nhr-67*(RNAi) on transcriptional and translational fluorescent reporters of a selection of genes that are expressed throughout LC migration (Blelloch and Kimble, 1999; Henderson et al., 1994; Lundquist et al., 2001; Vogel and Hedgecock, 2001; Zipkin et al., 1997). None of the genes that we tested, which were *lag-2::YFP* (n=30/30), *gon-1::GFP* (n=16/16), *mig-2::GFP* (n=29/29) and *him-4::YFP* (n=32/32), was affected by *nhr-67*(RNAi) (Fig. 3F). These observations suggest that *nhr-67* is not generally required to maintain gene expression in the LC, but instead regulates expression specifically during the mid-L3 through L4 stages.

nhr-67 regulates LC morphological change during the L3 to late L4 stages

We also identified morphological changes in the LCs of L4 larvae. Using a marker for LC cytoplasm, *lag-2::YFP*, we observed that the LC changes from a round to a polarized shape during the L3 and L4 stages (Fig. 4Aa-Af). We quantified these changes (Fig. 4C) in the early and late L3 stage and at four time points during the 10 hours of the L4 stage, classifying LC shape by the degree of polarization as 'round', 'pointed', 'elongated' or 'extended'. LCs with

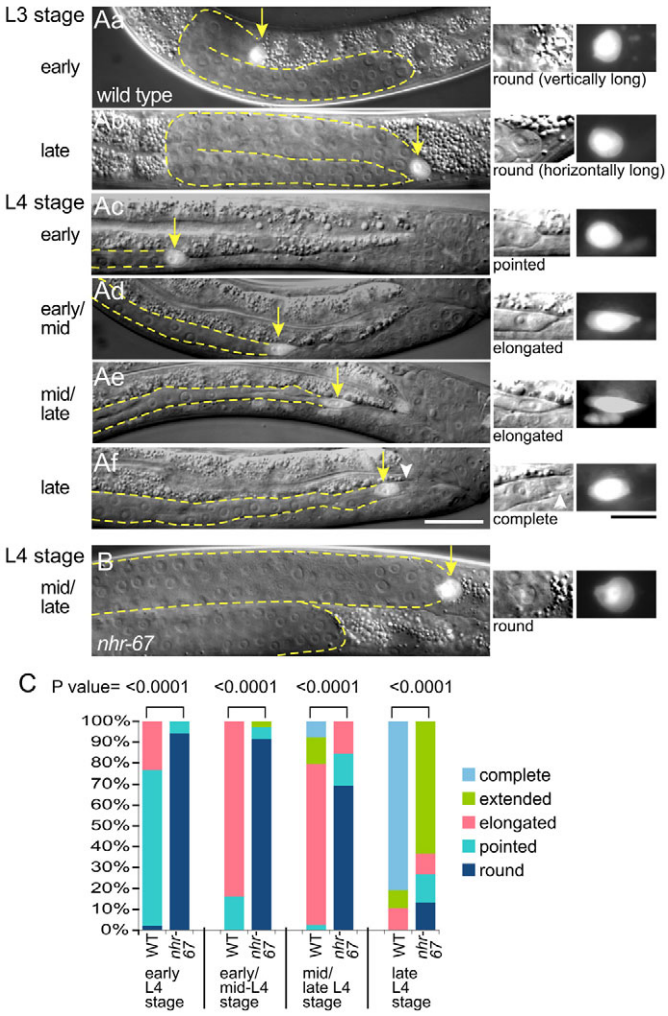


Fig. 4. Changes in LC morphology at L3 and L4 stages in wild-type and *nhr-67*(RNAi) animals. (**A**) A time series of LC migration during the L3 and L4 stages showing that the LC shape becomes increasingly polarized over time. In the left column are overlays of Nomarski and fluorescence images of the male gonad (outlined in yellow) expressing the LC marker *lag-2::YFP* (arrow). Enlarged Nomarski (middle column) and fluorescence (right column) images show LC shape at each stage. (**a**,**b**) The LC shape is round in the L3 stage, but changes from vertically (a) to horizontally (b) oblong. (**c**) In the early L4 stage, the LC leading edge develops a point. (**d**,**e**) During the L4 stage, the LC becomes increasingly elongated. (**f**) The LC completes its migration once it reaches the U.lp/U.rp cell (arrowhead) and becomes rounded as it is engulfed by U.lp/U.rp. Scale bars: 20 μm (left) and 10 μm (middle and right). (**B**) In mid/late L4 stage *nhr-67*(RNAi) animals, the LC still has a round morphology. (**C**) Quantification of LC shape during the L3 and L4 stages in wild-type and *nhr-67*(RNAi) animals. *P*-value calculated by Mann-Whitney U test.

'completed' migrations were typically round and engulfed. Wild-type L3 stage animals had predominantly round LCs that changed from being oblong in the vertical to horizontal direction (Fig. 4Aa,Ab). In L4 larvae, the rounded leading edges of the LCs became pointed, and LCs became more elongated (Fig. 4Ac-e). However, LCs rarely became more polarized than the elongated stage before they finished migrating (Fig. 4Af).

We examined whether *nhr-67* regulates LC morphology in L3 and L4 larvae. In *nhr-67*(RNAi) animals, the LC maintained a round shape, typical of the early L3 stage, throughout all of the L3 stage and most of the L4 stage (Fig. 4B,C). Over 90% of LCs in the first half of the L4 stage still had a round shape. However, these LCs converted to an extended shape, typical of late L4 stage larvae, at the normal time. Thus, there might be other factors besides NHR-67 that regulate the late L4 stage LC program. Our results show that *nhr-67* is required for LC shape polarization through the L3 and most of the L4 stages. *nhr-67* induces LC shape change by regulating other genes besides *unc-5* and *zmp-1*, as LCs in animals both overexpressing *unc-5* and mutant for *zmp-1* were able to undergo shape change (n=31/36).

Temporal cues play a primary role in the changes in LC morphology and gene expression

We have demonstrated that LCs of L4 stage *nhr-67*(RNAi) larvae resemble those of normal L3 larvae in their morphology, gene expression and position along the migratory path. NHR-67 might be required by the LC to correctly interpret temporal cues and synchronize its developmental program with the rest of the organism. Alternatively, because the LC has not reached the posterior body in *nhr-67*(RNAi) males by the L4 stage, the LC might be failing to receive spatially restricted cues from its environment. In this latter case, the changes in morphology and gene expression at the L4 stage would not be directly regulated by *nhr-67*, but would be secondary effects of LC position. To distinguish between these possibilities, we used a migration-defective mutant, *him-4* [which encodes the extracellular matrix (ECM) protein hemicentin], to test whether an incorrectly positioned L4 stage LC can still undergo changes in morphology and gene expression. In the absence of HIM-4, the LC meanders from its normal path; by the L4 stage, it is in either the head or mid-body region instead of in its normal posterior location (Vogel and Hedgecock, 2001). We found that LCs in *him-4(e1267)* mutants undergo the L4 stage-specific morphology change with correct timing, despite not having reached the posterior body: 100% (n=20) of the LCs in L3 larvae had a round shape, but by the mid-L4 stage 100% (n=14) of the LCs had a polarized shape (Fig. 5A-D). This is the same timing as observed for wild-type males, with 100% (n=35) having a round LC shape in the L3 stage and 100% (n=73) having a polarized shape in the mid-L4 stage. We defined a polarized LC shape to include all non-round shapes. We also found that the expression of ZMP-1 in L4 larvae is not regulated by positional cues. In *him-4; zmp-1::GFP* animals, *zmp-1* was correctly expressed in the LC at the L4 stage, despite the anterior positioning of the LC [95% (n=38) for *him-4* versus 98% (n=41) for wild type; Fig. 5E,F]. We conclude that *nhr-67* primarily regulates the timing of both L3 and L4 stage events by responding to either a cell-intrinsic clock or a global timing signal, rather than to spatial cues that originate from a posterior source in the animal.

However, positional cues might also contribute to *nhr-67*(RNAi) LC migration defects. Because it has been shown that MIG-2 localization in motile cells depends on ECM components and is required for cell shape change (Ou and Vale, 2009; Ziel et al., 2009), we examined MIG-2::GFP localization in the LC in wild-type and *nhr-67*(RNAi) males (Fig. 5G-J). In the LCs of L3 stage wild-type males, MIG-2::GFP was evenly distributed throughout the membrane, but during the L4 stage MIG-2 became polarized to the adherent, ventral side. In L4 stage *nhr-67*(RNAi) males, however, only five of 26 LCs showed MIG-2::GFP membrane polarization to the adherent side. It might be that MIG-2 does not localize properly in *nhr-67*(RNAi) males because the LC is not positioned in L4 to access spatially restricted ECM components.

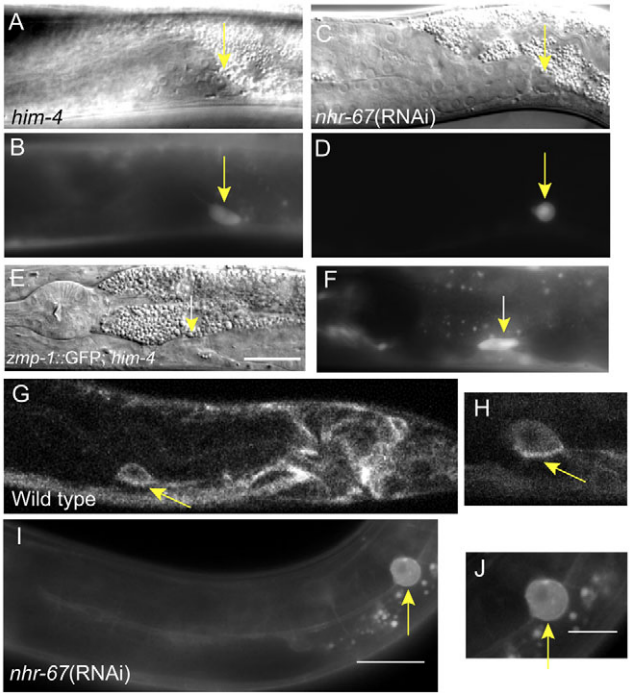


Fig. 5. Temporal cues are primarily responsible for changes in the LC at the L4 stage. (**A-D**) The LC in a mid-L4 stage *him-4* mutant (A,B) has a polarized shape similar to that of wild-type males (not shown), but different from a similarly positioned LC in an *nhr-67*(RNAi) male (C,D) of the same stage. (**E**,**F**) An L4 stage *him-4* mutant correctly expresses *zmp-1::GFP* in the LC, even though the LC is abnormally positioned near the pharynx (Nomarski, left; fluorescence, right). The LC also has the appropriate elongated morphology of the L4 stage. (**G**,**H**) Confocal image of L4 stage male posterior body and a high-magnification image of LC show MIG-2::GFP to be membrane localized to the ventral, adherent side (arrow). (**I**,**J**) Nomarski image of *nhr-67*(RNAi) male showing cytoplasmic and membrane localization of MIG-2::GFP (arrow) in the LC without polarization to the adherent side. Scale bars: 20 μm in E,I; 10 μm in J.

nhr-67 is required by the LC at different times throughout the L3 and L4 stages

We investigated whether *nhr-67* functions only at the start of the L3 stage to start a cascade of other genes that regulate later events in the L3 and L4 stages, or whether NHR-67 itself regulates events throughout these stages. We grew animals on *nhr-67*(RNAi) bacteria and then shifted them to non-RNAi-containing bacteria, such that they would be exposed to the effects of *nhr-67*(RNAi) during the L3, but not the L4, stage. If NHR-67 only acts at the early L3 stage, we would expect to see males with the full *nhr-67*(RNAi) gonadal defect, even if they had been removed from *nhr-67*(RNAi) after its initial effects. By contrast, if *nhr-67* acts at different times throughout the L3 and L4 stages, we would expect to see males with a hybrid phenotype of early *nhr-67*(RNAi) defects but later wild-

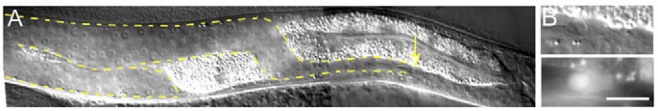


Fig. 6. *nhr-67* regulates changes in the LC at the L4 stage independently of those at the L3 stage. Animals with a hybrid gonadal phenotype consisting of the L3 stage *nhr-67* gonadal defect but a wild-type L4 stage gonad. (**A**) Nomarski image of a late L4 stage animal showing a gonad with the early characteristics of an *nhr-67*(RNAi) animal: the LC fails to turn from the dorsal to the ventral side in the mid-L3 stage and the LC (arrow) migrates slowly. (**B**) However, the same LC has wild-type *zmp-1::GFP* expression. Nomarski, top; fluorescence, bottom. Scale bars: 20 μm in A; 10 μm in B.

type characteristics. Indeed, we found hybrid phenotypes in the L3 and L4 stages when the animals were shifted from the *nhr-67*(RNAi) to non-RNAi-containing bacteria during the late L1/early L2 stage. In 11/44 males, the gonad failed to perform the turn from the dorsal to the ventral side during the mid-L3 stage, but properly expressed *zmp-1::GFP* by the late L4 stage (Fig. 6). These migrations were slower than in the wild type, but faster than in *nhr-67*(RNAi) animals. The hybrid phenotype further indicates that a shift in the levels of *nhr-67* can occur within ~6 hours. Our results support a role for NHR-67 at different times throughout the L3 and L4 stages in the execution of the LC developmental program.

DISCUSSION

Here we have described how the *C. elegans* male LC relies on timing cues to execute the various stages of its migration. Closer examination of LC migration revealed that the LC displays many complex behaviors, including migration over different body surfaces, the execution of two turns, and changes in cell shape and migration speed. Moreover, these behaviors occur at specific times during the migration, suggesting that dynamic, stage-specific gene regulation is involved. Three genes that we found to exhibit stage-specific expression in the LC are the *tailless* homolog *nhr-67*, the netrin receptor *unc-5* and the zinc metalloprotease *zmp-1*. *nhr-67* is expressed by the LC during the L3 and L4 stages and is discussed below. *unc-5* is expressed from the late L2 stage (Su et al., 2000) to the mid-L3 stage, and its downregulation is necessary for the LC to turn ventrally in the mid-L3 stage. *unc-5* is likely to be just one of several ECM receptors dynamically regulated in the LC, as judged by the fact that other migratory cells, such as the hermaphrodite DTCs in *C. elegans* and the neural crest and primordial germ cells in vertebrates, express several different ECM receptors required to navigate their complex course (Henderson and Copp, 1997; Meighan and Schwarzbauer, 2008). *zmp-1* is expressed only in the L4 stage. We found no defect in LC migration in *zmp-1* deletion mutants, but this might be due to redundancy of matrix metalloproteases in the LC (Meighan et al., 2004). In addition to these three genes, we uncovered stage-specific cell behaviors, such as the L4 stage LC shape change, that are not mediated by UNC-5 or ZMP-1, suggesting that the LC expresses many other genes stage-specifically.

One way to modulate complex cell behaviors is through instructional cues that are spatially restricted, such that a cell modifies its behavior upon reaching specific sites along its migratory route. Instead, the LC appears to implement these behaviors as part of its developmental program based on a temporal cue. We show, for instance, that changes in cell shape and *zmp-1* expression occur in L4 larvae even when the LC has not reached its normal position in the posterior body.

nhr-67 transcriptionally regulates a temporal subset of the LC developmental program

We have revealed a new function for *nhr-67* in controlling a time-dependent subset of events during LC migration. *nhr-67* acts cell-autonomously to regulate all the LC changes that we have identified within a specific time-window of the L3 and L4 stages, but none of the migratory changes either before or afterwards. This observation suggests that LC migration is assembled from temporal subprograms that might each be independently regulated by factors such as *nhr-67*.

In addition, *nhr-67* controls the timing of events during the subprogram it regulates. For each of the developmental events in the LC during the L3 and L4 stages, *nhr-67*(RNAi) animals display a phenotype indicative of the continuation of the early L3 stage and the delay of succeeding stages. There is evidence that *Tlx*, the mammalian homolog of *nhr-67*, regulates timing in mouse neurogenesis (Roy et al., 2004). In *nhr-67*(RNAi) animals, UNC-5, which in wild-type animals is downregulated in the LC by the mid-L3 stage, continues to be highly expressed in the late L3 stage and during the L4 stage, whereas *zmp-1*, a gene normally expressed in the LC at L4 stage, is never expressed. These roles of NHR-67 are consistent with previous findings that *tailless* acts as both a positive and negative regulator of gene expression (Hoch et al., 1992; Margolis et al., 1995; Pankratz et al., 1992). Also, in *nhr-67*(RNAi) animals, the LC does not turn from the dorsal to the ventral bodywall during the mid-L3 stage as in wild-type worms. We show that this abnormal guidance is due, at least in part, to the continued expression of UNC-5 in *nhr-67*(RNAi) animals past the mid-L3 stage. Finally, in contrast to the increasingly polarized morphology of the LC in wild-type worms, the LC of *nhr-67*(RNAi) males remains in the round, early L3 stage shape throughout most of its migration. Although an *nhr-67* null mutant might have a more severe phenotype than *nhr-67*(RNAi), the *nhr-67*(RNAi) phenotype is both penetrant and consistent in each animal and across trials.

An interesting question is how NHR-67 regulates events in the LC at diverse times during the L3 and L4 stages. One possibility is that NHR-67 confers specificity by binding to a heterodimeric partner or co-regulator (DeMeo et al., 2008; Nettles and Greene, 2005). Another possibility is that a temporal gradient is set by the level of expressed NHR-67, which would gradually accumulate over time. An example of this is seen with PHA-4, the master regulator of pharynx formation, which first activates genes whose regulatory regions have the highest affinity for PHA-4, and later activates genes whose regulatory regions have lower affinity (Gaudet and Mango, 2002).

Model for gene regulation in LC migration

We propose that LCs have both a basal migration program, which begins at LC specification and is used through the life of the LC, and at least three stage-specific programs that modify the basal program (Fig. 7). NHR-67 regulates one such program from the early L3 to mid-L4 stage. Since in *nhr-67*(RNAi) animals, LC migration before the early L3 stage and after the mid-L4 stage occurs with normal timing, there must be other transcriptional regulators acting in the LC. We hypothesize that each of these stage-specific regulators acts on a basal migration program consisting of genes that are expressed throughout LC migration. Some of the genes that we examined are part of the basal migration program, including *lag-2*, *gon-1*, *him-4* and *mig-2*; the latter three are required for normal gonadal migration (Blelloch and Kimble, 1999; Henderson et al., 1994; Lundquist et al., 2001; Vogel and Hedgecock, 2001; Zipkin et al., 1997). As the

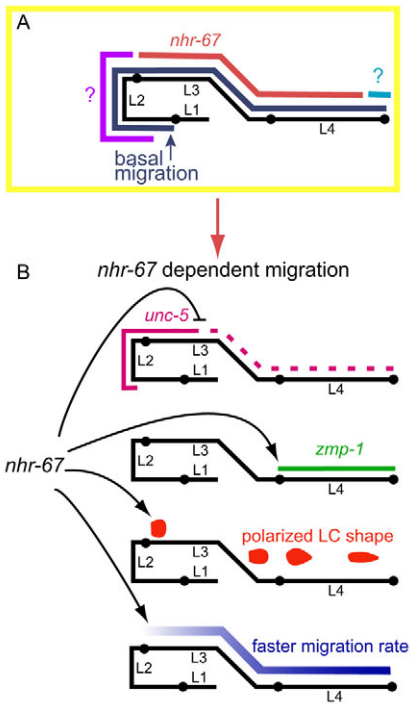


Fig. 7. Model for LC migration in *C. elegans*. (**A**) LC migration consists of two levels of regulation: a basal migration program and stage-specific modifiers such as *nhr-67*. The basic shape of the gonad during the L1 through L4 stages is indicated in black. The basal migration program (dark blue) is implemented at the start of LC migration in the L1-to-L2 molt and continues until LC death. The genes expressed throughout LC migration and used in the basal program include *lag-2*, *mig-2*, *gon-1* and *him-4*. *nhr-67* regulates changes in LC migration from the early L3 stage through to the mid/late L4 stage (red). There are likely to be other stage-specific regulators (purple and light blue) because LC development up until the L3 stage and in the late L4 stage occurs normally in *nhr-67*-deficient males. In the absence of *nhr-67*-dependent migration, the basal migration program continues and the LC migrates but remains in its early L3 stage form for an extended time. (**B**) *nhr-67*-dependent migration can be dissected into specific components. *nhr-67* negatively regulates *unc-5*, but positively regulates *zmp-1* expression, polarization of LC shape and migration speed.

expression of these genes was unaffected in *nhr-67*(RNAi) animals, we propose that the basal migration program continues even without the execution of stage-specific programs.

Timing cues and organogenesis

Cell migrations are often required during organogenesis and contribute to the shape and function of the final organ. In many of these cell migrations, spatially graded cues not only provide guidance but also regulate motility, cell morphology and gene expression. For example, in border cell migration in *Drosophila*, the migratory behavior is mediated through EGF and PDGF/VEGF receptors that bind signaling molecules from a spatially restricted origin (Duchek et al., 2001). Similarly, tracheal outgrowth in mouse or *Drosophila* requires FGF signaling from surrounding tissue, with the cells closest to the tissues being activated (Affolter and Caussinus, 2008; Cardoso and Lu, 2006). Temporal regulation of migration is less well characterized, perhaps because spatial cues have such a visible role in most cell migrations. Unlike other well-studied instances of cell migration, we have shown that the migration of the LC depends heavily on timing cues to execute different stages of its migration. We found that even when the LC is mispositioned in the body of the animal, and hence removed from its normal spatial environment, it is still capable of migrating and undergoing stage-specific programs. The delayed development of the LC in *nhr-67*(RNAi) animals also suggests that the LC uses NHR-67 to interpret timing information, the source of which might be a cell-intrinsic clock or a global timing cue. One hint that a global signal may be involved comes from the previous finding that the DAF-12 nuclear hormone receptor, which binds the global hormone dafachronic acid, is required by both the male LC and hermaphrodite DTCs for executing gonadal turns in L3 larvae and for arresting development in dauer larvae (Antebi et al., 1998; Motola et al., 2006).

Understanding temporal regulation during organogenesis should shed light on morphological differences between species, and between normal and diseased states. Since the gonads of different nematode species have different shapes (Chitwood and Chitwood, 1950), it is interesting to speculate that dramatic changes in gonadal shape could arise from subtle changes in temporal regulation of the LC. The existence of stage-specific regulators suggests the possibility that dramatic changes in organ shape can be achieved through the regulation of relatively few temporally regulated genes.

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