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Multiple roles for the E/Daughterless ortholog HLH-2 during *C. elegans* gonadogenesis

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Abstract

HLH-2 is the *Caenorhabditis elegans* ortholog of the *Drosophila* Daughterless and mammalian E basic helix–loop–helix (bHLH) transcriptional activators that function during diverse events during animal development. HLH-2 has been implicated in cell fate specification in different neural lineages and in the LIN-12/Notch-mediated anchor cell (AC)/ventral uterine precursor cell (VU) decision in the somatic gonad. Here, we show that *hlh-2* plays several distinct roles during somatic gonadogenesis. Our analysis suggests that *hlh-2* is required to endow specific somatic gonadal cells with the competence to undergo the AC/VU decision, as well as functioning in the AC/VU decision per se; this novel "proAC" role appears to be analogous to the proneural role of *Drosophila* Daughterless. In addition to its two distinct roles in the specification of the AC, *hlh-2* is also required for correct differentiation and function of the AC. *hlh-2* also acts at an independent point in the gonadal lineage both to specify distal tip cells (DTCs) and in DTC differentiation and function.

Keywords: HLH-2; Daughterless; LIN-12, Notch; Proneural

Introduction

Basic helix–loop–helix (bHLH) transcription factors have been implicated in many developmental processes including cell specification, differentiation, and sex determination (e.g., Campuzano and Modolell, 1992; Cronmiller and Cline, 1987; Engel and Murre, 2001). bHLH proteins have been assigned to seven different classes based on tissue distribution, dimerization preferences, and DNA binding specificities (Massari and Murre, 2000). Class I bHLH proteins, including *Drosophila* Daughterless and the mammalian E proteins, are typically transcriptional activators that function either as homodimers or as the obligate dimerization partners for Class II bHLH proteins. In *C. elegans*, there is one Class I bHLH protein, HLH-2 (Ledent and Vervoot, 2001; Ruvkun and Hobert, 1998). *hlh-2* has been shown to be important for neural development (Frank et al., 2003; Krause et al., 1997; Portman and Emmons, 2000) and in the regulation of a programmed cell death (Thellman et al., 2003). In this study, we focus on multiple roles of *hlh-2* in *Caenorhabditis elegans* gonadogenesis.

In wild-type hermaphrodites, two founder cells formed during embryogenesis, called Z1 and Z4, divide three times during the first larval (L1) stage to produce the 12 cells of the gonad primordium (Kimble and Hirsh, 1979, Fig. 1A). Ten cells of the primordium have invariant fates: eight precursors that later generate uterine and sheath/spermatheca cells, and two terminally differentiated distal tip cells (DTCs), which lead the growth of the U-shaped gonad and promote mitosis of the adjacent germline cells (Kimble and White, 1981). Two other cells of the primordium, named Z1.ppp and Z4.aaa, have variable fates: one cell will become the terminally differentiated anchor cell (AC), and the other will become a ventral uterine precursor cell (VU) (Kimble and Hirsh, 1979).

How Z1.ppp and Z4.aaa choose their fates—the "AC/VU decision"—has been the subject of much investigation. Although in every wild-type hermaphrodite, one of them becomes the AC and the other becomes a VU, which cell takes on which fate varies from individual to individual (Kimble and Hirsh, 1979) and depends on interactions

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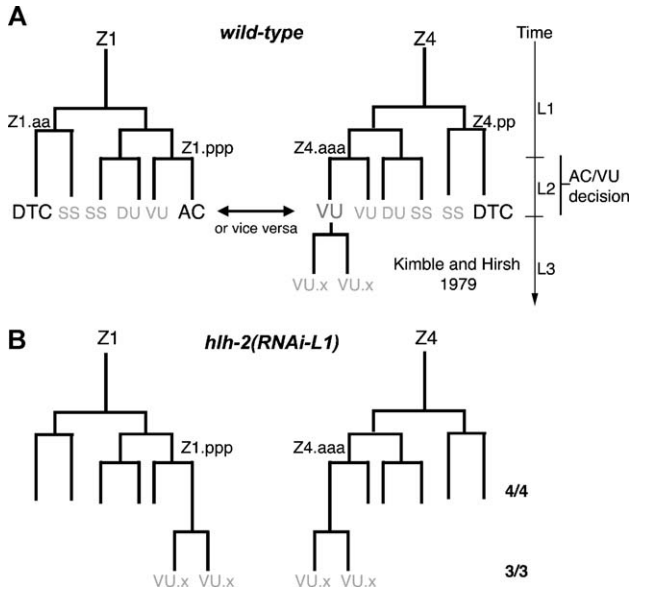

Fig. 1. Gonadal cell lineage through primordium formation of wild-type and *hlh-2(RNAi-L1)* hermaphrodites. (A) Wild-type hermaphrodite gonadal lineage (Kimble and Hirsh, 1979). Cell fates are written below each cell (DTC = distal tip cell; SS = sheath/spermathecal precursor cell; DU = dorsal uterine precursor cell; VU = ventral uterine precursor cell; AC = anchor cell; VU.x = daughter of a VU), where black lettering indicates a terminally differentiated cell type, and grey lettering indicates a cell that will eventually divide. Cells discussed in the text are labeled. In this diagram, Z1.ppp is pictured as becoming an AC and Z4.aaa is pictured as becoming a VU, but the opposite is equally likely. (B) *hlh-2(RNAi-L1)* hermaphrodite gonadal lineages. Four larvae were observed throughout the L1 and early L2 stages; all four exhibited a normal lineage pattern. Three additional larvae were observed from the beginning of the L2 stage until mid-L3, the time when a VU would normally divide. In 3/3 animals observed both Z1.ppp and Z4.aaa divided at this time (see Materials and Methods).

between the two cells during the L2 stage of hermaphrodite gonadogenesis (Kimble, 1981; Seydoux and Greenwald, 1989). These interactions are mediated by LIN-12, a receptor of the LIN-12/Notch family, and LAG-2, a ligand of the Delta-Serrate-LAG-2 (DSL) family. Z1.ppp and Z4.aaa initially express both *lin-12* and *lag-2*; a small variation in the level of *lin-12* activity becomes amplified by a feedback mechanism, such that the cell with higher *lin-12* activity continues to transcribe *lin-12* and ceases to transcribe *lag-2*, and the cell with lower *lin-12* activity continues to transcribe *lag-2* and ceases to transcribe *lin-12*. The *lin-12*-expressing cell becomes a VU, and the *lag-2* expressing cell becomes the AC (Greenwald et al., 1983; Lambie and Kimble, 1991; Seydoux and Greenwald, 1989; Wilkinson et al., 1994).

We have previously shown that *hlh-2* is required for *lag-2* transcription during the AC/VU decision, and have proposed that HLH-2 is post-transcriptionally downregulated in the presumptive VU upon LIN-12 activation as part of the negative feedback mechanism that leads to the cessation of *lag-2* transcription (Karp and Greenwald, 2003). Here, we show that the role of *hlh-2* in gonadogenesis is not limited to the AC/VU decision; rather, by combining functional analysis and expression data, we find that *hlh-2* plays multiple distinct roles in gonadogenesis, with discrete roles in the specification, differentiation, and function of both the AC and the DTCs.

Materials and methods

Genetics

All experiments were carried out at 20°C unless otherwise noted. The wild-type parent for all strains used was *C. elegans* var. Bristol strain N2 (Brenner, 1974). The key mutations used were *lin-12(n941) III* (Greenwald et al., 1983), *lag-2(q420ts) V* (Lambie and Kimble, 1991), and *smg-1(r861) I* (Hodgkin et al., 1989), which enables expression of certain transgenes (see below). Additional information about these alleles, and about incidental markers used for strain constructions mentioned in the text, can be found in Wormbase (www.wormbase.org).

To mark an AC, we used *arIs51[cdh-3::gfp]* IV (Karp and Greenwald, 2003) and *syIs49[zmp-1::gfp]* IV (Wang and Sternberg, 2000), both also carrying *dpy-20(+)*. To mark Z1, Z4, and their daughters, we used a transgene that requires a *smg* mutation for expression, *arIs13[lag-2::lacZ]*, which also carries *rol-6(d)* (Wilkinson et al., 1994). Another marker transgene, *arIs11[lin-12::lacZ]*, is mentioned in Fig. 5 but staining data are not shown.

Feeding RNAi performed on L1 or L2 larvae, as described (Timmons et al., 2001), was used to bypass lethality due to loss of embryonic *hlh-2* or *lag-2* activity. For *hlh-2*, results shown are from the full-length *hlh-2* cDNA (see below), but similar results were also obtained by introducing dsRNA corresponding to the 5′ half of the *hlh-2* cDNA or the 3′ half of the *hlh-2* cDNA (Karp and Greenwald, 2003, and data not shown), indicating that the phenotypes observed reflect reduced *hlh-2* activity and not a different gene with a fortuitous nucleotide sequence match.

Plasmids and transgenic lines

For RNAi, the following plasmids contain the given gene cloned into the ‘‘double T7’’ RNAi feeding vector pPD129.36 (Timmons and Fire, 1998): pPD128.110, *gfp* (Timmons et al., 2001), pKM1196, full-length *hlh-2* cDNA (M. Krause, unpublished), pNC41.1, *lag-2* genomic region from ATG to STOP (including introns) (Chen and Greenwald, 2004 and unpublished observations), pXK10, *lacZ* [from pPDXJ (derived from pPD21.68 and pPD16.43) (Fire et al., 1990)].

The cotransformation markers used for transgenes generated in this study were *ttx-3::gfp* (Hobert et al., 1997), pCW2.1 *ceh-22::gfp* (Okkema et al., 1997), and pMH86 *dpy-20(+)* (Han and Sternberg, 1991).

To rescue *lin-12(n941)*, p101i *lin-12(+)* (Fitzgerald et al., 1993) was used to generate the array *arEx250* [p101i *lin-*

12(+) 10 μg/ml, cosmid ZK112 *ncl-1(+)* (Miller et al., 1996) 45 μg/ml, pCW2.1 *ceh-22::gfp* 20 μg/ml]. The array was generated in a *ncl-1(e1865) unc-36(e251) lin-12(n941)* background, but the *ncl-1* mutation and rescuing sequences were irrelevant for the experiments described here.

To create transgenes carrying heat-inducible *hlh-2(+)* sequences, pKM1034 *hsp16-2::hlh-2* (Harfe et al., 1998) at 50 μg/ml was used along with transformation markers pMH86 *dpy-20(+)* and *ttx-3::gfp* each at 50 μg/ml. Two independent extrachromosomal arrays were generated, and one was chosen for integration, performed as described (Mello and Fire, 1995). The resulting integrated line *(arIs63)* was backcrossed at least five times before use for further strain construction.

Staged RNAi experiments

Two methods were used to divide larvae into "substages". In experiments where groups of larvae were divided into substages by timing, larvae were synchronized at hatching by starvation and then allowed to develop normally for varying numbers of hours before being transferred to RNAi bacteria. At the time of transfer, a few representative individuals were examined by Nomarski optics to determine the number of divisions of the cells in the somatic gonad (L1 larvae) or the extent of the gonad and of gonad primordium formation (L2 larvae) (Kimble and Hirsh, 1979). This method does not result in perfect synchronization. For more precise staging, individuals were picked directly without starvation and examined by Nomarski optics as above. Larvae of each substage (defined by number of somatic gonadal cells) were added to different RNAi plates.

Heat-shock experiments

A synchronized population of hermaphrodites of the genotype *arIs51*; *arIs63* was grown at 20°C, and subject to a 1-h heat shock at 33°C while most larvae were at the late L1 stage. They were then recovered to 20°C, and scored for DTC phenotypes when they reached the L3 stage.

Immunofluorescence

Larvae of the genotypes *smg-1(r861) unc-54(r293); arIs11[lin-12::lacZ]* and *smg-1(r861) unc-54(r293); arIs13[lag-2::lacZ]* (Wilkinson et al., 1994) were fixed and stained as in Karp and Greenwald (2003). For identifying the gonad in young L1 larvae, rabbit-anti-NID-1 (1:300 dilution) was added to mark the basement membrane which surrounds the gonad (Kang and Kramer, 2000). To count the number of cells in the gonads of older L1 larvae, we included propidium iodide (PI) (Molecular Probes #P-3566) in the staining procedure. In these cases, larvae were fixed and stained as usual, but PI was added to the washes; 0.5 μg/μl RNaseA was added to one wash, and 1 ng/μl PI was added to the following wash.

Lineage analysis

Lineage analysis was performed essentially as described (Sulston and Horvitz, 1977), using a Zeiss standard microscope with Nomarski optics at room temperature (22°C–25°C). To determine the presence of any early lineage defects in *hlh-2(RNAi-L1)* individuals, wild-type (N2) L1 stage larvae subject to *hlh-2(RNAi-L1)* were observed from Z1 to Z4 until the initial three cell divisions had occurred, usually in the early L2 stage. To determine whether Z1.ppp and Z4.aaa would both divide, wild-type larvae carrying the *arIs51* transgene were subject to *hlh-2(RNAi-L1)*. At the early L2 stage, larvae were selected in which Z1.ppp and Z4.aaa appeared morphologically normal. They were then observed until one or both divided. In all cases (3/3), both cells divided within 90 min of each other, their sisters, and the vulval precursor cells which typically divide around the same time as the VUs (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977).

Results

Null or strong loss-of-function alleles of *hlh-2* have eluded isolation, perhaps due to haploinsufficiency (M. Krause, personal communication). We therefore used RNA-mediated interference (RNAi) to reduce *hlh-2* activity. The "feeding" method was used because *hlh-2(RNAi)* causes an embryonic lethal phenotype when the double-stranded RNA is delivered to the parent by injection (Krause et al., 1997). To target different aspects of gonadogenesis, we exposed larvae to *hlh-2* double-stranded RNA at either the L1 or L2 stages, and we refer to such animals as *hlh-2(RNAi-L1)* or *hlh-2(RNAi-L2)* hermaphrodites.

We previously reported that some *hlh-2(RNAi-L2)* hermaphrodites have 2 ACs, and that negative regulation of HLH-2 protein accumulation may be a component of the negative feedback mechanism that reduces *lag-2* expression in the presumptive VU during the AC/VU decision (see Introduction) (Karp and Greenwald, 2003). Here, we describe RNAi results which indicate that *hlh-2* plays many different roles in the specification, differentiation, and function of the AC and the DTCs.

A role for hlh-2 in establishing the competence of Z1.ppp and Z4.aaa to undergo the AC/VU decision

When we performed RNAi on newly hatched larvae (denoted as *hlh-2(RNAi-L1)*), we found a highly penetrant 0 AC phenotype as assessed by three criteria: morphology, lack of expression of the AC markers *cdh-3::GFP* or *zmp-1::GFP*, and loss of vulval induction (Figs. 2C, D and data not shown). To assess whether the 0 AC phenotype is due to both cells adopting a VU fate, we performed lineage analysis of three *hlh-2(RNAi-L1)* hermaphrodites (see Materials and methods). This analysis revealed that both Z1.ppp

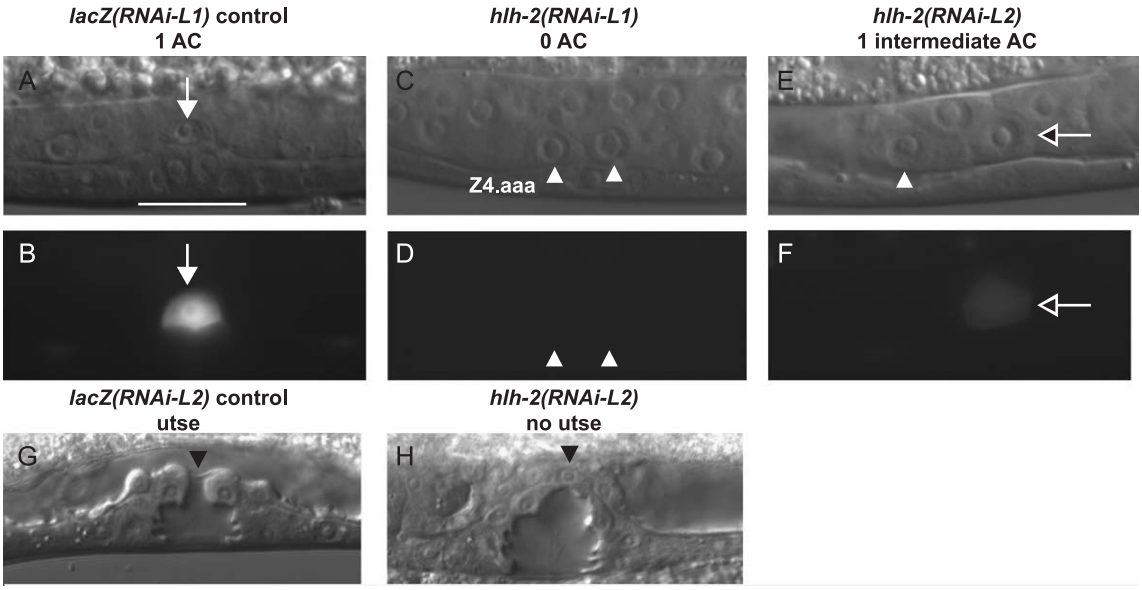

Fig. 2. *hlh-2(RNAi)* can cause multiple phenotypes with respect to the AC. Otherwise wild-type hermaphrodites carrying AC marker transgenes were subject to RNAi for either *lacZ* (A, B, G), or *hlh-2* (C–F, H). Abnormalities in the number of ACs (0 or 2) were seen, depending on the stage of gonadal development at which RNAi was performed. Abnormalities in the appearance of the ACs (cells with "intermediate" characteristics, that is, absent or lower AC marker gene expression or VU-like morphology with AC marker expression) were seen at timepoints that allowed for the generation of an AC. In this and all subsequent figures anterior is to the left and ventral is down. A normal AC (based on morphology and marker expression) is marked with a white arrow; an intermediate-AC is marked with an open arrow; a VU is marked with a white arrowhead. The utse (or place where the utse should be) is marked with a black arrowhead. The 2 AC phenotype was described previously (Karp and Greenwald, 2003) and is not shown here. (A, C, E) Nomarski images of L3 to early L4 stage larvae scored for the presence of an AC. (B, D, F) GFP images of the same larvae to detect the AC-marker *cdh-3*::*GFP*. (A, B) *lacZ(RNAi-L1)* control individual with 1 AC. 146/147 *lacZ(RNAi-L1* or *-L2)* larvae had 1 AC (five experiments). Vulval induction is evident by the multiple rounds of division of the vulval precursor cells below the AC (white line). (C, D) *hlh-2(RNAi-L1)* hermaphrodite with 0 ACs. Only Z4.aaa is visible in this focal plane; Z1.ppp also has characteristic VU morphology and position. 76/89 *hlh-2(RNAi-L1)* animals had 0 ACs (5 experiments). To score VU morphology this individual is shown at a stage before the time at which vulval induction can be scored. However, when scored later, larvae with a 0 AC phenotype almost always lacked any vulval formation (data not shown), suggesting that the transformation of the presumptive AC into a VU was complete. (E, F) *hlh-2(RNAi-L2)* hermaphrodite with one intermediate-AC (open arrow); this cell has morphological characteristics of a VU, but does express *cdh-3*::*GFP*. 27/120 *hlh-2(RNAi-L2)* hermaphrodites had an intermediate-AC, with or without another AC-like cell (three experiments). To score VU morphology this individual is shown at a stage before evidence of vulval induction. However, many larvae with an intermediate-AC displayed evidence of vulval induction (data not shown), suggesting that many intermediate-ACs possess this aspect of AC function. (G, H) Late L4 stage hermaphrodites scored for the presence of a utse. (G) *lacZ(RNAi-L2)* control hermaphrodite. Twenty-five out of 26 control hermaphrodites examined had a utse. (H) *hlh-2(RNAi-L2)* hermaphrodite lacking a utse. Twenty out of 22 such hermaphrodites exhibited thick tissue in the place of a utse.

and Z4.aaa divided at the correct time for a VU to divide, suggesting that both cells are specified as VUs (Fig. 1B).

The *hlh-2(RNAi-L1)* phenotype is opposite to the 2 AC phenotype produced by reducing *hlh-2* activity during the AC/VU decision in *hlh-2(RNAi-L2)* hermaphrodites (Karp and Greenwald, 2003), suggesting that the 0 AC phenotype caused by *hlh-2(RNAi-L1)* reflects a different activity of *hlh-2* before the AC/VU decision. Furthermore, it is opposite to the 2 AC phenotype observed in *lag-2(RNAi-L1)* or *lag-2* loss-of-function mutants (see below), suggesting that it reflects a transcriptional target of HLH-2 other than *lag-2*, its target during the AC/VU decision (Karp and Greenwald, 2003).

A role of the *Drosophila* ortholog of HLH-2, Daughterless, may provide a useful analogy for the 0 AC phenotype caused by *hlh-2(RNAi-L1)* in *C. elegans*. Daughterless and its dimerization partners are encoded by the proneural bHLH genes. These genes act during the development of the *Drosophila* microchaetes (small sensory bristles) to endow some ectodermal cells, called "proneural clusters", with the potential to undergo a neural/epidermal decision by making them competent to take on neural fate (Campuzano and Modolell, 1992; Simpson, 1990). By analogy, *hlh-2* may play a role in establishing the competence of Z1.ppp and Z4.aaa to undergo the AC/VU decision by endowing them with the ability to take on AC fate.

Within the proneural cluster, Delta/Notch-mediated interactions specify which cells become neural and which epidermal, where higher levels of *Notch* activity lead to epidermal fate and lower levels of *Notch* activity lead to neural fate. In the cells of proneural clusters, *Notch* activity is required for epidermal fate (Hartenstein and Posakony, 1990; Simpson, 1990), although it is not required outside of proneural clusters for the epidermal choice (Heitzler and Simpson, 1991). Genetic evidence supporting the requirement of proneural genes to establish competence to undergo a Notch-mediated neural/epidermal decision was provided by the observation that cells lacking proneural gene activity did not require *Notch* activity to adopt an epidermal fate (Heitzler and Simpson, 1991). If the proneural/proAC analogy holds, then we would expect that *hlh-2(RNAi-L1)* in a *lin-12(0)* or *lag-2(-)* background would cause the 0 AC phenotype associated with *hlh-2(RNAi-L1)*, rather than the

2 AC phenotype associated with loss of *lin-12* or *lag-2* activity. Indeed, we found this to be the case, indicating that the *hlh-2(RNAi-L1)* phenotype is epistatic to the *lin-12(0)* and the *lag-2(-)* phenotype (Table 1), and consistent with a "proAC" role for *hlh-2*, establishing the competence of Z1.ppp and Z4.aaa to undergo the AC/VU decision.

We attempted to assess whether *hlh-2* can be sufficient for AC fate in other gonadal cells by expressing *hlh-2* ectopically using a heat-shock promoter. Although this strategy worked well for the DTCs (see below), we found only variable defects in the number of ACs at low penetrance (data not shown), precluding a definitive answer. The variability may be due to technical factors such as the inefficiency of the heat shock promoter in Z1.ppp and Z4.aaa (data not shown) or post-transcriptional downregulation of HLH-2 in presumptive VUs (see Karp and Greenwald, 2003).

Roles for hlh-2 in AC differentiation and function

In addition to the 0 AC *[hlh-2(RNAi-L1)]* and 2 AC *[hlh-2(RNAi-L2)]* (Karp and Greenwald, 2003) phenotypes, we find that some *hlh-2(RNAi-L1)* and *hlh-2(RNAi-L2)* individuals display cells that have a mixture of AC and VU characteristics. Such "intermediate-ACs" may look morphologically like an AC, but fail to express the AC marker *cdh-3::GFP*, or may look morphologically like a VU, yet show expression of *cdh-3::GFP* (Figs. 2E, F). They also include cells with abnormal AC morphology and position in the gonad. Similar results have been obtained with another AC marker, *zmp-1::GFP* (data not shown). At least some of these intermediate-ACs can induce vulval development (data not shown). Furthermore, an intermediate-AC can be seen in a hermaphrodite that has an additional, seemingly normal AC, as well as in a hermaphrodite with no other AC-like cell. Such intermediate-ACs cells are never seen in control *lacZ(RNAi)* animals. These observations, together with the finding that HLH-2 protein accumulates in the AC but not the VU (Karp and Greenwald, 2003), suggest that *hlh-2* is necessary for the differentiation or function of the AC. This inference extends the analogy with proneural genes, which are also required for differentiation of the microchaetes (Dominguez and Campuzano, 1993).

hlh-2(RNAi-L2) hermaphrodites also display a failure in formation of the uterine seam cell (utse), a multinucleate uterine cell that forms a thin layer in between the

Table 1
The *hlh-2(RNAi-L1)* 0 AC phenotype is epistatic to the *lin-12(0)* and *lag-2(-)* 2 AC phenotype

Relevant genotype	RNAi	0 AC	1 AC	2 AC
wild-type	control	0	11/11	0
wild-type	*hlh-2-L1*	26/28	2/28	0
lin-12(0)	control	0	2/20	18/20
lin-12(0)	*hlh-2-L1*	22/29	1/29	6/29
lag-2(-)	control	0	0	48/48
lag-2(-)	*hlh-2-L1*	22/22	0	0

Normally *lin-12(0)* and *lag-2(-)* animals have a highly penetrant 2 AC phenotype (Greenwald et al., 1983; Lambie and Kimble, 1991, this table). However, when subject to *hlh-2(RNAi-L1)*, these animals show a 0 AC phenotype instead. Controls for RNAi experiments were either *gfp* (wild-type and *lin-12(0)*) or *lacZ* (*lag-2(-)*). The full genotype of the wild-type strain was *ncl-1(e1865) unc-36(e251) lin-12(n941); arIs51[cdh-3::gfp]; arEx250[lin-12(+), ceh-22::gfp]*. Larvae scored as "*lin-12(0)*" were derived from the wild-type strain, but had lost *ceh-22::GFP* expression, indicating loss of the *arEx250* rescuing array. However, it is possible that rare mosaic animals may have retained the array in gonadal cells. The full genotype of the *lag-2(-)* strain was *arIs51[cdh-3::gfp]; lag-2(q420ts)*. Experiments involving this strain were performed by shifting embryos to the non-permissive temperature (25°C).

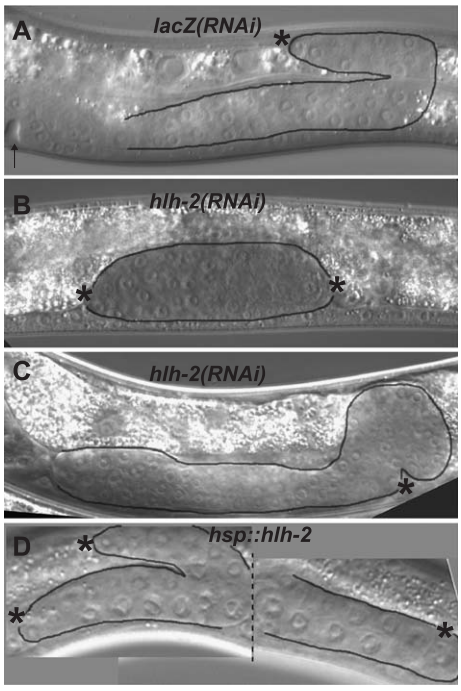

Fig. 3. *hlh-2* is important for DTC specification, differentiation, and/or function. (A–D) Nomarski images of L4 stage (A–C) or L3 stage (D) hermaphrodites. DTCs or the place where they would normally be are marked with an asterisk. Gonad arms are outlined for clarity; when gonad arms have extended (A, C, D) only one arm is visible in each focal plane. RNAi experiments presented in this figure were carried out at 25°C. (A) A *lacZ(RNAi-L2)* control hermaphrodite showing a normal DTC at the end of the extending gonad. The vulva is marked with an arrow. Twenty out of 20 gonad arms of *lacZ(RNAi-L2)* hermaphrodites and 50 out of 50 gonad arms of *lacZ(RNAi-L1)* hermaphrodites extended normally. (B) An *hlh-2(RNAi-L1)* hermaphrodite where both gonad arms completely failed to extend. Thirty-nine out of 74 gonad arms of *hlh-2(RNAi-L1)* hermaphrodites failed to extend. Most of the remaining 35 out of 74 gonad arms displayed defects described in (C). (C) An *hlh-2(RNAi-L1)* hermaphrodite showing a gonad arm that displays abnormal extension (compare to A). Of the 35 out of 74 gonad arms of *hlh-2(RNAi-L1)* hermaphrodites which did extend, 32 out of 35 arms showed defects such as those pictured here. (D) Gonad arms of one L3 stage hermaphrodite carrying a *hsp::hlh-2* construct and subject to heat-shock during the L1 stage. This individual displayed a supernumerary gonad arm extending anteriorly (top), indicating ectopic DTC specification, as well as two normal gonadal arms. To view all three DTCs simultaneously, two focal planes are shown in this picture, separated by a dashed line. 19/39 *hsp::hlh-2* hermaphrodites subject to heat shock during the L1 stage had three DTCs (one supernumerary arm) and 1 out of 39 such hermaphrodites had four DTCs (two supernumerary arms).

uterus and the vulva in the L4 hermaphrodite (Figs. 2G, H). Utse formation requires an inductive event in the L3 stage: LAG-2 from the AC activates LIN-12 in neighboring uterine cells (descendants of VUs) to specify the π fate, a prerequisite for utse formation (Newman et al., 1995, 2000). Since HLH-2 protein accumulates in the AC and since *hlh-2(RNAi-L2)* animals fail to express *lag-2* in the mature AC (Karp and Greenwald, 2003), we believe the utse defect is likely to reflect a failure of AC function.

Roles for hlh-2 in DTC specification, differentiation, and function

The DTCs function as leader cells that guide extension and turning of the gonad arms (Kimble and White, 1981, see Fig. 3A). In *hlh-2(RNAi-L1)* hermaphrodites, the gonad arms often do not extend, and morphologically distinguishable DTCs are not seen (Fig. 3B), consistent with a failure of DTC specification. In contrast, ectopic *hlh-2* expression provided during the L1 stage by expressing *hlh-2* under the control of a heat-shock promoter (see Materials and methods) causes the opposite phenotype, extra DTCs together with ectopic gonad arm extension (Fig. 3D). These observations suggest that *hlh-2* activity is necessary and sufficient for the specification of DTC fate.

Some *hlh-2(RNAi-L1)* and *hlh-2(RNAi-L2)* hermaphrodites display defects that are consistent with compromised DTC differentiation and function: gonad arms extend only partially, and the distal ends appear bloated and rounded (Fig. 3C). In these hermaphrodites, it appears that the DTCs were specified, allowing the outgrowth of the gonad arms, but that there was a failure in some aspect of DTC differentiation or function.

Evidence that the effects of hlh-2(RNAi) reflects multiple independent roles for hlh-2 in gonadogenesis

The observation that two different cell types of the gonad primordium—the AC/VU pair (Z1.ppp and Z4.aaa) and the DTCs (Z1.aa and Z4.pp)—have undergone fate transformations raised the possibility that the defects we observe in *hlh-2(RNAi-L1)* animals are due to an early somatic gonad lineage defect that affects multiple Z1/Z4 descendants, such as an altered interaction between Z1 and Z4 (e.g., Siegfried and Kimble, 2002). We therefore performed lineage analysis of *hlh-2(RNAi-L1)* hermaphrodites from the early L1 stage, before the division of Z1 and Z4, until all 12 gonadal cells of the somatic primordium were born. In 4/4 observed, the position and morphology of the cells appeared normal, as did the plane and number of cell divisions, indicating that there is no gross lineage defect as a result of *hlh-2(RNAi-L1)*. The first lineage defect observed is the apparent failure to specify either Z1.ppp or Z4.aaa as an AC described above. The larvae analyzed were affected by the RNAi treatment, as all four larvae analyzed subsequently displayed the 0 AC phenotype as well as defects in gonad arm extension consistent with failure to specify the DTC (1/4) or a failure in DTC differentiation or function (3/4). The lineage analysis suggests that the phenotype of *hlh-2(RNAi-L1)* hermaphrodites reflects specific and distinct roles during gonadogenesis.

Further support for the view that the effects of *hlh-2(RNAi-L1)* on the specification and function of the AC

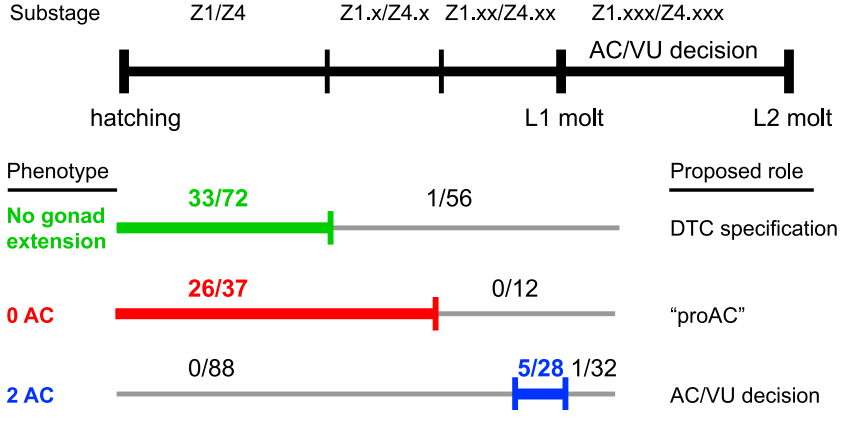

Fig. 4. The timing of *hlh-2(RNAi)* determines which phenotypes will occur. (top) A schematic view of somatic gonadal development from hatching until primordium formation at the L2 molt. Landmark timepoints are indicated. (bottom) Substages at which feeding larvae *hlh-2* RNAi bacteria results in the indicated phenotypes are denoted by thick colored lines; substages at which feeding larvae *hlh-2* RNAi bacteria does not result in the indicated phenotypes are denoted by thin grey lines. Numbers indicate either the number of larvae that displayed the phenotype over the total number of larvae scored at the indicated substages (AC phenotypes) or the number of gonad arms that failed to extend over the total number of gonad arms scored at the given substages (DTC phenotype). Larvae scored for the gonad extension and 0 AC phenotypes were precisely divided into substages by examining individual hermaphrodites by Nomarski optics before addition to RNAi bacteria. Larvae scored for the 2 AC phenotype were divided into substages by addition of groups of synchronized larvae grown for different times to RNAi bacteria (see Materials and Methods). The proposed wild-type role for *hlh-2* at the given substages is listed. Intermediate-ACs (included for this purpose as specified anchor cells) and aberrant gonad arm extension (included for this purpose as specified DTCs) were seen after feeding larvae with RNAi bacteria at all substages.

and of the DTCs reflect independent events comes from timed RNAi experiments, in which we fed hermaphrodites bacteria expressing double-stranded RNA corresponding to *hlh*-2 ("RNAi bacteria") during different "substages" based on the number of divisions of somatic gonadal cells (see Materials and methods and Fig. 1A). We observed the 0 AC phenotype when individuals were added to RNAi bacteria when Z1 and Z4 had either not yet divided, or had divided once, but not when Z1 and Z4 had divided more than once (Fig. 4). The 2 AC phenotype was only seen when larvae are added to RNAi bacteria at around the L1 molt, when Z1 and Z4 have divided at least twice (Fig. 4), implying that the "proAC" decision mediated by *hlh-2* occurs before the defect in lateral specification involving *hlh-2*. The apparent lack of DTCs, as manifested by complete failure of gonadal extension, was evident when individuals were added to RNAi bacteria before the division of Z1 and Z4, whereas aberrations in extension can occur at subsequent times.

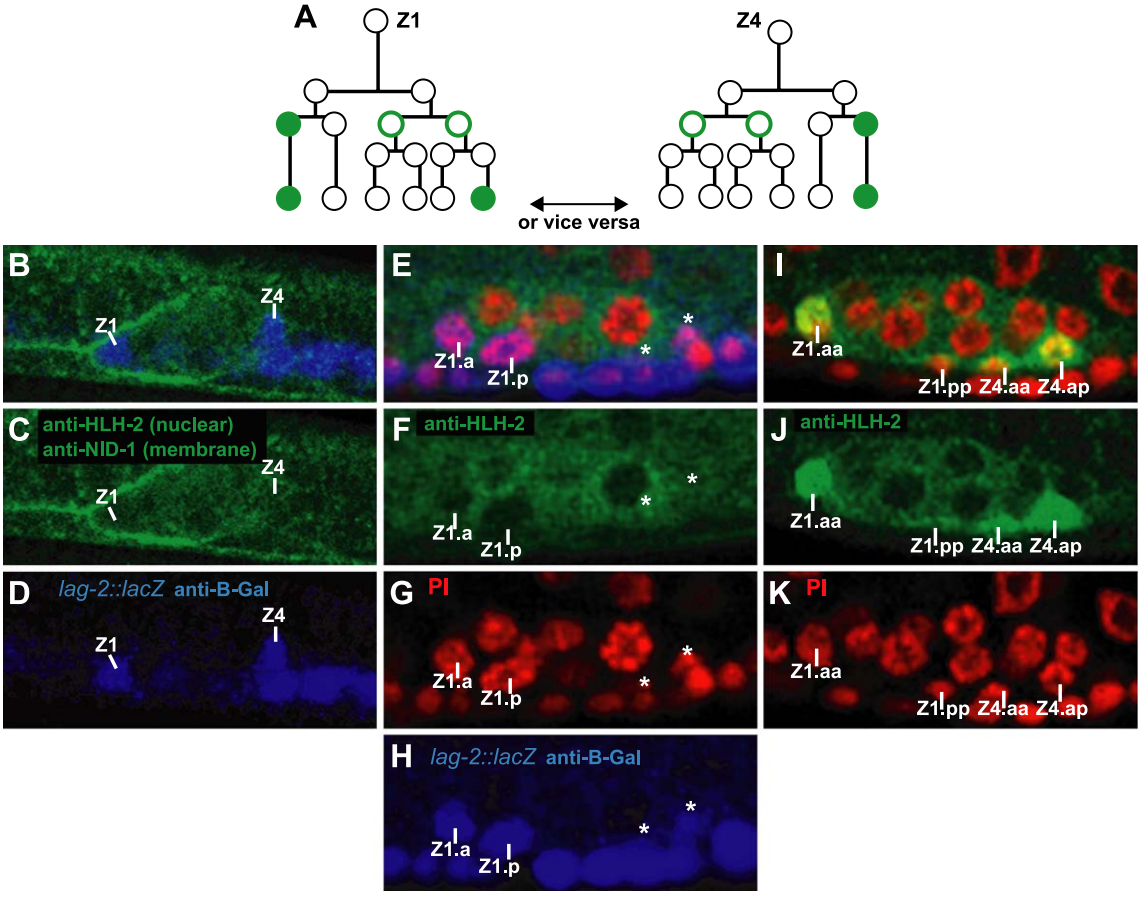

Fig. 5. HLH-2 protein accumulation during early gonadogenesis. We determined the pattern of HLH-2 accumulation with respect to the gonad cell lineage. Confocal micrographs of L1 hermaphrodites stained with anti-HLH-2 antibody and markers for specific cells (see below and Materials and Methods) are shown. All somatic gonadal cells in focus in the images are shown by name; white lines connect each cell to its name. Larvae have wild-type gonadal development but carried *arIs11* or *arIs13* marker *lacZ* transgenes and the *smg-1(r861)* allele required to express them (see Materials and Methods). (A) A schematic representation of the pattern of HLH-2 accumulation in the somatic gonad of L1 and L2 stage hermaphrodites, based on the data in this figure and in Karp and Greenwald (2003). The lineage diagram is as shown in Fig. 1. Solid green circles indicate consistent HLH-2 accumulation in that cell. Open green circles indicate variable HLH-2 accumulation in that cell. (B–D) A hermaphrodite before division of Z1 and Z4. *lag-2::lacZ* expression (anti-LacZ: blue, nuclear) marks Z1 and Z4 (as well as other, non-gonadal cells) and anti-NID-1 (green, membrane) marks the basement membrane surrounding the gonad. The combination of these markers allows the unambiguous identification of Z1 and Z4. HLH-2, if present, would be detected using anti-HLH-2 as green and nuclear staining. Zero out of 38 such hermaphrodites showed HLH-2 accumulation in Z1 or Z4. (B) The merged image. (C, D) Individual channels. (E–H) A hermaphrodite after Z1 and Z4 have divided (Z1.x/Z4.x stage). *lag-2::lacZ* expression (anti-LacZ: blue) marks the four daughters of Z1 and Z4 (and non-gonadal cells), propidium iodide (red) marks all nuclei, and HLH-2, if present, would be detected using anti-HLH-2 as green and nuclear staining. Zero out of 13 such hermaphrodites showed HLH-2 accumulation in the daughters of Z1 and Z4. In these images, Z4.a and Z4.p are in a different focal plane (asterisks indicate what can be seen of these cells); however, they show the same pattern of staining as Z1.a and Z1.p, shown here. (I–K) A hermaphrodite after Z1 and Z4 have undergone two rounds of division (the Z1.xx/Z4.xx stage), stained with anti-HLH-2 (green), and propidium iodide (PI) (red). PI was used to identify nuclei based on their position and morphology in the gonad. HLH-2 accumulation is evident in Z1.aa and Z4.pp (the DTCs) in twenty-four out of 28 such hermaphrodites, and is evident in at least one of Z1.pa, Z1.pp, Z4.aa, Z4.ap in twenty out of 28 such hermaphrodites. Anti-HLH-2 staining was observed variably in Z1.pa, Z1.pp, Z4.aa, and Z4.ap (see text). In this individual, HLH-2 is evident in the nuclei of Z1.aa and Z4.pp (out of focus), and in the cytoplasm as well as the nuclei of Z1.pp, Z4.aa (the parents of Z1.ppp and Z4.aaa), and Z4.ap.

The HLH-2 expression pattern appears to reflect distinct roles in gonadogenesis

An analysis of the expression pattern of HLH-2 during gonadogenesis is also consistent with the interpretation that *hlh-2* has distinct roles in the DTCs and the AC/VU pair, rather than a general role in gonadogenesis. Using an antibody against the endogenous HLH-2 protein (Krause et al., 1997), we observe that HLH-2 does not accumulate detectably in any cell of the gonad until Z1 and Z4 have undergone two rounds of division, near the end of the L1 stage (Fig. 5). HLH-2 was consistently observed in the DTCs (Z1.aa and Z4.pp) and in the presumptive AC and mature AC (Karp and Greenwald, 2003). HLH-2 was also weakly and inconsistently present in Z1.pp and Z4.aa (the parents of Z1.ppp and Z4.aaa), as well as their sisters, Z1.pa and Z4.ap. We believe the lack of consistency is due to the low-level of accumulation, but it is also possible that it reflects dynamic changes in HLH-2 accumulation at this stage. We note that an *hlh-2::lacZ* transcriptional reporter appears to be expressed in a similar pattern to HLH-2 protein during the L1 stage (data not shown). In addition, while HLH-2 accumulation in the DTCs is nuclear, as expected for a transcription factor, HLH-2 is detectable in the cytoplasm as well as the nucleus of other gonadal cells (e.g., Figs. 5I–K). The significance of this pattern of subcellular localization is not known.

Relationship between hlh-2(RNAi) defects and lag-2 activity

We previously provided evidence that HLH-2 is required for *lag-2* transcription during the AC/VU decision, accounting for the 2 AC phenotype that results when *hlh-2* activity is depleted during the L2 stage (Karp and Greenwald, 2003). *hlh-2* also appears to regulate *lag-2* expression in the mature AC (Karp and Greenwald, 2003), which can account for the observation presented here that the utse does not form.

To explore the relationship between other *hlh-2(RNAi)* defects and *lag-2* activity, we examined the phenotypes caused by loss of *lag-2* activity. Because *lag-2* activity is required for survival beyond the L1 stage (Lambie and Kimble, 1991), we shifted *lag-2(q420ts)* hermaphrodites from 15°C to 25°C during embryogenesis or performed *lag-2* "feeding RNAi" on newly hatched larvae *[lag-2(RNAi-L1)]* to generate hermaphrodites lacking *lag-2* activity during gonadogenesis. While reducing *lag-2* activity by both of these protocols can cause a 2 AC phenotype (Table 1 and data not shown), they did not cause the 0 AC or intermediate AC phenotypes: 32/33 *lag-2(RNAi-L1)* individuals and 28/28 *lag-2(q420ts)* shifted individuals had morphologically normal ACs. We note that *lag-2(RNAi-L2)* does not cause any phenotype in the AC/VU decision (data not shown). Similarly, loss of *lag-2* activity does not cause defects in DTC specification or migration, as 74/74 gonad arms of *lag-2(RNAi-L1)* individuals and 116/116 gonad arms of *lag-2(q420ts)* individuals shifted to 25°C extended normally. These results suggest that *lag-2* may not be the only relevant target gene underlying other defects in gonadogenesis that occur upon reduction of *hlh-2* activity.

Discussion

We have presented here evidence that *hlh-2* plays multiple and distinct roles during hermaphrodite gonadogenesis. In particular, we have shown that *hlh*-2 activity is necessary for the specification and function of the two terminally differentiated cell types, the DTCs and the AC, that are formed during the initial phase of gonadogenesis. As described in Results, *hlh-2* activity appears to be both necessary and sufficient to specify the DTCs, and is required for at least a subset of its functions, including its function as the leader of gonad arm extension. In this section, we focus on the roles of *hlh-2* in the genesis and function of the AC.

hlh-2 appears to play two distinct roles in the specification of the AC. One role, the "proAC" role, is to bestow competence on Z1.ppp and Z4.aaa to undergo the AC/VU decision. This apparent "proAC" role for *hlh-2* appears to precede a role for *hlh-2* in the "AC/VU decision", the LIN-12-mediated interaction that specifies the single cell that will differentiate as an AC (Karp and Greenwald, 2003). The "proAC" role for *hlh*-2 in endowing Z1.ppp and Z4.aaa with the potential to undergo the AC/VU decision was suggested by the observation that *hlh-2(RNAi-L1)* animals are unable to form an AC. That Z1.ppp and Z4.aaa no longer have the choice between the AC and VU fates is supported by the observation that *hlh-2(RNAi-L1)* hermaphrodites lack an AC even in the absence of *lin-12* or *lag-2* activity, a situation that, when Z1.ppp and Z4.aaa retain their choice, invariably leads to the AC fate (Greenwald et al., 1983; Lambie and Kimble, 1991; Seydoux and Greenwald, 1989).

The proAC role appears to be analogous to the proneural role of the *Drosophila* ortholog of *hlh-2*, *daughterless*, and its dimerization partners. The proneural genes endow ectodermal cells with the choice to adopt neural sense organ precursor (SOP) fate, and *Notch*-mediated interactions among proneural cells result in single cells becoming SOPs. The analogy between the AC/VU and SOP formation is particularly apt for the microchaete bristles, which, through Notch-mediated interactions, are evenly spaced in the epithelial field (Simpson, 1990). In both cases, feedback mechanisms involving modulation of *lin-12/Notch* activity ensure that the correct number and types of cells are generated (Heitzler and Simpson, 1991; Heitzler et al., 1996; Seydoux and Greenwald, 1989; Wilkinson et al., 1994).

Only two gonadal cells, Z1.ppp and Z4.aaa, manifest their competence to become ACs by having naturally variable fates: in a population of hermaphrodites, Z1.ppp will become the AC in half of the worms, and Z4.aaa will become the AC in the other half (Karp and Greenwald, 2003; Kimble and Hirsh, 1979). However, in wild-type

hermaphrodites, two other cells, Z1.ppa and Z4.aap (the sisters of Z1.ppp and Z4.aaa), also appear to have transient competence to become an AC (Seydoux et al., 1990), although they invariably become VUs. These cells do not accumulate HLH-2 protein, but do express an *hlh-2* transcriptional reporter (Karp and Greenwald, 2003). As *hlh-2* appears to play a proAC role in endowing cells with the competence to undergo the AC/VU decision, a simple hypothesis to explain these observations is that *hlh-2* activity in these cells or in their parents leads to their competence to be the AC, but that other factors squelch *hlh-2* activity, and hence the proAC potential, in Z1.ppa and Z4.aap. This hypothesis is suggested by the transient expression pattern of HLH-2 in Z1.pp and Z4.aa (Fig. 5) and the data from timed RNAi experiments (Fig. 4), suggesting that the role of *hlh-2* in establishing proAC potential may precede the AC/VU decision. An interesting challenge for the future will be to determine whether *hlh-2* is responsible for the AC-competence of Z1.ppa and Z4.aap, and to identify the factors that restrict the AC/VU decision to just Z1.ppp and Z4.aaa.

We also provided here evidence that *hlh-2* functions in the presumptive AC to promote differentiation, as evidenced by the intermediate AC phenotype, and in the differentiated AC, as suggested by the failure of utse formation. The observation that *hlh-2* has additional roles in the differentiation and/or function of the AC is also analogous with the roles of proneural genes, which are necessary for correct execution of neural fates. When proneural gene activity is reduced in *Drosophila* imaginal discs, defects in neuronal differentiation can be observed in addition to loss of neural cell fate (Dominguez and Campuzano, 1993). The AC has several important functions during larval development, including induction of vulval precursor cells to take on vulval fate (Kimble, 1981), and induction of a subset of uterine cells to take on π-cell fate (Newman et al., 1995). The signals expressed by the AC that mediate these inductive events are LIN-3, an EGF-related protein (Hill and Sternberg, 1992), which is responsible for vulval induction, and LAG-2, which is responsible for π cell induction (Newman et al., 1995, 2000). HLH-2 appears to be a direct transcriptional activator of both of these genes in the AC (Hwang and Sternberg, 2004; Karp and Greenwald, 2003).

We previously focused on the role of *hlh-2* in the LIN-12-mediated interaction that underlies the AC/VU decision (Karp and Greenwald, 2003). We suggested that post-transcriptional downregulation of HLH-2 in the presumptive VU in response to LIN-12 activation constitutes part of the negative feedback that downregulates *lag-2* transcription in the presumptive VU. We have now extended the roles of *hlh-2* during early gonadogenesis, identifying roles before the AC/VU decision and subsequently in the differentiation and function of the AC, as well as in the specification and function of a distinct cell type, the DTCs. It will be interesting to learn how these multiple roles are executed and kept temporally distinct.

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