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The *C. elegans* Hand gene controls embryogenesis and early gonadogenesis

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SUMMARY

The *C. elegans* genome encodes a single Hand bHLH transcription factor. Either *hnd-1(RNAi)* or a *hnd-1* deletion causes partially penetrant defects in viability and gonadogenesis. Dead embryos and young larvae are often misshapen at the posterior end. Our primary focus has been the role of *hnd-1* in gonadogenesis. Wild-type *C. elegans* has two somatic gonadal precursors and two primordial germ cells in stereotyped positions within its four-celled gonadal primordium. The *hnd-1* gene affects the presence and position of both the somatic gonadal precursors and primordial germ cells within the primordium, but does not appear to have any role in later gonadogenesis. *hnd-1* probably acts within the somatic gonadal precursors or their mesodermal predecessors; defects in primordial germ cells and germ line appear to be secondary. In *hnd-1* mutants, somatic gonadal precursors are generated normally, but are not maintained properly and sometimes die. A similar role in controlling the maintenance of precursor fates has been described for other genes governing early organogenesis, including the zebrafish Hand gene *hands off*. We also report the discovery of two genes, *ehn-1* and *ehn-3*, that have overlapping functions with *hnd-1* in embryogenesis and gonadogenesis.

Key words: *C. elegans*, Gonadogenesis, HAND, Organ primordium, *hnd-1*, *ehn*

INTRODUCTION

An organ primordium consists of precursor cells that generate all the diverse cell types of the mature organ. For some organs, ‘organ selector’ genes control all precursor cells within the organ primordium, regardless of cell type (Gaudet and Mango, 2002; Gehring and Ikeo, 1999). By contrast, precursor cells in other organ primordia are specified by the intersection of global patterning genes and may not rely on a single organ selector gene (Bradley et al., 2001; Lockwood and Bodmer, 2002). However, the generality of these mechanisms remains unknown.

We have focused on developmental controls of the gonadal primordium in the nematode *Caenorhabditis elegans*. This organ primordium is unusually simple: it is composed of two somatic gonadal precursor cells (SGPs) and two primordial germ cells (PGCs). The SGPs generate all somatic tissues of the gonad proper (i.e. ovary or testis), as well as genital ducts (e.g. uterus, vas deferens), whereas the PGCs give rise to all germ cells, including gametes. The SGPs and PGCs arise from distinct embryonic blastomeres and assemble into the gonadal primordium midway through embryogenesis (Fig. 1A,B) (Sulston et al., 1983). Within the mature primordium, the SGPs (Z1 and Z4) reside at the distal poles and the PGCs (Z2 and Z3) are situated proximally (Fig. 1C) (Kimble and Hirsh, 1979). In addition to this proximal-distal polarity, the primordium displays left-right and dorsal-ventral polarity (Fig. 1C). Therefore, the four-celled gonadal primordium is patterned in three axes.

Somatic and germline precursors originate from distinct embryonic lineages during gonadogenesis in other organisms as well. In *Drosophila*, SGPs are specified by global patterning genes that subdivide the mesoderm into discreet regions (Boyle et al., 1997; Boyle and DiNardo, 1995). Once specified, the *clift* (*eyes absent* – FlyBase) gene, which encodes a novel nuclear protein, maintains the SGP fate (Boyle et al., 1997). The mammalian gonadal mesoderm may similarly rely on genes that pattern the embryo as a whole (Capel, 2000). In addition, several transcription factors affect development of the gonadal mesoderm in mice: *Wt1*, *Sf1*, *Lim1* (*Lhx1* – Mouse Genome Informatics) and *Emx2* control both gonadal and non-gonadal development (Kreidberg et al., 1993; Miyamoto et al., 1997), whereas *Lhx9* appears specific for gonadogenesis (Birk et al., 2000). PGCs, on the other hand, are often formed outside the gonad and later migrate to the developing gonad (reviewed by Starz-Gaiano and Lehmann, 2001; Wylie, 2000). Once there, the PGCs depend on the somatic gonad for survival and for cell fate decisions within the germ line (Kimble and White, 1981; McCarter et al., 1997; Starz-Gaiano and Lehmann, 2001).

Several genes have been identified that control early gonadogenesis in *C. elegans* (Fig. 1C) (Hubbard and Greenstein, 2000). For example, *gon-2* and *gon-4* control the onset and timing of gonadal cell divisions (Friedman et al., 2000; Sun and Lambie, 1997), and *lin-17*, *sys-1*, *wrm-1*, *lit-1* and *pop-1* govern the asymmetric division of the SGPs (Siegfried and Kimble, 2002; Sternberg and Horvitz, 1988).

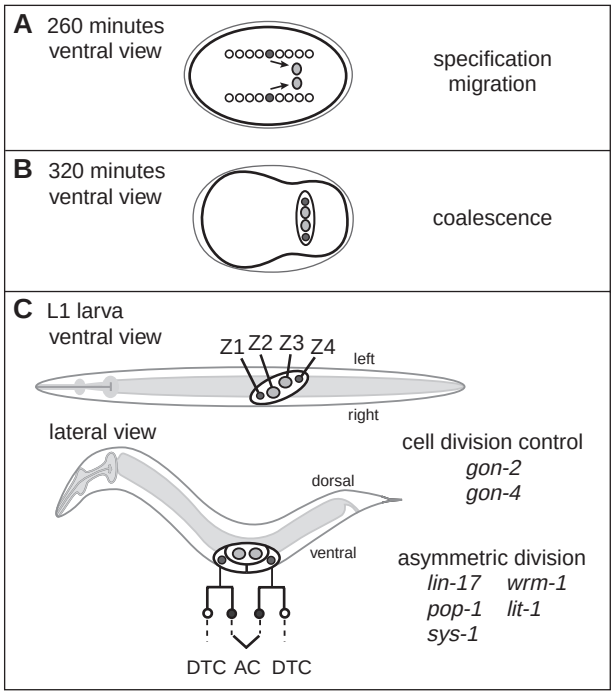

Fig. 1. Early gonadogenesis in *C. elegans*. Somatic gonadal precursors (SGPs: Z1 and Z4), dark gray; primordial germ cells (PGCs: Z2 and Z3), light gray. (A) SGPs are specified within the mesodermal layer (white circles) and then migrate to meet PGCs. (B) SGPs and PGCs coalesce into the gonadal primordium, which at this stage has a left-right orientation. (C) During embryo morphogenesis, the gonadal primordium shifts to an anterior-posterior orientation, and acquires left-right and dorsal ventral axes. The first SGP division is asymmetric and segregates the potential to make two regulatory cells: anchor cells (AC) and distal tip cells (DTC). Genes crucial for early SGP divisions are noted.

For germline development, *pie-1* and *nos-2* control PGC fate and influence their incorporation into the gonadal primordium (Seydoux et al., 1996; Subramaniam and Seydoux, 1999; Tenenhaus et al., 2001). In this paper, we report that the *C. elegans* Hand bHLH transcription factor *hnd-1* is important for early gonadogenesis as well as for embryogenesis. Specifically, *hnd-1* influences the number and position of SGPs in the gonadal primordium, and affects body shape in the embryo. The *hnd-1* gene is expressed broadly in the embryonic mesoderm and then more specifically in the SGPs. Our results suggest that *hnd-1* governs maintenance of SGP fate and SGP survival. We also report the discovery of two genetic enhancers of *hnd-1*, named *ehn-1* and *ehn-3* (for enhancer of Hand), that have overlapping functions with *hnd-1* in embryogenesis and gonadogenesis.

MATERIALS AND METHODS

Strains

Animals were grown at 20°C unless otherwise noted. All strains were derivatives of Bristol strain N2 (Sulston and Horvitz, 1977). The following mutations are described by Hodgkin (Hodgkin, 1997) or cited references. *LGI*: *gon-2(q388)* (Sun and Lambie, 1997) and *sys-1(q544)* (Miskowski et al., 2001). *LGII*: *hlh-1(cc450)* (Chen et al., 1994); *unc-104(e1265)*; *rol-6(e187)*; and *mnDf93* (Sigurdson et al., 1984). *LGIV*: *ced-2(e1752)*; *ced-3(n717)*; *gon-4(q519)* (Friedman et al., 2000); *unc-24(e138)*; *unc-5(e53)*; *dpy-13(e184)*; and *nDf41*. *LGX: unc-9(e101)*. Dominant GFP balancers: *mIn1[mIs14]* for *LGII* (Edgley and Riddle, 2001); *hT2[qIs48]* for *LGI*; and *nT1[qIs50]* for *LGIV*. *qIs48* and *qIs50* are insertions of *ccEx9747* onto *hT2* and *nT1*, respectively. Molecular markers: *qIs55* [*hnd-1(N)::GFP*]; *qIs69* [*hnd-1::GFPlacZ]*; *qIs56* [*lag-2::GFP*] (Siegfried and Kimble, 2002); *leIs129* [*pes-1::GFP*] (Molin et al., 2000); *qIs61* [*pes-1::GFP*]; *ayIs7* [*hlh-8::GFP*] (Harfe et al., 1998); and *qIs77* [*unc-122::GFP*] (Miyabayashi et al., 1999). *qIs56* and *qIs61* were generated by microparticle bombardment (Praitis et al., 2001).

Plasmids and transgenes

All cloning was performed by standard methods (Sambrook et al., 1989). PCR products were sequenced. Primer sequences are available upon request. Transgenes were generated as simple arrays unless otherwise noted.

hnd-1 cDNA (pJK849 and pJK901)

Using a probe from the coding region of C44C10.8, we isolated a *hnd-1* cDNA from an embryonic *C. elegans* cDNA library (a gift from P. Okkema) and subcloned it to make pJK849. The *hnd-1* 5′ end was cloned by RT-PCR using embryonic total RNA, a primer to the SL1 trans-spliced leader and internal *hnd-1*-specific primers. A full-length *hnd-1* cDNA (pJK901) was assembled from the SL1 RT-PCR product and pJK849.

hnd-1(FL)::GFP (pJK850)

GFP coding sequences were amplified by PCR from pPD95.81 (a gift from A. Fire) and subcloned into a *hnd-1* genomic fragment (pJK906). pJK850 includes 1568 bp of the *hnd-1* sequence upstream of the 5′UTR and 182 bp downstream of the 3′UTR. pJK850 was injected with pRF4[Rol] (Mello et al., 1991) into *hnd-1* to generate *qEx486*; this array rescued *hnd-1* gonadal defects completely (n=136) and reduced lethality from 28% to 7**%** (n=190).

hnd-1(N)::GFP (pJK848)

The first two exons and 1540 bp upstream of the *hnd-1* 5′UTR were PCR amplified and cloned into pPD95.81 (a gift from A. Fire). pJK848 was injected into *unc-4(e120)* with the co-injection marker pNC4-21[*unc-4*+] (Miller and Niemeyer, 1995) and N2 DNA to create *qEx447* and, subsequently, *qIs55*. With the exception of SGPs, *hnd-1(N)::GFP* was detected in cells that also express *hlh-1*, a marker for body muscle (Krause et al., 1990).

hnd-1::GFPlacZ (pJK900)

The *hnd-1* promoter (plus 11 N-terminal codons) was PCR amplified and cloned into pPD96.04 (a gift from A. Fire). pJK900 was injected with pRF4[Rol+] to create *qEx492* and, subsequently, *qIs69*. pJK850 and pJK900, but not pJK848, express GFP in several head cells that we have not identified.

HS-hnd-1 (pJK902)

The *hnd-1* cDNA from pJK901 was cloned into pPD49.78 (a gift from A. Fire) to generate pJK902, which was injected into *qIs61* with the co-injection marker pRF4[Rol+] to make *qEx493*. Embryos were subjected to two 30-minute heat pulses at 33°C, with a one hour recovery interval. Resulting L1 larvae were scored for extra SGPs using *pes-1::GFP*.

hlh-1::hnd-1GFP (pJK904)

A *hnd-1::GFP* fusion was generated by inserting GFP into the *Rsr*II site of the full-length *hnd-1* cDNA (pJK901). *hnd-1::GFP* was then cloned into pPD51.45 (Krause et al., 1990) to generate pJK904, which was injected into *hnd-1* with the co-injection marker pRF4[Rol+] to make *qEx496*; this array rescued *hnd-1* gonadal defects and marginally rescued lethality (20%, n=372).

lag-2::hnd-1GFP (pJK905)

A *hnd-1::GFP* fusion (see above) was cloned into pJK590 (Blelloch et al., 1999) to generate pJK905, which was injected into *hnd-1* with the co-injection marker pRF4[Rol+] to make *qEx497*; this array partially rescued *hnd-1* gonadogenesis defects and did not rescue lethality (24%, *n*=192).

hnd-1 genomic DNA (pJK906)

A plasmid carrying *hnd-1* genomic DNA was amplified by PCR; it contained the same upstream and downstream sequences as in *hnd-1(FL)::GFP*. pJK906 was injected with pPD136.64 [*myo-3::YFP*] (a gift from A. Fire) and pJK907 [*pes-1::CFP*] into *hnd-1* to make *qEx495*; this array was used for mosaic analysis.

pes-1::CFP (pJK907)

The *pes-1* promoter from pUL#MJA1 (Molin et al., 2000) was cloned into pPD136.64 (a gift from A. Fire).

hnd-1 RNA interference and deletion

Double-stranded *hnd-1* RNA was generated, using pJK849 as template, and injected at 1 mg/ml. The *hnd-1(q740)* deletion was isolated essentially as described by Kraemer et al. (Kraemer et al., 1999), and backcrossed eight times. To test for maternal effects, *hnd-1* females, generated by *fog-1* RNAi (Jin et al., 2001), were crossed with N2 males [17% of the cross-progeny died as embryos or young larvae (*n*=313), and all adult progeny had normal gonads (*n*=260)]. To test for zygotic lethality, we scored progeny of *unc-9 hnd-1/++* mothers [6% died as embryos or larvae (*n*=235)]. To investigate whether *hnd-1(q740)* was a null allele, RT-PCR was performed on mutant and wild-type worms, using primers to a region retained in the *hnd-1* deletion. Template RNA was prepared from 20 gravid adults using TRI reagent (Molecular Research Center). A PCR product was obtained only from wild-type worms.

Tests for *hnd-1* genetic interactions

hlh-1

Progeny of *hlh-1/+; hnd-1* mothers had 55% embryonic and larval lethality, compared with 25% defects for *hlh-1/+* (Chen et al., 1994) and 28% defects for *hnd-1* (this work).

The following were evaluated using number of gonadal arms as a measure:

sys-1

100% of *sys-1/+; hnd-1/+* worms had two arms (*n*=89). *sys-1/+; hnd-1* had 68% gonadal arms, compared with 70% for *hnd-1* alone and <1% for *sys-1/+*. *sys-1* dominantly enhances other Sys mutants (K. Siegfried, unpublished).

gon-4

100% of *gon-4/+; hnd-1/+* worms had two arms (*n*=112). *gon-4; hnd-1* double mutants had 30% gonadal arms (*n*=46), compared with 43% for *gon-4* (Friedman et al., 2000) and 70% for *hnd-1*.

gon-2

At 20°C, the progeny of *gon-2; hnd-1* worms had 78% gonadal arms (*n*=89), compared with 70% for *hnd-1* and 100% for *gon-2* (Sun and Lambie, 1997). The progeny of *gon-2; hnd-1* worms shifted to 25°C as L4s resembled *gon-2* alone (most had no visible gonad).

Antibody staining

Embryos were fixed essentially as described by Miller and Shakes (Miller and Shakes, 1995), and then stained with α-PGL-1, a component of P granules (Kawasaki et al., 1998), α-HLH-1 (Krause et al., 1990) or α-UNC-54 (Miller et al., 1983) for body muscle. Secondary antibodies were used at 1:400 (Jackson Labs, West Grove, PA). DAPI staining was performed as described by Kadyk and Kimble (Kadyk and Kimble, 1998).

ehn-1 and *ehn-3* genetics

ehn-1(q638) was identified in a EMS mutagenesis screen for gonadogenesis mutants (L.D.M., K. Siegfried, F.-H. Markussen, unpublished). *ehn-1(q690)* and *ehn-3(q689)* were obtained in an *ehn-1* non-complementation screen of 2251 haploid genomes: EMS mutagenized males were crossed to *ehn-1(q638) rol-6* hermaphrodites, and F2 progeny screened for gonadal defects. By three-factor mapping, we positioned *ehn-1* between *unc-104* and *rol-6* on linkage group *II*, and *ehn-3* between *dpy-13* and *unc-5* on linkage group *IV*. *ehn-1* is almost maternally and zygotically sufficient for gonadogenesis. From an *ehn-1(q638) rol-6* mother, 1% of heterozygous cross progeny had defects (*n*=221); from an *ehn-1(q638) rol-6/++* mother, none of the *ehn-1 rol-6* homozygous progeny had defects (*n*=199). *ehn-3* has minor dominance: <1% of *ehn-3 unc-5/++* had gonadogenesis defects (*n*=222).

RESULTS

The *C. elegans* Hand gene affects embryogenesis and gonadogenesis

The *C. elegans* genome encodes a single Hand bHLH protein (Ledent and Vervoort, 2001; Ruvkun and Hobert, 1998). We initially investigated *hnd-1* (C44C10.8) because of its sequence similarity to vertebrate proteins implicated in Notch signaling (Saga et al., 1997). The *hnd-1* gene is predicted to have four exons encoding a 226 amino acid protein with one bHLH domain and no other motif (Fig. 2A,B). We confirmed these four exons in a single cDNA and identified the 5′ end using RT-PCR with the trans-spliced leader SL1 (Fig. 2A; Materials and Methods). During the course of this work, we isolated *hnd-1(q740)*, a deletion mutant that removes 674 bp from the *hnd-1* 5′ flanking region, as well as its first two exons and introns (Fig. 2A). Consistent with *hnd-1(q740)* being a null allele, we did not detect *hnd-1* mRNA in *hnd-1* mutants (Materials and Methods). Furthermore, *hnd-1(RNAi)* and the *hnd-1* deletion caused similar defects (Table 1), suggesting that both represent strong and perhaps complete loss-of-gene function.

To explore *hnd-1* function, we examined both *hnd-1(RNAi)* animals and the *hnd-1*-deletion mutant. Similar defects were observed, and penetrance, recessivity and maternal effects were analyzed in the mutant (Materials and Methods). The *hnd-1* phenotype includes a partially penetrant lethality (28%, *n*=909), as well as partially penetrant gonadal defects in

Table 1. Penetrance of *hnd-1*/EHN adult gonadal defects

	Gonadal morphology (%)*				
Genotype	Two arms	One arm	Abnormal	None visible	*n*
Wild type	100	0	0	0	>1000
hnd-1(q740)	48	41	6	5	656
hnd-1(RNAi)	41	51	4	5	332
ehn-1(q638)	89	10	0	0	638
ehn-1(q638)/mnDf93	88	11	1	0	361
ehn-1(q690)	87	12	1	1	351
ehn-1(q690)/mnDf93	86	12	2	0	524
ehn-3(q689)	82	15	3	0	1031
ehn-3(q689)/nDf41	75	20	5	1	197

*Percentage of surviving adults (*n*).

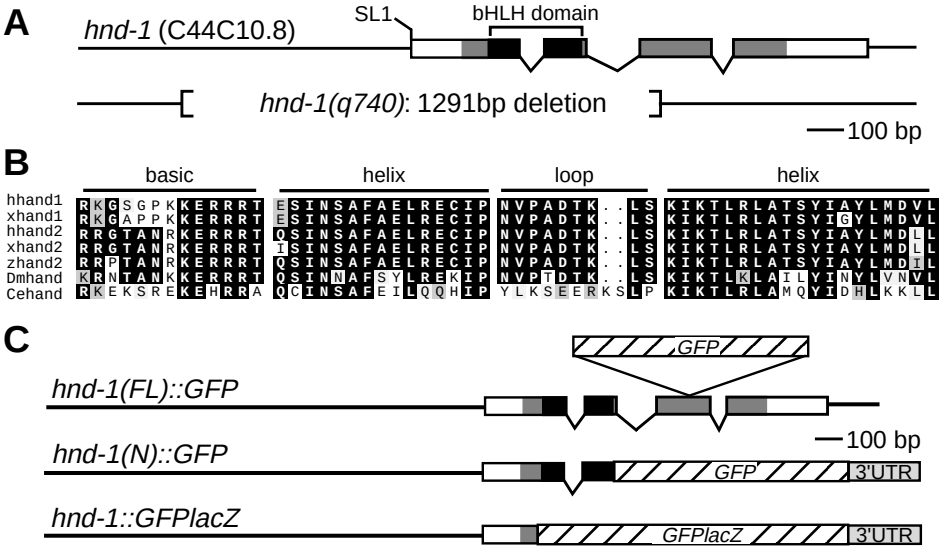

Fig. 2. The *hnd-1* gene encodes the *C. elegans* Hand transcription factor. (A) *hnd-1* (formerly C44C10.8) genomic organization. White, untranslated regions; gray and black, coding region; black, basic helix-loop-helix (bHLH) domain. Brackets below mark end points of *hnd-1* deletion. (B) Amino acid sequence alignment of bHLH domains of human (h), *Xenopus* (x), zebrafish (z), *Drosophila* (Dm) and *C. elegans* (Ce) HAND proteins. (C) *hnd-1* reporters. Top, *hnd-1(FL)::GFP* inserts GFP after amino acid 163. Middle, *hnd-1(N)::GFP* fuses *GFP* to the N terminus of HND-1 and uses the *unc-54* 3′UTR (light gray). Bottom, *hnd-1::GFPlacZ* replaces most of the *hnd-1* coding region with a *GFPlacZ* fusion and the *unc-54* 3′UTR (light gray). *GFP* and *GFPlacZ* are not to scale.

surviving adults (52%, n=656). Aside from gonadal defects and minor body wall abnormalities, *hnd-1* survivors appeared normal. The *hnd-1* mutant was recessive: the gonadal defects had no maternal effect, but both maternal and zygotic *hnd-1* activities were important for viability.

hnd-1 affects embryo morphogenesis

hnd-1 mutants can die as embryos or young larvae with variable body shape defects, typically in the posterior (Fig. 3A,B). Most *hnd-1* embryos contained pharynx and gut (Fig. 3C,D), as well as muscle, as evidenced by twitching. Because *hnd-1::GFP* is expressed in mesodermal precursors (see below), we compared body wall muscles in wild-type and *hnd-1* embryos using an α-myosin antibody (Miller et al., 1983). Both wild-type and *hnd-1* late-stage embryos have four quadrants of body muscle (Fig. 3E,F) (Miller et al., 1983), although muscle fibers were sometimes disorganized in mutants (Fig. 3F).

hlh-1 has striking similarities to *hnd-1*. The *hlh-1* gene encodes a MYOD-like bHLH protein, and *hlh-1* mutants have severe defects in embryo morphogenesis but generate body muscle normally (Chen et al., 1994). We examined *hnd-1; hlh-1* double mutants to determine whether these two bHLH proteins might have overlapping functions, but double mutants made body muscle (not shown). Furthermore, we found no significant genetic interaction between *hlh-1* and *hnd-1* (Materials and Methods). Therefore, *hnd-1* and *hlh-1* appear to function independently.

hnd-1 governs SGP number and position

Wild-type hermaphrodites possess two gonadal arms. By contrast, adult hermaphrodites depleted for *hnd-1* displayed a range of gonadal shapes: two gonadal arms (Fig. 4A); a single

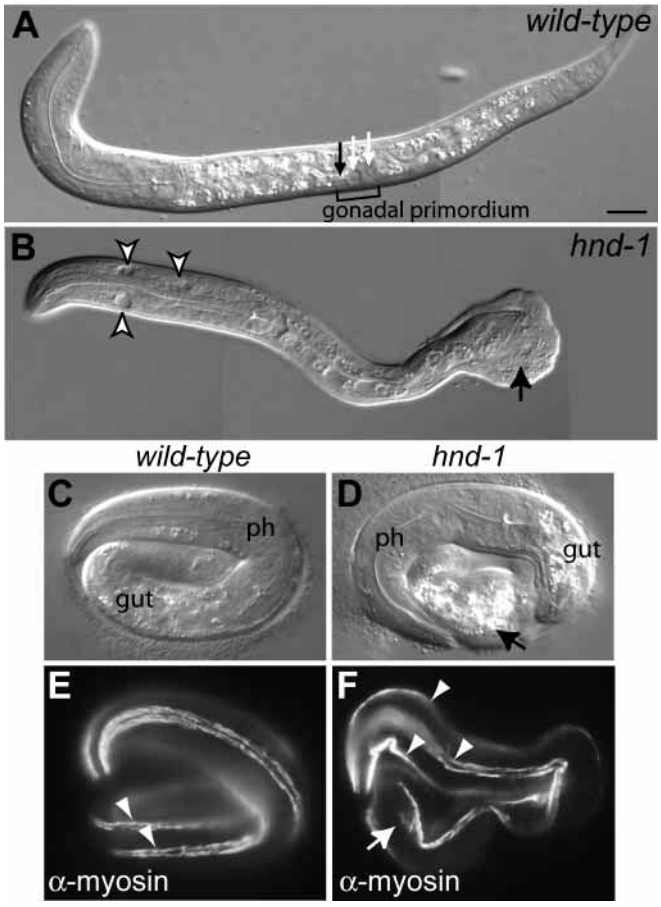

Fig. 3. *hnd-1* morphogenesis defect. (A,C,E) wild type, (B,D,F) *hnd-1(q740)*. (A) Wild-type L1 with normal body morphology. The gonadal primordium is bracketed. Three cells are visible: Z1, black arrow; PGCs, white arrows; Z4 is in a different focal plane. (B) *hnd-1* L1 with typical body shape defect (black arrow). Arrested larvae often have vacuoles in the head (arrowheads). (C) Wild-type pretzel-stage embryo is elongated and contains pharynx (ph) and gut. (D) *hnd-1* pretzel-stage embryo has not elongated posteriorly (arrow), but has a fully developed pharynx and gut tissue, which can be disorganized. (E) Wild-type embryos stained with α-myosin antibodies. Two muscle quadrants are visible in this plane (arrowheads). (F) *hnd-1* embryos generate four muscle quadrants, which can be disorganized posteriorly (arrow); three quadrants are visible in this plane (arrowheads). Scale bar: 10 μm.

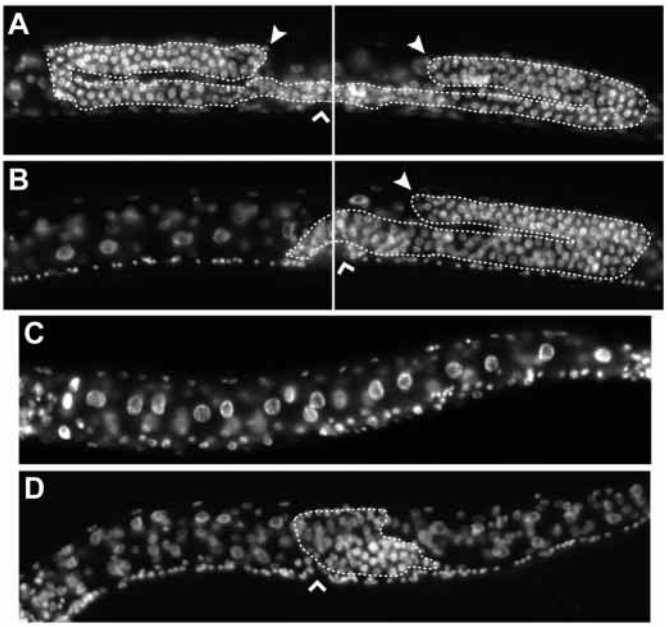

Fig. 4. *hnd-1* gonadal defects. (A-D) L4 *hnd-1(RNAi)* hermaphrodites, DAPI stained to highlight nuclei. Dashed line delineates the extent of the gonad. Arrowhead, distal end; carat, center of gonad (vulva). (A,B) Anterior half of animal is on the left, posterior on the right. (A) Two-armed gonad. (B) One-armed gonad. (C) No apparent gonad. (D) Abnormal gonad.

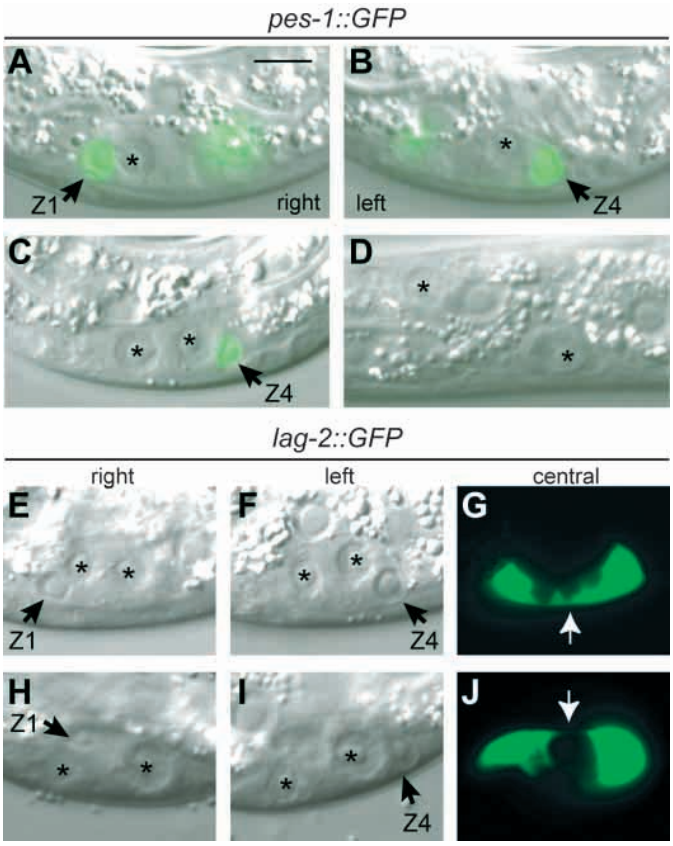

Fig. 5. Gonadal primordia in *hnd-1* L1 larvae. All images are *hnd-1(RNAi)*. Black arrow, SGP with name of cell; asterisk, PGC. (A-C) *pes-1::GFP* marks SGPs, GFP overlays DIC image. (A,B) Primordium with two SGPs. Right focal plane shows Z1 (A). Left focal plane shows Z4 (B). (C) Primordium with one SGP, left focal plane. (D) Primordium with no SGPs, two PGCs are present, but are separated. (E-J) *lag-2::GFP* marks SGPs. (E-G) Two SGPs in normal positions. Z1 is at anterior pole on right (E), Z4 is at posterior pole on left (F). Z1 and Z4 extend cytoplasmic processes to meet mid-ventrally (G, white arrow). (H-J) Primordium with one misplaced SGP. Z1 is displaced dorsally (H), Z4 is located at the posterior pole (I). Z1 and Z4 meet mid-dorsally via a thin cytoplasmic process (J, white arrow). Scale bar: 5 μm.

gonadal arm (Fig. 4B); no apparent gonad (Fig. 4C); or abnormal gonads (Fig. 4D). 'Abnormal gonads' include a variety of shapes, most typically an amorphous mass (Fig. 4D). One-armed and two-armed gonads were frequent and were usually fertile, whereas absent and abnormal gonads were less common and were always sterile (Table 1). A similar, but less penetrant effect was seen in males (not shown). We conclude that *hnd-1* is important, but not essential, for gonadogenesis.

The gonadal morphologies in *hnd-1* mutants suggested a defect early in gonadogenesis. Therefore, we examined SGPs in *hnd-1* gonadal primordia, using either nuclear *pes-1::GFP* (Molin et al., 2000) (Fig. 5A-C) or cytoplasmic *lag-2::GFP*, which reveals cellular processes (Blelloch et al., 1999) (Fig. 5G,J). Whereas all wild-type gonadal primordia had two SGPs (Table 2A), *hnd-1* primordia could have two (Fig. 5A,B), one (Fig. 5C), zero (Fig. 5D) or even three SGPs (Table 2A). Therefore, *hnd-1* is important for determining SGP number.

SGP position was also affected in *hnd-1* mutants. In wild type, the two SGPs reside at the distal poles of the primordium, flanking the PGCs and extending cytoplasmic processes to meet mid-ventrally. In most *hnd-1* mutants, SGPs occupied similar polar positions (Table 2B), and extended ventral processes (Fig. 5E-G). However, in some *hnd-1* mutants, one or both SGPs were not at the pole, but instead were found more centrally in the primordium (Table 2B). When an SGP was misplaced dorsally, it extended cytoplasmic processes along the dorsal surface of the primordium (Fig. 5H-J). Finally, in some *hnd-1* mutants, SGPs were observed ectopically (Table 2B). These ectopic SGPs could be in animals with either two or three total SGPs; as predicted, ectopic gonadal arms have been observed in rare *hnd-1* mutants. Therefore, *hnd-1* can affect the position of the SGPs within the primordium and within the animal.

SGP number and position are crucial for gonadogenesis

We used *hnd-1* mutants born with aberrant gonadal primordia to investigate how organization of that primordium affected gonadogenesis. Specifically, we used *pes-1::GFP* to score SGPs in *hnd-1* L1 larvae, permitted the animals to develop and then examined them again as L4s. Our results (Table 3) led to three conclusions. First, most *hnd-1* primordia with a wild-type appearance (two SGPs placed at the poles) generated wild-type appearing adults with two gonadal arms (93%, n=70). Therefore, *hnd-1* appears to play little or no role in gonadogenesis after formation of the gonadal primordium. Second, most primordia containing one SGP generated adult gonads with only a single arm (98%, n=53); none made two

Table 2. Effect of *hnd-1*/EHN mutants on SGP number and position

A SGP number

Genotype*	Percentage of animals: Three SGPs	Two SGPs	One SGP	No SGP	*n*
Wild type	0	100	0	0	64
hnd-1(q740)	2	57	39	3	103
hnd-1(RNAi)	2	61	36	1	87
ehn-1(q638)	0	90	10	0	106
ehn-3(q689)	0	89	8	2	171
ehn-1(q638); ehn-3(q689)	0	5	35	60	43
ehn-3(q689); hnd-1(RNAi)	0	4	29	67	49

*All contain *qIs61*, which is an integrated *pes-1::GFP*.
n, number of animals scored.

B SGP position

Genotype*	Percentage of SGPs in each position: Pole Position†	Central‡	Ectopic§	*n*
Wild type	100	0	0	128
hnd-1(q740)	87	9	4	162
hnd-1(RNAi)	83	15	3	143
ehn-1(q638)	90	9	1	196
ehn-3(q689)	96	4	0	320

*All contain *qIs61*, which is an integrated *pes-1::GFP*.
†SGP at its normal position within the primordium.
‡SGP misplaced centrally within the primordium.
§*pes-1::GFP*-expressing cells outside of the gonadal primordium.
n, number SGP cells scored.

arms. Finally, primordia with wild-type SGP number but aberrant SGP position often generated defective gonads (26%, *n*=23). Therefore, SGP position within the primordium may be important for gonadogenesis.

Effects on germline development in *hnd-1* mutants

The gonadal primordium in *hnd-1* mutants sometimes lacked PGCs. In primordia with two SGPs, 7% lacked one or both PGCs (*n*=59), and in primordia with one SGP, 40% were missing at least one PGC (*n*=47). To investigate whether the two PGCs were made in *hnd-1* mutants, we stained ~100-cell embryos with a germline-specific antibody, α-PGL-1 (Kawasaki et al., 1998): all had two PGL-1-staining cells (*n*=237). Therefore, *hnd-1* does not affect the generation of PGCs, but instead affects their maintenance within the gonadal primordium. A simple interpretation is that *hnd-1* acts in SGPs, which in turn are essential for germline survival and positioning.

hnd-1 expression during wild-type development

To investigate *hnd-1* expression, we constructed three GFP reporters (Fig. 2C). *hnd-1(FL)::GFP* inserts GFP coding sequences into the third exon of the full-length HND-1 protein; this reporter rescued *hnd-1* mutants (Materials and Methods). *hnd-1(N)::GFP* inserts GFP more N-terminally and replaces the *hnd-1* 3′UTR with the *unc-54* 3′UTR. *hnd-1::GFPlacZ* replaces most of the *hnd-1* coding region with GFP and β-galactosidase coding sequences, and the *unc-54* 3′UTR. All three *hnd-1* reporters expressed GFP in largely the same cells, but *hnd-1(N)::GFP* and *hnd-1::GFPlacZ* expressed GFP at a higher level and expression persisted longer. The rescue by *hnd-1(FL)::GFP* suggests that its expression is relevant to *hnd-1* function.

The *hnd-1* reporters expressed GFP in the MS, C and D embryonic lineages (Fig. 6A). Expression was first observed in four MS great-granddaughters, four C great-granddaughters and two D daughters (Fig. 6B). These MS descendants give rise to the SGPs and other mesodermal cells (Fig. 6A) (Sulston et al., 1983); the C- and D-expressing cells all generate body wall muscle (Sulston et al., 1983). Expression continued through one cell division (Fig. 6C) and then became difficult to detect using *hnd-1(FL)::GFP*. *hnd-1(N)::GFP* remained detectable in some cells within these MS and C lineages (Fig. 6D), but disappeared from most body muscle cells by the comma stage of embryogenesis (Fig. 6E). Then, the *hnd-1* reporters were expressed in the SGPs (Z1 and Z4) as they approached the PGCs to form the gonadal primordium (Fig. 6E,F). Shortly after the primordium was assembled, *hnd-1(N)::GFP* expression was reduced or disappeared (Fig. 6G). GFP was not detected in the SGPs at hatching or post-embryonically (not shown). Therefore, *hnd-1* appears to be expressed during embryogenesis in mesodermal precursor cells that generate predominantly body wall muscle, and then in SGPs.

The *hnd-1* gene is not required for specification of the SGP fate

The most common gonadal defect in *hnd-1* mutants is a missing SGP (Table 2A). To investigate whether both SGPs are made in *hnd-1*-mutant embryos, we used the *hnd-1(N)::GFP* reporter, which is an early SGP marker. We found both SGPs

Table 3. Correlation of gonadal primordium and adult gonad in *hnd-1* mutants

Genotype*	Gonadal primordium: Number of SGPs	SGP position‡	Adult gonad (%): Two arms	One arm	Abnormal	None visible	*n*
Wild type	2	Both at poles	100	0	0	0	64
hnd-1†	2	Both at poles	93	6	1	0	70
hnd-1†	2	One at pole, one misplaced	74	9	17	0	23
hnd-1†	1	Pole	0	98	2	0	53
hnd-1†	1	Misplaced	0	71	29	0	7
hnd-1†	0	Not applicable	0	0	0	100	4

*All contain *qIs61*, an integrated *pes-1::GFP*.
†Includes both *hnd-1(RNAi)* and *hnd-1(q740)*.
‡Presence and position of Z1 and Z4 were scored by *pes-1::GFP* expression.

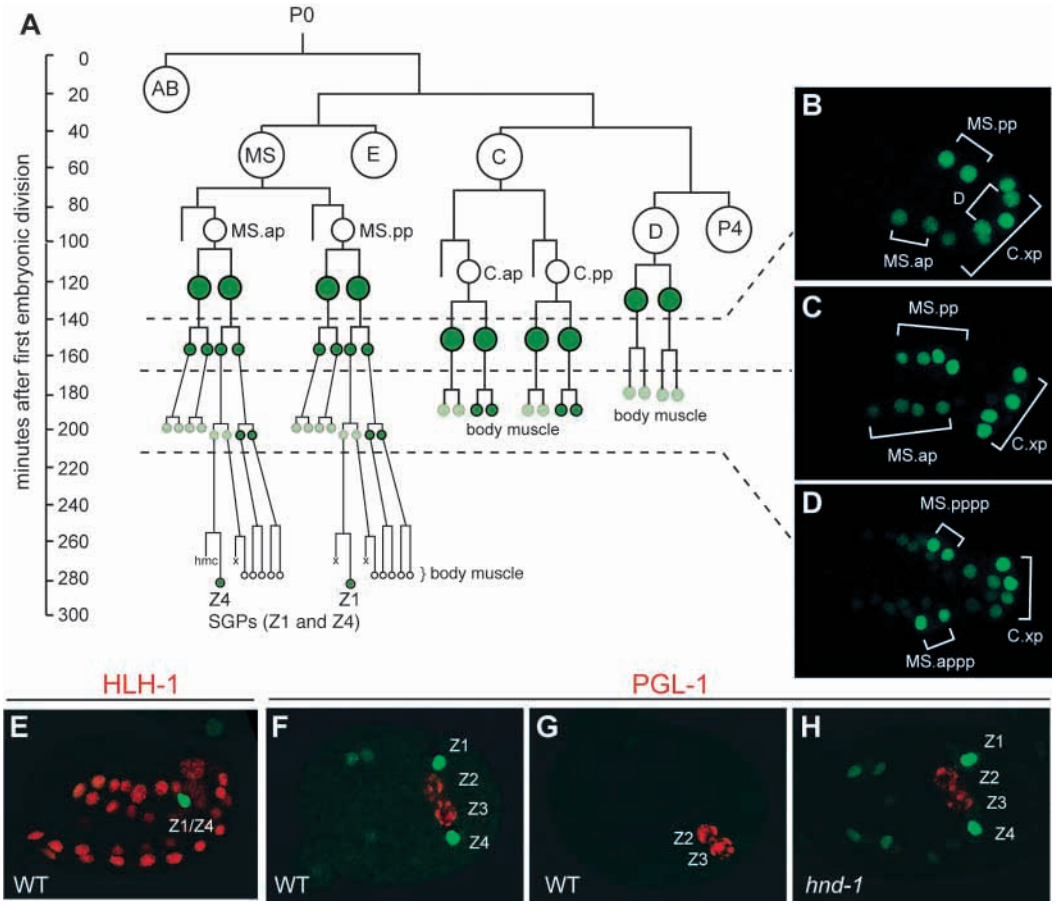

Fig. 6. *hnd-1::GFP* expression. (A) Lineage diagram depicting cells that express *hnd-1::GFP* (green). Dashed lines indicate approximate stage of embryos in panels B-D. (B-H) Confocal images of embryos expressing *hnd-1::GFP* (green). (B-D) Projections of z-series; embryos were observed over time to identify cells. (B) *hnd-1(N)::GFP* expression is first detected in four granddaughters of MS, descendants of MS.ap and MS.pp, in four granddaughters of C, descendants of C.ap and C.pp (C.xp), and in two daughters of D. (C) *hnd-1(FL)::GFP* expression in granddaughters of MS.ap/MS.pp and daughters of C.ap/C.pp. (D) *hnd-1(N)::GFP* expression fades after the next division of most *hnd-1*-expressing cells but is retained in daughters of MS.appp and MS.pppp, and in daughters of C.ppp and C.app. (E-H) Embryos fixed and stained with α-HLH-1 or α-PGL-1 (red). (E) Unlike HLH-1 (red), which is detected in body muscle lineages throughout embryogenesis (Krause et al., 1990), *hnd-1(N)::GFP* is absent from body muscles by the comma-stage of embryogenesis; at this time, expression is seen in Z1/Z4. (F) *hnd-1(N)::GFP* is detected in Z1 and Z4 as they meet the PGCs (Z2, Z3), marked by PGL-1 (red). (G) Shortly after, expression is absent from Z1 and Z4. (H) *hnd-1* embryos express *hnd-1(N)::GFP* in Z1/Z4.

present and in their normal position near the PGCs (Fig. 6H; n=29). Therefore, *hnd-1* is not necessary for SGP specification or SGP migration to the PGCs.

After formation of the gonadal primordium, SGPs remained associated with PGCs in *hnd-1* embryos; none were seen detaching. Instead, SGP nuclei sometimes became smaller and *hnd-1* reporter expression faded prematurely (38%, n=13). The simplest hypothesis is that *hnd-1* is required for maintenance of SGP fate and possibly SGP survival.

We next investigated whether ectopic *hnd-1* expression could transform other cells to the SGP fate. To this end, we used a heat-inducible promoter to express *hnd-1* during embryogenesis but found no ectopic SGPs, as assayed by *pes-1::GFP* (Materials and Methods). We also expressed a full-length HND-1::GFP fusion protein under control of either of two mesodermal promoters (see below), but again did not observe ectopic SGPs. These results are consistent with the proposed role for *hnd-1* in controlling SGP maintenance or survival.

What becomes of SGPs in *hnd-1* mutants?

The missing SGPs in *hnd-1* mutants might be explained by transformation to a different cell type, or by cell death. To explore the first idea, we reasoned that the most likely transformation would be to a different mesodermal cell type. We tested this using *hlh-8::GFP* to mark the M mesoblast (Harfe et al., 1998) and *unc-122::GFP* to mark coelomocytes (Miyabayashi et al., 1999). All wild-type L1s had a single *hlh-8::GFP*-expressing M mesoblast, as expected (n=61). Similarly, most *hnd-1* mutants had a single M cell, but a few had two M cells (5%, n=63) or no M cell (2%, n=63). Those with an additional M cell had two SGPs, suggesting that extra M cells were not transformed SGPs. Likewise, occasional extra coelomocytes were seen, but overall *hnd-1* mutants had marginally fewer coelomocytes than wild type (5.5 versus 5.9 on average per animal, n>30). Importantly, the extra coelomocytes could be in worms with two gonadal arms. Therefore, *hnd-1* appears to have a low-penetrance effect on M cells and coelomocytes, but this is unlikely to account for the missing SGPs.

To determine whether SGPs are lost as a result of programmed cell death in *hnd-1* mutants, we examined *hnd-1; ced-3* double mutants using the *pes-1::GFP* marker. The *ced-3* gene is required for all programmed cell deaths (Ellis and Horvitz, 1986). In *hnd-1* single mutants, 42% were missing at least one SGP (Table 2A), and, in *ced-3; hnd-1* double mutants, 45% lacked at least one SGP (n=69). Therefore, SGP loss does not appear to rely on *ced-3*-dependent programmed cell death.

Next, we investigated whether SGPs died in *hnd-1* mutants. In *C. elegans*, cell corpses resulting from either programmed or necrotic cell death are engulfed by their neighbors (Chung

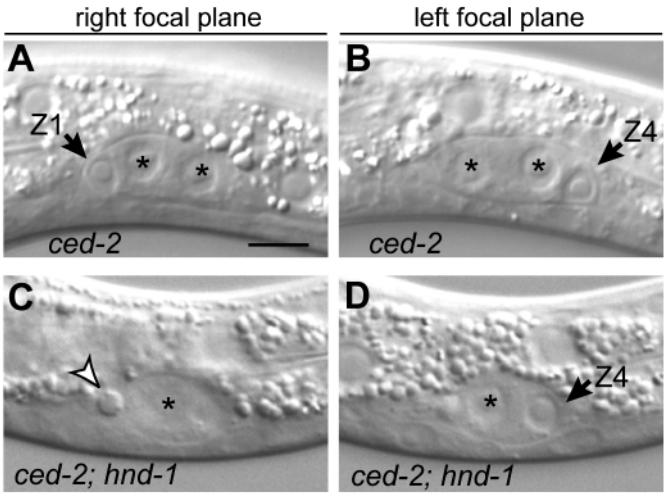

Fig. 7. Cell death in *hnd-1* mutants. Gonadal primordia: black arrow, SGP with name of cell; asterisk, PGC. (A,B) *ced-2* mutants have no corpses near gonad. Z1 is in the right plane (A) and Z4 is in the left plane (B). (C,D) *ced-2; hnd-1* double mutant. Z1 is missing, but a cell corpse occurs in its place at the anterior pole of the gonadal primordium (C, open arrow). Z4 is present (D). Scale bar: 5 μm.

et al., 2000; Ellis et al., 1991). The engulfment of cell corpses relies on several genes, including *ced-2* (Ellis et al., 1991). In *ced-2* single mutants, no cell corpses were evident near the gonad (Fig. 7A,B; n=54); however, in *ced-2; hnd-1* double mutants, cell corpses were found near the gonad (Fig. 7C,D; 28%, n=50). Importantly, the presence and site of corpses correlated with SGP absence. We observed no cell corpses near gonads with two SGPs (n=53), mostly anterior or right cell corpses near gonads missing Z1 (4/5; Fig. 7C), and only posterior or left cell corpses in those missing Z4 (8/8). Indeed, in one cell corpse, *hnd-1::GFP* was faintly expressed, indicating that it had been specified originally as an SGP (data not shown). Therefore, SGPs appear to die in *hnd-1* mutants.

hnd-1 activity acts in somatic tissues to control gonadogenesis

Both SGPs and PGCs are affected in *hnd-1* mutants. To learn where *hnd-1* functions, we used a combination of mosaic analysis and transgenic experiments driving the *hnd-1* coding region with tissue-specific promoters.

In *C. elegans*, mosaic animals can be made by loss of extra-chromosomal arrays that carry transgenes and that are transmitted with varying fidelity at each cell division (Herman, 1984). For this study, we created an extra-chromosomal array that carries a rescuing *hnd-1* genomic fragment and two fluorescent markers (*myo-3::YFP* to mark body muscle and *pes-1::CFP* to mark SGPs). We then identified 'germline mosaics', animals that retained the array in somatic tissues but failed to transmit it to their progeny; such animals have lost the array in divisions generating the germline blastomere P4 (see Fig. 6). All six germline mosaics had a wild-type gonadal primordium, which suggests that *hnd-1* activity acts in somatic tissues rather than in the germ line.

To further explore where *hnd-1* acts, we used either of two promoters: *hlh-1*, which is expressed in body muscle and not in SGPs (Krause et al., 1990); or *lag-2*, which is first expressed in the AB and MS lineages (Moskowitz and Rothman, 1996), and then in SGPs (Miskowski et al., 2001). Each promoter was fused to a full-length, rescuing *hnd-1::GFP* cDNA and expressed in *hnd-1* mutants. Expression of HND-1::GFP by the *hlh-1* promoter rescued the *hnd-1* gonadogenesis defects, from 52% to 5% defective (n=42). By contrast, HND-1::GFP driven from the *lag-2* promoter, which is expressed in the two SGPs (Fig. 5G), did not appreciably rescue *hnd-1* gonadogenesis defects (36% defective, n=108). The latter experiment has the caveat that this promoter is switched on after SGPs assemble into the gonadal primordium and it may not be expressed in dying SGPs. From the *hlh-1::hnd-1GFP* result, we suggest that HND-1 acts in early mesodermal lineages.

hnd-1 acts independently of other early gonadogenesis genes

The *hnd-1* SGP defects are the earliest observed to date among any genes controlling *C. elegans* gonadogenesis. To investigate whether *hnd-1* might function with other early gonadogenesis genes, we explored genetic interactions between *hnd-1* and two mutant classes. The first type, represented by *gon-2* and *gon-4* (Friedman et al., 2000; Sun and Lambie, 1997), controls the onset of cell divisions in the gonad but not in other tissues (Fig. 1C). Gonadal divisions are delayed in *gon-2* or *gon-4* single mutants (Friedman et al., 2000; Sun and Lambie, 1997), but not in *hnd-1* mutants (n=5). Moreover, *hnd-1; gon-2* and *hnd-1; gon-4* double mutants have additive phenotypes (Materials and Methods). Therefore, *hnd-1* does not affect the onset or timing of gonadal divisions and acts independently of *gon-2* and *gon-4*.

The second class of early gonadogenesis genes, represented by *sys-1* (Miskowski et al., 2001), is required for SGPs to produce daughter cells with different developmental potential (Fig. 1C). In wild type, each SGP generates one distal tip cell (DTC), whereas in *sys-1* mutants they make no DTCs (Miskowski et al., 2001). Most *hnd-1* SGPs that were properly positioned generated DTCs (96%, n=193; Table 3), and no genetic interactions were found with *sys-1* (Materials and Methods). Therefore, *hnd-1* does not appear to affect SGP asymmetric divisions, but instead ensures that two SGPs are present and properly positioned in the gonadal primordium.

Identification of genetic enhancers of *hnd-1*

To identify additional genes controlling SGP development, we screened for EMS-induced mutants with a *hnd-1*-like gonadogenesis phenotype and discovered loss-of-function mutations of *ehn-1* and *ehn-3* [for enhancer of Hand (Materials and Methods)]. The *ehn-1* and *ehn-3* mutants had low-penetrance gonadal defects (Table 1). For *ehn-1*, gonadal defects could be rescued either maternally or zygotically, but *ehn-3* exhibited no maternal effect (Materials and Methods). Furthermore, *ehn-1* had low-penetrance lethality, but lethality was negligible in *ehn-3* mutants (Table 4).

The gonadal primordia of *ehn-1* and *ehn-3* mutants had absent or misplaced SGPs, as described above for *hnd-1* mutants (Table 2A,B). However, *hnd-1*, *ehn-1* and *ehn-3* displayed subtle differences in their spectrum of defects. For example, ectopic SGPs were seen in *ehn-1* but not *ehn-3* mutants, and only *hnd-1* mutants generated extra SGPs (Table 2A). Despite these minor differences, the primary defects were similar among the three mutants, which suggests that they may

Table 4. Genetic interactions between the *hnd-1*/EHN genes

Genotype	Gonadal morphology (%)*			Lethality (%)†		
	Two arms	None visible	n_L	Larval lethal	Dead embryos	n_T
hnd-1(q740)	48	5	656	20‡	8	909
ehn-1(q638)	89	0	638	5§	0	671
ehn-1(q690)	87	0	351	5§	9	413
ehn-3(q689)	82	0	1031	0	1	1046
ehn-1(q638); ehn-3(q689)	4	20	779	2§	6	853
ehn-1(q690); ehn-3(q689)	2	36	474	4§	0	495
ehn-1(q690); hnd-1(q740)	15	26	114	47‡,§	11	651
ehn-3(q689); hnd-1(q740)	0	82	376	6‡	8	437
ehn-1(q638); ehn-3(q689); hnd-1(RNAi)	0	81	63	48‡,§	24	130

*Percentage of living adults (n_L) with two gonadal arms (Two arms) or no visible gonad (None visible).
†Lethality is a percentage of total progeny (n_T).
‡Arrested larvae often had severe body shape defects.
§Larvae died as L1s with no obvious morphological defects.

function in a common pathway to control early gonadal development.

Functional relationships between the *hnd-1* and EHN genes

To investigate the functional relationships between the *hnd-1* and EHN genes, we first investigated double and triple mutants (Table 4). Although *hnd-1*, *ehn-1* and *ehn-3* single mutants all had relatively low-penetrance gonadal defects, the double and triple mutants showed increased penetrance (Table 4). For example, 80-90% of *ehn-1* and *ehn-3* single-mutant adults had two gonadal arms, but almost none of the *ehn-1; ehn-3* double mutants had two gonadal arms (2-4%; Table 4). Similarly, only 5% of the double mutants had two SGPs at hatching (Table 2A). By contrast, larval lethality did not increase in the *ehn-1; ehn-3* double mutant. Therefore, *ehn-1* and *ehn-3* may be partially redundant for SGP development.

The *ehn-1; hnd-1* and *ehn-3; hnd-1* double mutants were also more defective than any of the single mutants, but each double mutant was unique. For the gonadogenesis defects, *ehn-3* enhanced *hnd-1* more strongly than did *ehn-1*. Thus, some *ehn-1; hnd-1* double mutants made two gonadal arms and only about one-third had no apparent gonad. By contrast, no *ehn-3; hnd-1* double mutants had two gonadal arms, and most had no visible gonad (Table 4). Intriguingly, this situation was reversed for lethality: *ehn-1* enhanced *hnd-1* more strongly than did *ehn-3* for both embryonic and larval lethality. One simple explanation is that the three genes are all partially redundant, but that each has acquired an individual role in the repertoire of activities normally carried out by *hnd-1*/EHN genes (see Discussion).

The *ehn-1; ehn-3; hnd-1* triple mutant appears additive for the *ehn-1; hnd-1* and *ehn-3; hnd-1* defects. Thus, the penetrance of the triple mutant with respect to lethality is similar to that of the *ehn-1; hnd-1* double mutant, and the penetrance of the triple mutant with respect to gonadal defects is similar to that of the *ehn-3; hnd-1* mutant (Table 4). The fact that the triple mutant is not fully penetrant may suggest the existence of one or more additional genes involved in the process, or it may indicate that the *ehn-1* or *ehn-3* mutant is not a null.

To begin addressing relationships between the *ehn* genes and *hnd-1* at a molecular level, we examined expression of *hnd-1(N)::GFP* to mark *hnd-1* transcription and SGP formation. The *ehn-1* and *ehn-3* single mutants both expressed *hnd-1(N)::GFP* in two SGPs (*ehn-1*, n=35; *ehn-3*, n=31). Therefore, *ehn-1* and *ehn-3* do not control *hnd-1* transcription in SGPs, which is consistent with the idea that they function in parallel to *hnd-1*. Furthermore, *ehn-3; hnd-1* double mutant embyros made two SGPs (n=8), but few possessed SGPs at hatching (4%; Table 2A). Therefore, like *hnd-1*, the *ehn-1* and *ehn-3* genes do not affect SGP specification but instead influence SGP fate or survival.

DISCUSSION

In this paper, we investigate the controls governing development of the *C. elegans* gonadal primordium. Our primary focus is the *hnd-1* gene, which encodes the single Hand transcription factor in the *C. elegans* genome (Ledent and Vervoort, 2001; Ruvkun and Hobert, 1998). Animals lacking *hnd-1* activity have partially penetrant defects in gonadogenesis and embryogenesis (this work). In addition, we have found two genetic enhancers of *hnd-1* that have overlapping functions. We discuss the roles played by these genes in *C. elegans* organogenesis and compare our findings to similar studies in other organisms.

hnd-1 and control of SGPs in the gonadal primordium

Wild-type *C. elegans* has four gonadal precursors, two SGPs and two PGCs, in stereotyped positions within the primordium. *hnd-1* mutants affect the presence and position of these precursors in the primordium and within the animal. Thus, *hnd-1* mutants can possess fewer than normal, as well as mispositioned, SGPs or PGCs. The *hnd-1* gene probably acts cell autonomously in the SGPs or their precursors to control early gonadogenesis. However, *hnd-1* does not affect SGP specification, because the correct number of SGPs is generated in all *hnd-1*-mutant embryos. Nor does it cause SGPs to be transformed into either of two mesodermal types (coelomocytes and the M mesoblast), although it remains possible that they are transformed into muscle cells. Instead, we suggest that *hnd-1* is required for SGP survival.

What happens to SGPs in *hnd-1* mutants? We used two classes of cell death mutants: *ced-3*, which eliminates all programmed cell death (Ellis and Horvitz, 1986), and *ced-2*, which is defective in cell corpse engulfment (Ellis et al., 1991). At first glance, our results appear contradictory: we observed extra cell corpses in *hnd-1; ced-2* double mutants, but saw no increase in the number of SGPs in *hnd-1; ced-3* double mutants. One simple explanation is that SGPs die by a *ced-3*-independent pathway. Alternatively, if the SGPs no longer expressed markers of their fate (e.g. *pes-1*), they would not have been identified in our analysis of *hnd-1; ced-3* mutants. Therefore, it remains possible that *hnd-1* mutant SGPs fail to maintain their fate and die via programmed cell death. In either case, the correlation between missing SGPs and extra cell corpses strongly supports the idea that *hnd-1* is required for SGP survival.

Why might SGPs die in *hnd-1* mutants? One simple explanation is that inhibition of apoptosis is part of the normal developmental program, as has been suggested for the winged-helix transcription factor Fork head in *Drosophila* salivary gland development (Myat and Andrew, 2000). Alternatively, cells may be programmed to die when they receive ambiguous developmental cues. This idea is supported by the extensive apoptosis seen in many developmental mutants [e.g. *Pax6*/*eyeless* mutants (Halder et al., 1998)]. Because *hnd-1* SGPs initially show evidence of their fate (they express SGP markers and migrate to the PGCs), we favor the idea that *hnd-1* is required for maintenance of cell fate and that in its absence the SGPs die. Similarly, *Pax6/eyeless* mutants generate eye primordia that express early markers of their fate (e.g. *ey*-eye enhancer *lacZ*) and later undergo programmed cell death (Halder et al., 1998).

hnd-1 and embryonic viability

In addition to gonadal defects, *hnd-1* mutants can die as embryos or young larvae with body morphogenesis defects. Elongation of the embryo is driven largely by cell shape changes in the hypodermis (Priess and Hirsh, 1986). However, mutants affecting muscle development also disrupt the process (e.g. Bejsovec and Anderson, 1988; Chen et al., 1994; Waterston, 1989). Of particular interest to this work is the *hlh-1* gene, which encodes the *C. elegans* myoD homolog (Krause et al., 1990); its loss disrupts development of body wall muscles and causes a characteristic morphogenesis defect (Chen et al., 1994). We explored the possibility that *hnd-1* may similarly be involved in body muscle development. However, *hnd-1; hlh-1* double mutants still make body muscle, suggesting that these bHLH proteins control different aspects of muscle development. Although speculative at the current time, we suggest that *hnd-1* may play a role in muscle fate that parallels its role in controlling SGP fate.

Three genes with overlapping functions in SGP development

The *hnd-1* deletion has incompletely penetrant gonadal and embryonic defects. Yet, the mouse and zebrafish Hand mutants are completely penetrant (Firulli et al., 1998; Riley et al., 1998; Srivastava et al., 1997; Yelon et al., 2000). Why might a *hnd-1*-null mutant exhibit partially penetrant defects? One simple explanation is genetic redundancy. We have identified two genes, *ehn-1* and *ehn-3*, that enhance the *hnd-1* phenotype. All three single mutants have partially penetrant gonadal defects, and mutations in two of the three, *hnd-1* and *ehn-1*, also affect viability. Each of the double mutants has a more severe gonadogenesis defect, which suggests at least two pathways control SGP survival. Redundancy frequently results from gene duplication (Ohno, 1970). However, only one Hand homolog exists in the *C. elegans* genome, and neither *ehn-1* nor *ehn-3* maps to a region containing any predicted bHLH protein. Therefore, the *hnd-1* and EHN genes redundantly control SGP development, but they are unlikely to represent paralogous pathways.

Intriguingly, *ehn-1* enhances *hnd-1* lethality more strongly than it enhances the *hnd-1* gonadal defect, whereas *ehn-3* enhances the *hnd-1* gonadal defect but not its lethality. The identity of *hnd-1* as a putative bHLH transcription factor provides a molecular framework for considering the enhancement of *hnd-1* by *ehn-1* and *ehn-3*. One idea is that *ehn-1* and *ehn-3* might encode, or control the activity of, transcription factors that cooperate with *hnd-1* in the regulation of partially overlapping sets of target genes. Regardless of the molecular mechanism, the *hnd-1*/*ehn* genes clearly have overlapping, but non-equivalent, functions in embryonic development and gonadogenesis.

Regulation of mesoderm development by Hand transcription factors

The *hnd-1* gene encodes the single Hand transcription factor in the *C. elegans* genome (Ledent and Vervoort, 2001). Higher vertebrates contain two Hand genes (*eHand/Hand1* and *dHand/Hand2*), whereas a single family member has been identified in zebrafish (Yelon et al., 2000), ascidians (Dehal et al., 2002) and flies (Moore et al., 2000). Vertebrate *dHand* is expressed in lateral plate mesoderm and is important for development of mesodermal organs, including heart and limbs (Firulli et al., 1998; Riley et al., 1998; Srivastava et al., 1997; Yelon et al., 2000). The *Drosophila* Hand gene is expressed in the dorsal vessel (heart) and visceral mesoderm, but its function is not known (Moore et al., 2000). The *C. elegans* Hand gene, *hnd-1*, is first expressed broadly in mesodermal precursors that generate striated muscles, and then is restricted to the somatic gonadal precursors; its function appears to affect both muscle and gonadal development. Therefore, all Hand genes explored to date are expressed in mesodermal cells and, where studied, are important for mesoderm development.

The defects in *hnd-1* have intriguing similarity to the defects in the zebrafish Hand gene called *hands off* (*han)*. Thus, *han* mutants generate the normal number of precardiac cells, but these cells cannot differentiate and a midline heart tube fails to form (Yelon et al., 2000). Similarly, SGPs are specified correctly in *hnd-1* mutants, but they often fail to maintain their fate and can subsequently die. The fate of the cardiac precursors in zebrafish *han* mutants is not known (Yelon et al., 2000). We suggest that the zebrafish and nematode Hand genes may play parallel roles in controlling cardiac and gonadal precursor cells, respectively. Interestingly, zebrafish *han* may also be important for gonadogenesis: *han* mutants have defects in migration of germ cells to the gonad as well as abnormalities in *pax2.1* expression in the putative gonadal mesoderm (Weidinger et al., 2002). Therefore, zebrafish *han* mutants, like *hnd-1* mutants, might have defects in development of the gonadal mesoderm. Our identification of *hnd-1* as a regulator of somatic gonadal

development in *C. elegans* raises the possibility that Hand genes are ancient regulators of gonadogenesis.

Genetic controls of early gonadogenesis

How does the control of SGP development by *hnd-1*/*ehn* genes compare to the genetic regulation of early gonadogenesis in other animals? Although genes have been identified that govern formation of the early gonad in both *Drosophila* and vertebrates, the genetic control of early gonadogenesis remains relatively uncharted territory. Perhaps most analogous to *C. elegans hnd-1*/*ehn* genes is *Drosophila clift*, which encodes a novel nuclear protein required for both SGP development in the gonad and for photoreceptor survival in the eye (Boyle et al., 1997). The *clift* effect on SGPs is remarkably similar to that of the *hnd-1*/*ehn* genes: SGPs are generated in *clift* mutants, but they do not coalesce into a gonadal primordium and are lost over time (Boyle et al., 1997). Furthermore, ectopic *clift* expression, like ectopic *hnd-1* expression, did not increase SGP number (Boyle et al., 1997). Therefore, like *hnd-1*, *clift* may not be sufficient to direct SGP development on its own. In mice, several transcription factors have been implicated in development of the genital ridge, a mesodermal swelling destined to generate the somatic gonad (Birk et al., 2000; Capel, 2000). In *Sf1* and *Wt1* knockout mice, the genital ridge forms initially but it does not develop further; instead, the ridge regresses because of programmed cell death (Kreidberg et al., 1993; Luo et al., 1994). Therefore, although these genes all encode different transcription factors, the similarities in mutant phenotype suggest parallels in the genetic control of somatic gonadal precursors in flies, mammals and worms.

Controls of early organogenesis

How does *C. elegans* gonadogenesis compare with the development of other organs? Some organs rely on 'selector' genes, which regulate (directly or indirectly) all the genes needed to generate a particular organ. One simple example of an organ selector gene is *C. elegans pha-4*, which encodes a forkhead transcription factor that appears to regulate most, and perhaps all, pharyngeal genes (Gaudet and Mango, 2002; Horner et al., 1998). Another example is that of the *Drosophila Pax6/eyeless* gene, which encodes a paired homeodomain transcription factor that is crucial for eye development (Quiring et al., 1994). Dramatically, *Pax6/eyeless* induces extra eyes when expressed ectopically (Halder et al., 1995). Our data suggest that *hnd-1* does not fit into the organ selector model. Although loss of *hnd-1* function can cause the complete loss of gonadal development, global expression of *hnd-1* did not induce ectopic gonadal development. Therefore, gonad 'identity' in *C. elegans* might rely on the coordinate regulation of several genes. Based on our analysis of *hnd-1*/*ehn* double mutants, the *ehn* genes represent likely candidates for additional regulators of the gonadal fate. Similarly, during *Drosophila* salivary gland development, several genes, including the winged-helix transcription factor encoded by *fork head* (*fkh*), are independently regulated and required for the development of specific salivary gland cell types (reviewed by Bradley et al., 2001). Interestingly, one aspect of *fkh* function is to inhibit apoptosis in the salivary gland primordia (Myat and Andrew, 2000). Therefore, like *hnd-1*, *fkh* acts after the salivary gland primordia are specified and is required for the survival of specific salivary gland cell types. We suggest that the role of *hnd-1* is to maintain the somatic gonadal fate and thereby prevent the death of the somatic gonadal precursors. It remains to be seen whether the maintenance of cell fate and cell survival are intimately linked during the development of other organs.

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