TRA-1/GLI controls development of somatic gonadal precursors in *C. elegans*

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Summary

TRA-1/GLI is best known as a master regulator of sex determination in the nematode *C. elegans*, but its fly and vertebrate homologs (e.g. Ci, GLI) regulate embryonic patterning and cell proliferation. In this paper, we show that TRA-1/GLI controls development of the two somatic gonadal precursors (SGPs) in both XX and XO animals, in addition to its role in sex determination. Normally, SGPs reside at the poles of the gonadal primordium and divide according to intrinsic gonadal axes. In *tra-1*-null mutants, however, SGPs assume non-polar positions and the polarity of one SGP is reversed. Consistent with its SGP function, TRA-1 protein is present in SGPs during embryogenesis and early larval development. Previous studies have shown that the *ehn-3* gene also affects SGP positions, and we report here that *tra-1* and *ehn-3* interact genetically. Whereas SGPs in *tra-1* and *ehn-3* single mutants are largely normal and generate many descendants, those in *tra-1; ehn-3* double mutants do not mature or divide. Furthermore, *tra-1* is a dominant enhancer of the *ehn-3* gonadal defect, which includes the enhancement of a weak sexual transformation in the gonad. We cloned *ehn-3*, and found that it encodes a C2H2 zinc-finger protein. A rescuing EHN-3::GFP reporter is predominantly nuclear and expressed specifically in SGPs. The EHN-3 protein is therefore likely to regulate gene expression. We propose that TRA-1/GLI and EHN-3 have overlapping roles in regulation of multiple steps of SGP development. We speculate that regulation of SGP development may be an evolutionarily ancient role of TRA-1/GLI in nematode development.

Key words: TRA-1, GLI, EHN-3, Cell polarity, Cell proliferation, Gonadogenesis, *C. elegans*

Introduction

TRA-1 is the single *C. elegans* homolog of the Ci/GLI family of zinc-finger transcription factors (Zarkower and Hodgkin, 1992). In flies and vertebrates, GLI acts in the *hedgehog* signal transduction pathway, which controls cell proliferation and pattern formation (reviewed by Ingham and McMahon, 2001). By contrast, in *C. elegans*, TRA-1/GLI acts in a highly divergent *hedgehog*-related pathway to control sex determination, and specifically to promote female fates (Aspöck et al., 1999; Hodgkin, 1987; Kuwabara et al., 2000; Zarkower and Hodgkin, 1992). Indeed, TRA-1/GLI also promotes female fates in *Pristionchus pacificus*, a nematode that has diverged from *Caenorhabditis elegans* by 200-300 million years (Pires-daSilva and Sommer, 2004). Therefore, its function as a sex-determination regulator is likely to be ancient in nematode evolution. We report that TRA-1/GLI is also a key regulator of somatic gonadal precursor cells (SGPs) as part of a common gonadogenesis program controlling SGPs in both XX and XO animals.

In *C. elegans*, XX embryos develop as hermaphrodites (essentially females that transiently make sperm), and XO embryos develop as males (Hubbard and Greenstein, 2000). Most adult tissues are sexually dimorphic. Of particular importance to this paper are the gonads: hermaphrodite adults have two U-shaped gonadal arms that are related to each other by two-fold rotational symmetry, but males have only a single J-shaped gonadal arm that is asymmetric. Each gonadal arm, whether hermaphrodite or male, possesses a proximodistal axis that is established in early gonadogenesis. At the distal end of each arm reside either one (hermaphrodites) or two (males) distal tip cells, which govern germline proliferation; more proximal are the germline and sex-specific somatic gonadal structures (e.g. uterus in hermaphrodites, seminal vesicle in males). The proximodistal axis of the gonad departs from the anteroposterior body axis and instead reflects coordinates internal to the organ.

When a wild-type first stage larva (L1) hatches from its eggshell, it possesses a four-celled gonadal primordium with two somatic gonadal precursors (SGPs) and two primordial germ cells (PGCs) (Hubbard and Greenstein, 2000). The anterior and posterior SGPs (Z1 and Z4, respectively) reside at the poles of the primordium and the PGCs are cradled

between them (Fig. 1A). This primordium assembles during embryogenesis and appears identical in the two sexes. The genetic control of SGP development and assembly of the gonadal primordium is not well understood. We previously described three genes that affect SGP development. The *hnd-1* gene encodes a Hand bHLH transcription factor and influences SGP survival and SGP position within the primordium; the *ehn* genes are genetic enhancers of *hnd-1* (Mathies et al., 2003). The vertebrate Hand transcription factor has been implicated in controlling development of several organs, including the gonad in zebrafish (Weidinger et al., 2002).

The SGPs divide asymmetrically to generate daughters with different fates. Their polar daughters generate distal tip cells (DTCs), and their central daughters give rise to a cell with the potential to become an anchor cell (AC) in hermaphrodites or a linker cell (LC) in males (Kimble and Hirsh, 1979). This asymmetric division requires components of a Wnt/MAPK signal transduction pathway (Miskowski et al., 2001; Siegfried and Kimble, 2002; Sternberg and Horvitz, 1988). How the SGPs are directed to divide with opposite polarities within the gonadal primordium is not known.

The *tra-1* gene is best known for its role in specifying female fates: XX *tra-1* null mutants are sexually transformed from hermaphrodite to male (Meyer, 1997). In addition, *tra-1* affects gonadogenesis: 20-50% of both XX and XO null mutants possess small and variably mis-shapen gonads (Hodgkin, 1987; Schedl et al., 1989). However, the role of *tra-1* in gonadogenesis had not been explored. In this paper, we demonstrate that *tra-1* controls both SGP position and polarity in XX and XO animals, and that TRA-1 protein is expressed in SGPs in male and hermaphrodite gonadal primordia. We also find that *tra-1* acts redundantly with *ehn-3* to control SGP maturation and division. Finally, we report that *ehn-3* encodes a nuclear zinc-finger protein that is expressed specifically in the SGPs. We conclude that TRA-1/GLI and EHN-3 are key regulators of SGP development. This role for TRA-1 in the control of SGP development is a major departure from its better-known role in sex determination, and may be an ancient role of TRA-1/GLI in nematode development.

Materials and methods

Strains

Animals were grown at 20°C. All strains were derivatives of Bristol strain N2 (Sulston and Horvitz, 1977). The following mutations are described by Hodgkin (Hodgkin, 1997) or cited references.

LGII: *ehn-1(q690)* (Mathies et al., 2003), *tra-2(ar221ts)*

LGIII: *dpy-18(e364)*, *tra-1(e1099)*, *tra-1(e1834)*, *unc-119(ed3)*, *eDp6*

LGIV: *ced-2(e1752)*, *ced-3(n717)*, *dpy-13(e184sd)*, *ehn-3(q689)* (Mathies et al., 2003), *unc-5(e53)*, *nDf41*, *mDf4*, *fem-3(e1996)*

LGV: *fog-2(q71)*, *him-5(e1490)*

LGX: *hnd-1(q740)* (Mathies et al., 2003), *xol-1(y9)*

Dominant GFP balancers: *hT2[qIs48]* for *LGI* and *LGIII*, and *nT1[qIs50]* for *LGIV* and *LGV*.

Molecular markers: *qIs47* [X-linked GFP], *qIs55* [*hnd-1(N)::GFP*] (Mathies et al., 2003), *qIs56* [*lag-2::GFP*] (Blelloch et al., 1999), *qIs61* [*pes-1::GFP*] (Molin et al., 2000), *qIs74* [*GFP::POP-1*] (Siegfried et al., 2004), *qIs76* [*tra-1::GFP*], a transcriptional reporter of tra-1 expression (Chang et al., 2004), *qIs77* [*unc-122::GFP*] (Miyabayashi et al., 1999), and *rdEx1* [*GFP::TRA-1*], a translational TRA-1 reporter with partial rescuing activity.

Phenotypic analysis and laser ablation

XO *tra-1* males were identified as non-GFP animals from a cross of *tra-1(e1099)/hT2[qIs48]* females to *tra-1(e1099)/hT2[qIs48]; qIs47* XO males. *qIs47* is an X-linked GFP marker and *hT2[qIs48]* is a GFP balancer chromosome. *tra-1/hT2[qIs48]* females were generated using *fog-1(RNAi)* (Jin et al., 2001). Cell lineages were followed as described (Sulston and Horvitz, 1977). Laser ablations were performed using a Micropoint Ablation Laser System (Photonic Instruments) as described (Bargmann and Avery, 1995). For SGP daughter isolation, Z1 or Z4 was ablated, the animal rescued, then one SGP daughter ablated. All ablations were validated. The fate of the remaining SGP daughter was assayed in L3-L4 using *lag-2::GFP*.

tra-1 and *ehn-3* RNAi

Double-stranded RNA was synthesized using Megascript T7 kit (Ambion) and injected at 1 mg/ml. Template for RNA synthesis contained over 500 nucleotides and targeted all *ehn-3* and *tra-1* transcripts.

ehn-3 molecular biology

We mapped *ehn-3* between *dpy-13* and *unc-5* on chromosome *IV*: 1/34 Unc non-Dpy animals from *ehn-3(q689)/unc-5 dpy-13* were Ehn; *mDf4* complemented and *nDf41* failed to complement *ehn-3(q689)*. We cloned *ehn-3* by transformation rescue. Two independent transgenic lines carrying a PCR-generated fragment from 1817 bp upstream to 1024 bp downstream of the predicted ZK616.10-coding region rescued *ehn-3(q689)* completely (n=66). Using RT-PCR with the SL1 trans-spliced leader, we confirmed the predicted gene structure; this transcript is *ehn-3B1*. We also identified a minor splice variant (*ehn-3B2*) that skips the second exon. The entire rescuing region was sequenced from *ehn-3(q689)* genomic DNA, and found to carry only one change 788 bp upstream of the *ehn-3B1* ATG. We isolated an *ehn-3* deletion by a PCR-based screen (Kraemer et al., 1999): *ehn-3(q766)* removes 1074 bp upstream of the *ehn-3B* ATG plus DNA encoding the N-terminal 14 amino acids of EHN-3B. Using semi-quantitative RT-PCR to a region contained in all three transcripts, we examined ZK616.10 expression in *ehn-3* mutants: *hlh-2* was the template control and amplifications were performed within the linear range (32 cycles for *ehn-3*, 27 cycles for *hlh-2*). Both *ehn-3(q689)* and *ehn-3(q766)* mutants expressed about tenfold less ZK616.10 RNA than did wild-type. Since *ehn-3(q689)* appeared to be the stronger allele, we searched for additional transcripts by RT-PCR and found one that included two unpredicted upstream exons trans-spliced to SL1; *ehn-3(q689)* causes a frame-shift mutation in the second exon of this larger transcript (*ehn-3A*); *ehn-3(q766)* removes the second exon and part of the first exon common to *ehn-3A* and *ehn-3B*.

TRA-1 antibody staining

We generated two antisera against the TRA-1 N-terminus: α-TRA-1(DZ) against a peptide (Segal et al., 2001), and α-TRA-1(AS) against a GST fusion to an N-terminal TRA-1 fragment. Both antibodies detected TRA-1 in SGPs of L1 larvae; α-TRA-1(AS) also detected TRA-1 in embryonic SGPs. Recognition was specific, as antigen was not seen in *tra-1(e1834)* larvae (not shown) (Segal et al., 2001). Embryos were fixed by freeze-cracking (Miller and Shakes, 1995), and larvae by a modified Finney-Ruvkun protocol (Finney and Ruvkun, 1990). Primary antibodies were α-TRA-1 (pre-absorbed against *tra-1(e1834); eDp6* fixed worms), α-PGL-1 (Kawasaki et al., 1998) and α-GFP (Clontech). Secondary antibodies were α-rabbit Cy3 and α-mouse FITC (Jackson ImmunoResearch). To distinguish between males and hermaphrodites in L1s, we relied on sexually dimorphic coelomocyte positions: they flank the gonad in males, but are anterior in hermaphrodites. *unc-122::GFP* marked coelomocytes

and *him-5* increased male frequency. We used *tra-2(ts); xol-1* to generate XX L1 pseudomales (Miller et al., 1988), and a *fog-2* male/female strain to produce 50% XO progeny (Schedl and Kimble, 1988).

EHN-3::GFP (pJK939)

The *ehn-3* genomic region, including 429 bp of upstream sequence and all six exons of *ehn-3*, was PCR amplified, cloned into pT7blue (Novagen), sequenced and cloned into pPD95.79 (a gift from A. Fire) to make pJK939; this construct fuses GFP in frame to the last amino acid of EHN-3. pJK939 was injected with pRF4 (Mello et al., 1991) to make *qEx488*, and integrated to make *qIs68*. EHN-3::GFP rescues *ehn-3(q689)* (from 18% to 1% defective, *n*=604) and *tra-1; ehn-3(q766)* double mutants (from 97% to 8% absent gonad, *n*=37).

GFP::TRA-1 (pJK946)

The *tra-1* 3′ end (*Bam*HI to *Apa*I) was cloned from pDZ53 (Zarkower and Hodgkin, 1992) into pJK876 (Chang et al., 2004), replacing GFP and β-gal-coding sequences. GFP-coding sequences were cloned into the *Bam*HI site. Finally, the *tra-1* cDNA 5′ end was cloned downstream of GFP to make GFP::TRA-1. pJK946 was injected with pRF4 (Mello et al., 1991) to make *rdEx1*. *rdEx1* partially rescues *tra-1(e1099)*: rescued animals had a feminized tail and two-armed gonad, but were sterile. GFP::TRA-1 localization mirrors that of TRA-1 antibodies in L1 gonads, but is not localized properly in all tissues. For example, GFP::TRA-1 accumulates in intestinal nuclei of both males and hermaphrodites, while α-TRA-1 antibodies detect TRA-1 protein only in hermaphrodite intestinal nuclei.

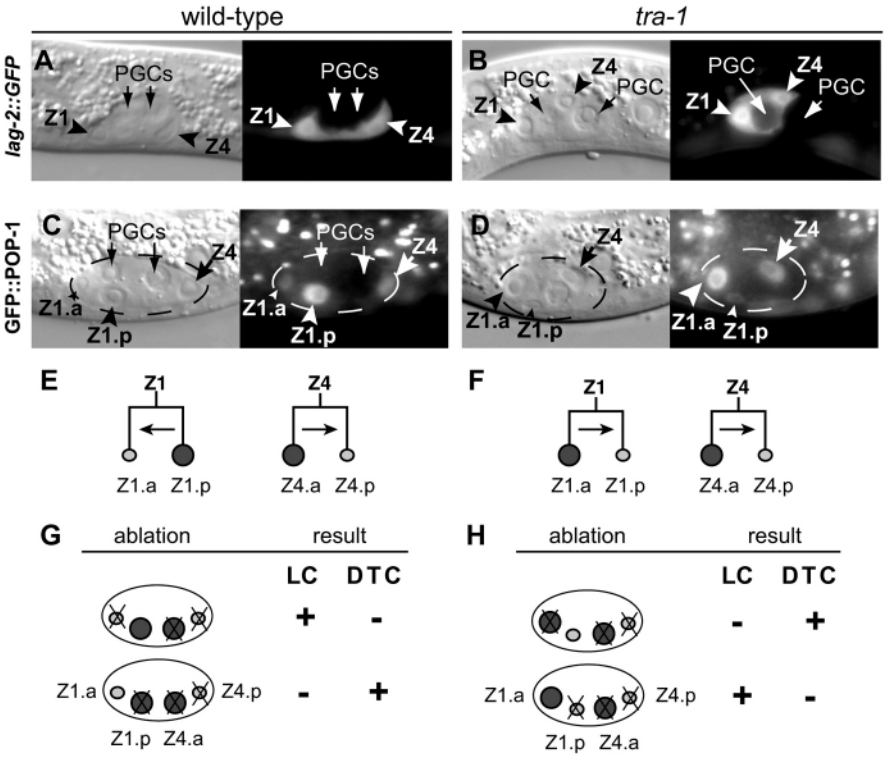

Fig. 1. *tra-1* controls gonadal symmetry. (A-D) Left, DIC images; right, immunofluorescence of same gonad in which (A,B) *lag-2::GFP* marks SGPs (Blelloch et al., 1999) and (C,D) GFP::POP-1 marks SGPs and their daughters. (A) Gonadal primordium in wild-type XO male with SGPs (Z1 and Z4) at poles and PGCs located centrally. (B) Gonadal primordium in *tra-1(e1099)* XX pseudomale, Z4 is dorsal and more anterior than normal. (C) Wild-type XO male, Z1 divides asymmetrically to produce Z1.a, which is smaller and has less nuclear GFP::POP-1 (small arrowhead); and Z1.p, which is larger and has more GFP::POP-1 (large arrowhead), a phenomenon also seen in hermaphrodites (Siegfried et al., 2004). In this gonad, Z4 has not divided. (D) *tra-1(RNAi)* XX pseudomale, polarity of Z1 division is reversed: Z1.a is larger and has more nuclear GFP::POP-1 (large arrowhead), while Z1.p, which is smaller, has less GFP::POP-1 (small arrowhead). In this gonad, Z4 has not divided and is located more dorsal and anterior than normal. (E,F) Z1/Z4 asymmetric divisions. (E) In wild-type males, central daughters (Z1.p/Z4.a, dark) are larger than polar daughters (Z1.a/Z4.p, light). (F) In *tra-1* mutants, the polarity of Z1 division is reversed, so its polar daughter is larger (Z1.a, dark) than its central daughter (Z1.p, light), but the Z4 division has its normal polarity. (G,H) Laser ablation experiments test the fate of SGP daughters. In each experiment, three out of four SGP daughters were ablated (X), leaving one daughter to be assessed for fate. (G) In wild-type males, isolated Z1.p generated a linker cell (LC, *n*=1), while isolated Z1.a generated a distal tip cell (DTC, *n*=1). (H) In *tra-1* mutants, isolated Z1.p generated a DTC (*n*=2), while isolated Z1.a generated a LC (*n*=6).

Results

tra-1 governs SGP position in the L1 gonadal primordium

To understand the *tra-1* defect in gonadogenesis, we began by examining the gonadal primordium in *tra-1* mutants. For these experiments, we used either the *tra-1(e1099)* nonsense mutation or the *tra-1(e1834)* internal deletion, both of which are strong loss-of-function and putative null alleles (Zarkower and Hodgkin, 1992). In both XX and XO animals (Fig. 1; not shown), one or both SGPs were not located in their normal polar position, but instead were located more centrally within the primordium (Fig. 1B). We used *hnd-1::GFP*, an SGP marker expressed in the embryo just prior to and during primordium assembly (Mathies et al., 2003), to determine whether SGPs were properly positioned as the embryonic gonadal primordium assembles. In *tra-1* embryos, SGPs assembled normally into the primordium (*n*=15). Therefore, SGP mis-positioning in *tra-1* L1 primordia is not due to a defect in primordium assembly. Instead, SGPs must move from or fail to maintain their normal positions. By continuous observation of *tra-1* L1 mutants, we found that Z4 usually migrated anteriorly from its posterior pole (22/24), and that Z1 sometimes moved posteriorly from its anterior pole (7/24). Therefore, *tra-1* has a highly penetrant effect on SGP positions in the L1 gonad. We conclude that *tra-1* is important for maintenance of SGPs at the poles of the primordium.

tra-1 governs polarity of the Z1 division

In wild-type primordia, SGPs divide asymmetrically, producing daughters with distinct sizes and fates (Fig. 1C,E) (Kimble and Hirsh, 1979). Moreover, the polarities of these asymmetric divisions are opposite to each other: smaller SGP daughters are polar and larger ones are central. In *tra-1*

mutants, SGPs divided asymmetrically, but the polarity of the Z1 division was reversed compared with wild type (Fig. 1D,F): the larger daughter was generated at the pole and the smaller daughter was more central (15/17). Thus, *tra-1* regulates the polarity of the Z1 division.

Asymmetry of the SGP division is controlled by Wnt/MAPK signaling (Siegfried and Kimble, 2002), including POP-1, the *C. elegans* TCF/LEF transcription factor (Lin et al., 1995). In wild-type hermaphrodites, the central SGP daughters display more nuclear GFP::POP-1 than their polar sisters (Siegfried et al., 2004). We find that SGP daughters in wild-type males similarly exhibit GFP::POP-1 asymmetry: the larger central daughters have more nuclear GFP::POP-1 than do smaller polar daughters (Fig. 1C, right). In *tra-1* mutants, the polarity of GFP::POP-1 asymmetry was reversed in Z1, such that the larger polar daughter had more nuclear GFP::POP-1 than its smaller central sister (Fig. 1D, right). Therefore, the *tra-1* Z1 polarity reversal affects both cell size and abundance of nuclear GFP::POP-1.

We next asked if the Z1 polarity reversal affected daughter cell fates. To this end, we isolated SGP daughters from other somatic gonadal cells using laser ablation and then assayed fates using functional and morphological criteria. In males, the larger daughter normally generates a linker cell (LC), which is a large, round cell that expresses *lag-2::GFP* brightly and guides gonadal elongation; by contrast, the smaller daughter becomes a male DTC, which is a small elongate cell that stimulates germline proliferation and expresses *lag-2::GFP* dimly (Fig. 1G) (Blelloch et al., 1999). In *tra-1* mutants, the smaller central daughter of Z1 expressed *lag-2::GFP* dimly and stimulated germline proliferation, and the larger polar daughter generated a cell with typical LC morphology that expressed *lag-2::GFP* brightly and led gonadal elongation (Fig. 1H). Therefore, the fate of each Z1 daughter correlated with its size and GFP::POP-1 level. We conclude that the polarity of the Z1 division is fully reversed in *tra-1* mutants. The polarity of Z4, by contrast, was normal in *tra-1* mutants.

TRA-1 localization in SGPs

To assess *tra-1* expression in SGPs, we examined TRA-1 protein using α-TRA-1 antibodies (Segal et al., 2001) (this work) and a GFP::TRA-1 translational reporter (see Materials and methods). In wild-type XX embryos, TRA-1 was predominantly nuclear in SGPs from formation of the primordium (Fig. 2A,B), through embryogenesis (Fig. 2C), and into the first larval stage (Fig. 2D). The nuclear location suggests that TRA-1 is active in SGPs, because the active state of TRA-1 has been correlated with nuclear localization (Segal et al., 2001).

Next, we examined embryos from a *fog-2* male/female strain, which produces 50% XX and 50% XO progeny (Schedl and Kimble, 1988), to determine if TRA-1 is similarly expressed in male SGPs. In twofold embryos, most SGPs possessed nuclear TRA-1 (27/30). Therefore, TRA-1 is present in SGP nuclei of both XX hermaphrodite and XO male embryos, consistent with the idea that TRA-1 is active in SGPs of both sexes.

After hatching, TRA-1 was still detected in SGPs, but its level and subcellular distribution were distinct in the two sexes. In hermaphrodite L1 SGPs, TRA-1 was largely nuclear (Fig. 2E), but in male L1 SGPs, TRA-1 was present at a lower level and was uniformly distributed between nucleus and cytoplasm (Fig. 2F). We observed a similar sexually dimorphic difference

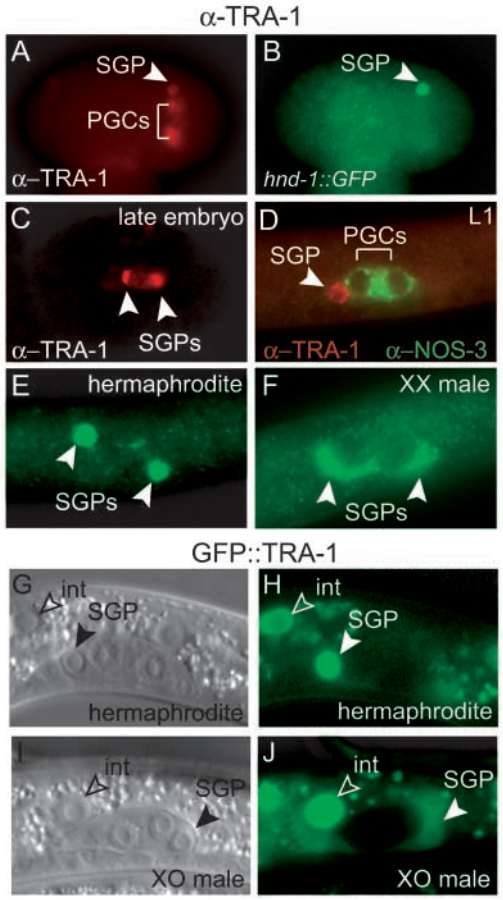

Fig. 2. TRA-1 expressed in embryonic and larval SGPs. (A-F) Embryos or larvae stained with α-TRA-1 antibodies; (A-C,E,F) α-TRA-1(AS) polyclonal antibody; (D) α-TRA-1(DZ) peptide antibody. (A,B) Wild-type XX embryo at ~320 minutes, when gonadal primordium has just formed; this embryo carries a *hnd-1::GFP* reporter to identify SGPs. (A) TRA-1 is present in SGPs (only one is visible) of the newly formed gonadal primordium and appears to be predominantly nuclear. SGP staining is reproducible. PGC staining with α-TRA-1 is variable and may be non-specific. (B) *hnd-1::GFP* identifies SGP shown in A. (C) Wild-type XX threefold embryo with strong nuclear TRA-1 staining. (D) Wild-type XX L1 gonadal primordium. TRA-1 (red) in SGP nuclei (only one is visible); PGCs are identified by α-NOS-3 (green). (E) Wild-type XX L1 gonadal primordium; TRA-1 in SGPs is predominantly nuclear. (F) *tra-2; xol-1* XX pseudo-male L1 gonadal primordium. TRA-1 in masculinized SGPs is expressed at a lower level and is distributed between nucleus and cytoplasm; a similar pattern is observed in wild-type XO males (not shown). (G-J) Larvae expressing a TRA-1 translational reporter (GFP::TRA-1). (G,I) DIC images; (H,J) immunofluorescence. (G,H) Wild-type L1 XX hermaphrodite. GFP::TRA-1 is expressed in SGPs (only one is visible) and intestinal cells (int). This rescuing fusion protein is predominantly nuclear. (I,J) Wild-type L1 XO male; GFP::TRA-1 is expressed at a lower level in male SGPs than in hermaphrodite SGPs, and it is distributed through both nucleus and cytoplasm. The exposure in J is longer than that of H to highlight SGP cytoplasmic expression. Expression of GFP::TRA-1 in intestinal cells is not regulated in a sex-specific manner; it therefore serves as a measure of relative exposure between H and J.

in the distribution of GFP::TRA-1 in L1 SGPs (Fig. 2G-J). The GFP::TRA-1 reporter partially rescued *tra-1(0)* mutants, indicating that it produces a functional protein (see Materials and methods). After the first SGP division, TRA-1 continues to be expressed in hermaphrodite gonads, but is no longer detectable in males (not shown). Therefore, after hatching, TRA-1 is predicted to be more active in hermaphrodite gonads than in male gonads, consistent with its role in promoting female fates.

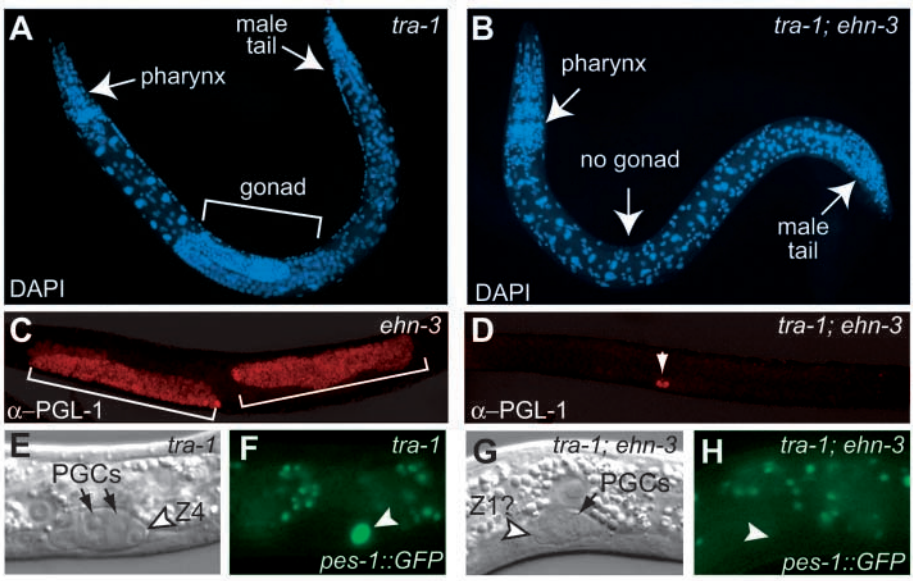

Fig. 3. *tra-1* and *ehn-3* redundantly control SGP development. (A,B) Young adults, DAPI stained; male tail and pharynx are indicated. (A) *tra-1(e1099)* single mutant with a normally shaped male gonad (bracket). (B) *tra-1(e1099); ehn-3(q766)* double mutant with no visible gonad (arrow). (C,D) L4/young adults, stained with antibodies to germ cell marker PGL-1. (C) *ehn-3(q766)* single mutant with normal two-armed gonad (brackets). Actual genotype is *tra-1/+; ehn-3*. (D) *tra-1(e1099); ehn-3(q766)* double mutant with only a few germ cells. (E-H) L1 larvae expressing *pes-1::GFP*, an SGP-marker (Molin et al., 2000). (E,G) DIC; (F,H) immunofluorescence of same animal shown in E and G, respectively. (E,F) *tra-1(e1099)* mutant L1. (E) SGP (Z4, white arrow) has typical morphology. (F) Z4 expresses *pes-1::GFP*; (G,H) *tra-1(e1099); ehn-3(q766)* double mutant L1. (G) A small cell is associated with PGCs (white arrow); (H) this small cell does not express *pes-1::GFP*.

tra-1 and *ehn-3* redundantly control gonadal development

The *hnd-1*, *ehn-1* and *ehn-3* genes affect SGP position in a manner reminiscent of *tra-1*: in *hnd/ehn* mutants, SGPs are assembled correctly into the primordium, but they can lose their polar position (Mathies et al., 2003). We examined double mutant combinations of *tra-1* with *hnd-1*, *ehn-1* or *ehn-3* to explore their relationships. To our surprise, *tra-1; ehn-3* double mutants had a striking synergistic effect on gonadogenesis: whereas most *tra-1* and *ehn-3* single mutants possessed easily detectable gonadal tissue by Nomarski microscopy and DAPI staining (Fig. 3A), very few *tra-1; ehn-3* double mutants had a detectable gonad (Fig. 3B; Table 1). We examined the gonad in more detail using PGL-1, a germ cell marker that detects many cells in wild-type (Kawasaki et al., 1998), *ehn-3* (Fig. 3C) and *tra-1* late larval gonads. By contrast, *tra-1; ehn-3* double mutants consistently had only a few PGL-1-positive cells (Fig. 3D). The interaction appears specific to *tra-1* and *ehn-3*, as we observed only a weak synergistic interaction for *tra-1; hnd-1* and *tra-1; ehn-1* double mutants (Table 1). We conclude that *tra-1* and *ehn-3* have an overlapping role in generation of gonadal tissue.

Because *tra-1* is a sex-determining gene, we asked whether mutations in other sex-determining genes might interact genetically with *ehn-3*. Specifically, we used *fem-3* and *tra-2* to represent feminizing and masculinizing activities, respectively (Meyer, 1997). If production of gonadal tissue is controlled by the sex-determination pathway, one might expect *tra-2* (like *tra-1*) to have a synergistic effect with *ehn-3*, and *fem-3* to have an antagonistic effect. However, no genetic interaction was observed in either *tra-2; ehn-3* or *ehn-3 fem-3* double mutants (Table 1). We conclude that *tra-1* acts independently of the sex determination pathway to control generation of gonadal tissue.

SGPs in *tra-1; ehn-3* double mutants

Why do *tra-1; ehn-3* double mutants have so little gonadal tissue? An examination of L1 larvae by Nomarski microscopy revealed the probable cause – cells with morphology typical of SGPs were not seen (n=16). Whereas all *tra-1* and *ehn-3* single mutants had normal SGPs (Fig. 3E) (Mathies et al., 2003), *tra-1; ehn-3* double mutants had either one or two tiny cells associated with PGCs (Fig. 3G). To determine whether the tiny PGC-associated cells were indeed SGPs, we used *pes-1::GFP* and *lag-2::GFP*, which are molecular SGP markers (Blelloch et al., 1999; Molin et al., 2000). The SGPs in *tra-1* single mutants expressed *pes-1::GFP* (Fig. 3F, 19/20), as did SGPs in *ehn-3* single mutants (53/53). However, the tiny cells in *tra-1; ehn-3* double mutants did not detectably express *pes-1::GFP* (Fig. 3H; 0/16) or *lag-2::GFP* (not shown). The possibility of faint *lag-2::GFP* expression by the tiny PGC-

Table 1. Genetic interactions between *tra-1* and *hnd-1/ehn*

Genotype	Gonad (%)* Present	Absent	*n*
tra-1(e1099) XX	100	0	282
ehn-3(q766) XO	98	2	41
tra-1(e1099); ehn-3(q766) XX	7	93	56
ehn-3(q689) XO	97	3	88
tra-1(e1099); ehn-3(q689) XX	6	94	94
tra-2(e1095); ehn-3(q689) XX	98	2	213
ehn-3(q689) fem-3(e1996) XX	98	2	56
hnd-1(q740) XO	97	3	88
tra-1(e1099); hnd-1(q740) XX	87	13	90
ehn-1(q690) XO	99	1	134
ehn-1(q690); tra-1(e1099) XX	88	12	179

*Gonadal morphology scored by DIC optics.

associated cells could not be ruled out, because of bright expression in adjacent ventral neurons. However, we searched for *lag-2::GFP* cells with SGP morphology elsewhere in the animal and did not find them. Similarly, *pes-1::GFP*-expressing cells were not found ectopically. We conclude that typical SGPs are missing from *tra-1; ehn-3* L1 larvae.

To determine whether SGPs were generated in *tra-1; ehn-3* embryos, we used *hnd-1::GFP*, which is first expressed in SGPs as they assemble into the gonadal primordium (Mathies et al., 2003). In *tra-1(RNAi); ehn-3(q689)* embryos, two *hnd-1::GFP*-expressing SGPs were generated as normal, and they assembled into the gonadal primordium (n=11). Therefore, *tra-1; ehn-3* SGPs express SGP-specific markers in embryos, but not in L1 larvae.

One simple explanation for the missing SGPs might have been cell death. The *hnd-1* gene is crucial for SGP survival (Mathies et al., 2003), and *ehn-3* mutants enhance the SGP survival defect of *hnd-1(RNAi)* (L.M., unpublished). We used cell death mutants to visualize cell corpses or prevent programmed cell death in *tra-1; ehn-3* mutants. SGP corpses are easily seen in *ced-2* mutants, which are defective in corpse engulfment (Ellis et al., 1991; Mathies et al., 2003), and cells that normally die by programmed cell death are able to survive in *ced-3* mutants, which are defective in programmed cell death (Ellis and Horvitz, 1986; Yuan et al., 1993). We examined newly hatched *tra-1; ehn-3(RNAi); ced-2* L1 animals, but found no gonad-associated corpses (n=35), and we examined *tra-1; ehn-3(RNAi); ced-2 ced-3*, but found no increase in SGP number (n=15). Therefore, SGPs do not appear to undergo programmed cell death in *tra-1; ehn-3* mutants.

We suggest that the tiny PGC-associated cells in *tra-1; ehn-3* mutants are, in fact, the missing SGPs. This idea is based on three lines of evidence. First, SGPs are born and assembled into the primordium normally in the embryo. Second, SGPs did not appear to die or be ectopically placed. Third, the tiny PGC-associated cells are in the right position and number to be SGPs (most animals had one or two tiny cells, but the cells were so small that one may have been missed). Therefore, we consider these tiny PGC-associated cells to be aberrant SGPs. We followed the aberrant SGPs of *tra-1; ehn-3* mutants during the first three larval stages (L1-L3). During this interval, wild-type SGPs generate 10-12 daughter cells, depending on sex. The aberrant SGPs, however, did not grow in size or divide, but they did remain associated with the germ cells (n=2). Therefore, the SGPs appear to be present in *tra-1; ehn-3* mutants, but they fail to enlarge, express L1-specific SGP markers and divide.

Table 2. *tra-1* mutations dominantly enhance *ehn-3*

Genotype	Gonadal morphology (%)*		
	Wild type	Abnormal†	*n*
tra-1(e1099)/+	100	0	282
ehn-3(q766)	97	3	187
tra-1(e1099)/+; ehn-3(q766)	86	14	161
ehn-3(RNAi)	90	10	103
tra-1(e1099)/+; ehn-3(RNAi)	40	60	120
ehn-3(q689)	82	18	1031
tra-1(e1099)/+; ehn-3(q689)	35	65	254
ehn-3(q689) fem-3(e1996)/ehn-3(q689)	81	19	195
tra-2(e1095)/+; ehn-3(q689)	74	26	156

*Gonadal morphology scored by DIC optics.
†Includes single-armed, abnormal morphology, and absent gonads.

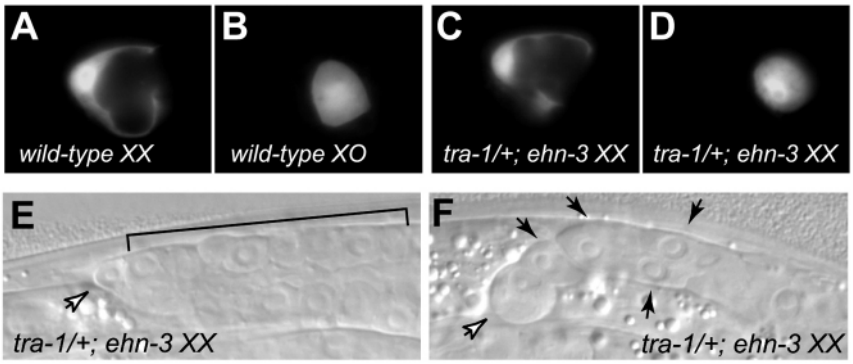

Fig. 4. Gonadal masculinization in *tra-1/+; ehn-3*. (A-D) Gonadal leader cells marked with *lag-2::GFP* (Blelloch et al., 1999). (A) Wild-type XX hermaphrodite leader cell (DTC) is crescent shaped. (B) Wild-type XO male leader cell (LC) is round. (C,D) *tra-1/+; ehn-3(q689)* XX leader cells. (C) Leader cell with typical DTC crescent shape (most common). (D) Leader cell with typical LC round shape. A similar low-penetrance gonadal masculinization was also observed in *tra-1/+; ehn-3(RNAi)*. (E,F) *tra-1/+; ehn-3(q689)* XX larvae, DIC. (E) Gonadal arm with normal hermaphrodite organization: DTC (white arrow) is adjacent to germ cells (bracket) and separated from other somatic gonadal cells (out of photo). (F) Gonadal arm with male organization: LC (white arrow) is adjacent to somatic gonadal cells (black arrows) and separated from germ cells.

A dose sensitive interaction between *tra-1* and *ehn-3*

In constructing *tra-1(e1099); ehn-3(q689)*, we found that *tra-1* is a dominant enhancer of the *ehn-3* gonadogenesis defects. Only 18% of *ehn-3(q689)* mutants had gonadal defects and none were seen in *tra-1(e1099)/+* heterozygotes; however, 65% of *tra-1(e1099)/+; ehn-3(q689)* mutants had defects (Table 2). A similar, but less dramatic, effect was seen for *ehn-3(q766)* (Table 2). To determine whether other sex determination regulators behaved similarly, we examined *ehn-3(q689)* for enhancement by *tra-2/+* and suppression by *fem-3/+*, but found no striking interaction (Table 2). We conclude that gonadogenesis is sensitive to *tra-1* dosage in *ehn-3* mutants.

While examining *tra-1(e1099)/+; ehn-3(q689)* mutants, we noticed a low-penetrance gonadal masculinization (Fig. 4). In wild-type hermaphrodites, the leader cell, called a distal tip cell (DTC), is crescent-shaped (Fig. 4A) and resides next to germ cells (Fig. 4E), whereas the male leader cell, called a linker cell (LC), appears round and lies next to somatic gonadal cells (Fig. 4B). We examined leader cell shape using *lag-2::GFP*, which is strongly expressed in DTCs and LCs. In *tra-1(e1099)/+; ehn-3(q689)* hermaphrodites, most leader cells had normal hermaphrodite morphology (Fig. 4C), but some were round and male-like (3/20; Fig. 4D). By contrast, in *tra-1(e1099)/+* heterozygotes, all leader cells had typical hermaphrodite morphology (0/52) and *ehn-3(q689)* homozygotes had a very low-penetrance masculinization (3/486). Masculinized leader cells were also seen in *tra-1(e1099)/+; ehn-3(RNAi)* (7/64), suggesting that sexual transformation is due to loss of *ehn-3*

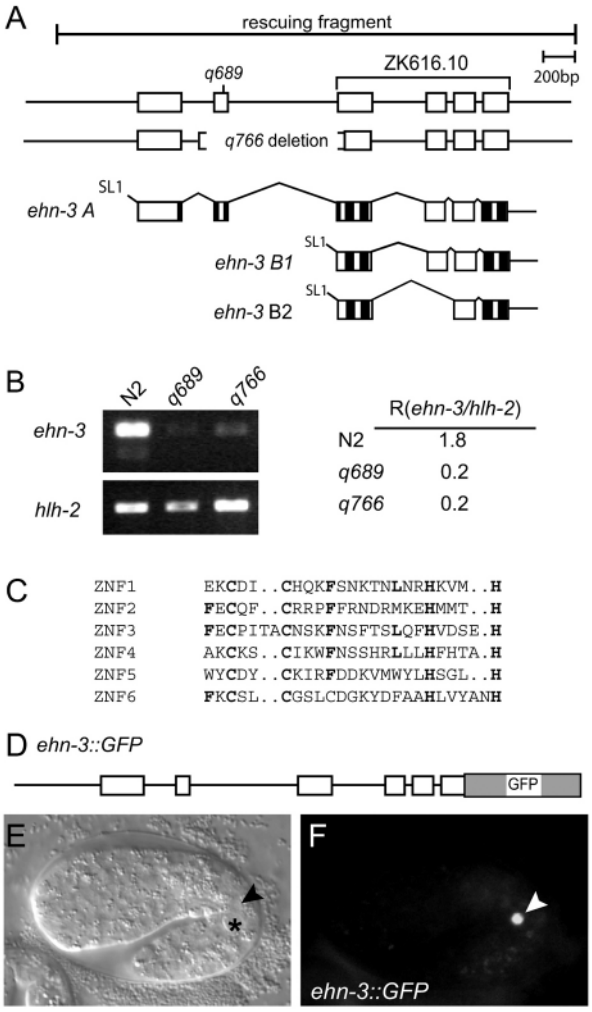

Fig. 5. *ehn-3* encodes an SGP-specific protein with paired zinc fingers. (A) The *ehn-3* locus. cDNA analyses identify three transcripts, each predicted to encode a protein with multiple zinc fingers (black boxes). All cDNAs are transpliced to the SL1 leader. *ehn-3A* encodes six zinc fingers; *ehn-3B1* and *B2* encode four zinc fingers. The gene predicted by the sequence consortium, called ZK616.10, corresponds to *ehn-3B*. *ehn-3(q689)* is associated with an insertion (T) and a C→T transition in exon 2 of *ehn-3A*. *ehn-3(q766)* deletes a region including exon 2 from *ehn-3A*, the next intron and part of the first common exon, including coding sequence for the first 14 amino acids of EHN-3B1 and B2. (B) Semi-quantitative PCR used to assess transcript levels in *ehn-3* mutants. (Left) *ehn-3* PCR products were less abundant when amplified from *ehn-3* mutants than from wild type (lower band in N2 is *ehn-3B2*); by contrast, *hlh-2* PCR product was similar in wild-type and *ehn-3* mutants. (Right) Ratio of *ehn-3* to *hlh-2* PCR products. (C) Alignment of EHN-3 putative zinc fingers. ZNF1-ZNF3 and ZNF6 were predicted by Pfam; ZNF4 and ZNF5 were identified by eye. (D-F) SGP-specific expression of EHN-3::GFP. (D) EHN-3::GFP transgene composition: *ehn-3* genomic DNA, including 429 bp of 5′ flanking sequence plus the entire EHN-3-coding region, was fused in frame to the GFP-coding sequence. (E) Wild-type embryo expressing EHN-3::GFP. SGP (arrow) has assembled into the gonadal primordium; PGC, asterisk. (F) EHN-3::GFP is expressed in SGP (arrowhead) shortly after gonadal primordium is formed.

function. Moreover, the *tra-1(e1099)/+; ehn-3(q689)* male-like leader cells were often adjacent to other somatic gonadal cells and separated from germ cells (Fig. 4F). We have not seen masculinization of non-gonadal tissues (e.g. tail), suggesting a gonad-specific effect. The *tra-1* enhancement of *ehn-3* masculinization raises the possibility that *ehn-3* and *tra-1* normally work together to specify gonadal sexual fate.

EHN-3 is a nuclear Zn finger protein in embryonic SGPs

We cloned *ehn-3* to gain molecular insight into its role in gonadogenesis and its genetic interaction with TRA-1. Briefly, *ehn-3* includes the predicted gene ZK616.10 (Fig. 5). Four lines of evidence support this identification. First, the ZK616.10 genomic region rescued *ehn-3* (Fig. 5A, top; see Materials and methods). Second, RNAi to ZK616.10 mimics the *ehn-3* mutant phenotype and acts synergistically with *tra-1* (Table 2). Third, a ZK616.10::GFP rescuing transgene is expressed specifically in embryonic SGPs (see below), a time and place consistent with *ehn-3* function. Finally, the *ehn-3(q689)* allele bears a frame shift mutation in an unpredicted upstream exon (see below) and *ehn-3(q766)* deletes part of ZK616.10 (Fig. 5A). Both mutations dramatically reduce expression of the ZK616.10 transcript, and are likely to be loss-of-function mutations (Fig. 5B).

Analysis of *ehn-3* cDNAs by RT-PCR revealed three *ehn-3* transcripts that generate proteins with paired C2H2 zinc fingers and no other recognizable motif (Fig. 5C). The longer isoform (EHN-3A) is predicted to have six zinc fingers, while the shorter isoforms (EHN-3B1 and EHN-3B2) encode four zinc fingers; the B1 and B2 isoforms differ only in number of amino acids between their paired zinc fingers (99 for B1; 51 for B2; Fig. 5A). The *ehn-3(q689)* allele causes an early termination of EHN-3A, while *ehn-3(q766)* removes one *ehn-3A*-specific exon, a large intron and part of the first exon common to all three *ehn-3* transcripts (Fig. 5A). *ehn-3(q689)* is phenotypically more severe than *ehn-3(q766)*, both when assayed over a deficiency (Table 3) and with *tra-1(0)/+* (Table 2). In addition, *ehn-3(RNAi)* resembles *ehn-3(q689)* when assayed with *tra-1(0)/+* (Table 2). Therefore, we suggest that *ehn-3(q689)* is a strong loss-of-function allele.

The presence of zinc fingers in EHN-3 suggests a role in controlling gene expression. To learn where EHN-3 is expressed, we constructed EHN-3::GFP, a reporter transgene with the *ehn-3* 5′ flanking region, all exons and introns, and GFP fused in frame to the C terminus of all EHN-3 isoforms (Fig. 5D). This reporter rescued *ehn-3* and *tra-1; ehn-3* double mutants (see Materials and methods), suggesting that its

Table 3. *ehn-3* allelic series

Genotype	Gonadal morphology (%)*		
	Wild type	Abnormal†	*n*
ehn-3(q766)	97	3	187
ehn-3(q766)/nDf41	96	4	100
ehn-3(q766)/ehn-3(q689)	89	11	132
ehn-3(q689)‡	82	18	1031
ehn-3(q689)/nDf41‡	74	26	197

*Gonadal morphology scored by DIC optics.
†Includes single-armed, abnormal morphology, and absent gonads.
‡Mathies et al., 2003.

expression reflects that of the endogenous gene. EHN-3::GFP was first seen shortly after assembly of the gonadal primordium (Fig. 5E,F), was faint at the twofold stage of embryogenesis and disappeared by hatching (data not shown). Furthermore, EHN-3::GFP was largely nuclear (Fig. 5F). Based on its paired zinc fingers, nuclear location and SGP-specific expression, we suggest that EHN-3 is likely to be an SGP-specific regulator of gene expression.

Interdependence of *tra-1*, *hnd-1* and *ehn-3* expression

The *hnd-1*, *tra-1* and *ehn-3* genes are expressed in SGPs at overlapping, but different, times of embryogenesis (Fig. 6; this work) (Mathies et al., 2003). Because all three encode putative transcription factors, we asked if any of them might regulate expression of the others. As described above and in a previous work (Mathies et al., 2003), the *hnd-1::GFP* transcriptional reporter was expressed normally in *tra-1* and *ehn-3* mutants. Similarly, the EHN-3::GFP translational reporter was expressed in all SGPs in wild-type (Fig. 5D,E) and *tra-1* embryos (*n*=20). In *hnd-1* embryos, about half the normal number of SGPs expressed EHN-3::GFP (*n*=47), consistent with the reduced number of SGPs seen in *hnd-1* L1s. We conclude that the expression of *hnd-1* and *ehn-3* appear to be initiated independently of each other and of *tra-1*.

tra-1 expression was reduced in *ehn-3(q689)* mutants when compared with wild type. Using either TRA-1 antibodies or a *tra-1::GFP* transcriptional reporter (Chang et al., 2004), about a third of the *ehn-3(q689)* SGPs failed to express *tra-1* in L1 larvae (*tra-1::GFP*, 24/76; TRA-1, 19/60). By contrast, no difference was found in the expression of a *tra-1::GFP* transcriptional reporter between wild-type (Chang et al., 2004) and *hnd-1* SGPs (*n*=34). Therefore, *ehn-3* appears to be important for *tra-1* expression.

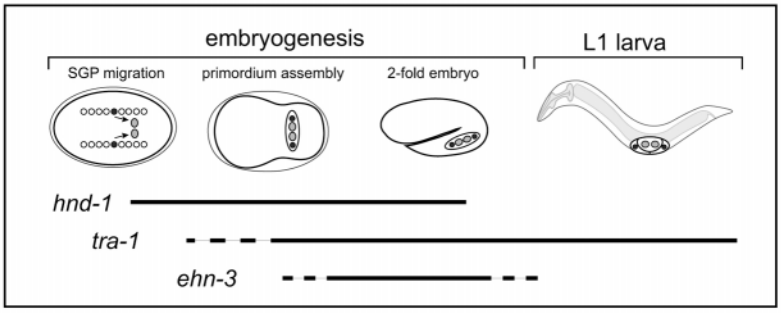

Fig. 6. Overlapping expression of *ehn-3*, *tra-1* and *hnd-1* in embryonic SGPs. *hnd-1*, *tra-1* and *ehn-3* are expressed in the SGPs at different, but overlapping, times during embryogenesis. *hnd-1::GFP* is first detected in SGPs migrating towards germ cells and disappears about the two-fold stage of embryogenesis. TRA-1 is detected in newly formed gonadal primordium and may be expressed earlier in migrating SGPs (M.S. and A.S., unpublished). TRA-1 continues expression in SGPs during embryogenesis and into the first larval stage, when its level and distribution are modulated in the two sexes. EHN-3::GFP is expressed exclusively in SGPs shortly after gonadal primordium forms; it disappears by the threefold stage of embryogenesis.

Discussion

In this paper, we demonstrate that the *C. elegans* TRA-1/GLI transcription factor is a key regulator of SGP development and gonadal symmetry in both XX and XO animals. TRA-1 controls SGP position and polarity within the gonadal primordium, and therefore is crucial for establishing gonad-specific axes. In addition, TRA-1 acts redundantly with another zinc-finger protein, called EHN-3, to promote SGP maturation and division. We speculate that SGP regulation may be an evolutionarily ancient role of TRA-1/GLI in nematode development.

TRA-1 controls symmetry in the gonadal primordium

The wild-type *C. elegans* gonadal primordium is symmetrical, and that symmetry is maintained in both sexes through the first SGP division (Kimble and Hirsh, 1979). Thus, the two SGPs reside at the poles of the primordium, and they divide with opposite polarity to establish the opposing gonadal axes. The more central SGP daughters are larger and have proximal fates, while polar daughters are smaller and have distal fates. In *tra-1* mutants, the gonadal primordium is not symmetrical: *tra-1* SGPs often move from their normal polar positions, and they divide with the same polarity with respect to the anteroposterior axis. Thus, their anterior SGP daughters are larger and have proximal fates, while their posterior daughters are smaller and have distal fates. This same defect is seen in XX and XO *tra-1* mutants. Therefore, in wild-type animals, the activity of TRA-1 controls symmetry of the gonadal primordium.

The molecular mechanism by which TRA-1 governs gonadal symmetry is not known. One possibility is that the TRA-1 transcription factor regulates expression of specific cellular constituents in the SGPs that generate internal signals to create a gonad-specific polarity. For example, TRA-1 might direct assembly of a junction between Z1 and Z4 that polarizes these two cells with respect to each other rather than the anteroposterior axis. Now that TRA-1 has been identified as a key regulator of SGP position and polarity, it will be possible to identify target genes governing gonadal polarity and to learn the molecular mechanism by which proximodistal axes are organized within the early gonad.

Is *tra-1* control of gonadal symmetry related to its sex-determining function?

TRA-1 is well known for specifying female fates in *C. elegans* (Meyer, 1997), and female gonadogenesis is a symmetrical process (hermaphrodites are essentially females that make sperm transiently). The gonadal primordium is also symmetrical in males, even though it develops into a one-armed asymmetric gonad. We have found that TRA-1 promotes symmetry in the gonadal primordium of both XX and XO animals. One idea is that the symmetrical primordium represents a female aspect of early gonadogenesis in both sexes. According to this view, TRA-1 control of primordium symmetry is simply another manifestation of its sex determination function to promote female fates.

A symmetrical gonadal primordium is thought to be an ancient feature of nematode gonadogenesis (Chitwood and Chitwood, 1950; Félix and Sternberg, 1996). In virtually all nematodes examined, this same morphology is observed,

despite a range of adult gonadal morphologies. For example, some female nematodes possess asymmetrical adult gonads, but nonetheless gonadogenesis begins with a symmetrical primordium (Félix and Sternberg, 1996). Conversely, some male nematodes possess symmetrical adult gonads (Chitwood and Chitwood, 1950). Therefore, the symmetry or asymmetry of adult gonads is not a conserved sexual trait.

Why might an asymmetric adult gonad, such as that in *C. elegans* males, begin development from a symmetrical primordium? In *tra-1* mutants, the SGPs divide asymmetrically, and produce proximal and distal daughters. However, the positions of those proximal and distal daughters are abnormal in *tra-1* mutants, and the resultant gonads are usually mis-shapen and sterile (Hodgkin, 1987). Perhaps SGP position at the pole of the primordium is crucial for the correct placement of proximal and distal daughters within the developing gonad. That placement appears to be important, but not essential, for generation of mature gonads with appropriate axes. Based on this reasoning, we suggest that TRA-1 control of SGP positions within the gonadal primordium may reflect a primitive role in patterning (see below) rather than in specification of the female fate.

TRA-1 and EHN-3: redundant regulators of SGP maturation

The *tra-1* and *ehn-3* genes act redundantly to promote SGP maturation. In *tra-1* and *ehn-3* single mutants, the SGPs express markers and divide, albeit abnormally. By contrast, in *tra-1; ehn-3* double mutants, the SGPs do not mature and appear arrested in development. Thus, whereas SGPs appear normal in *tra-1; ehn-3* embryos, they are abnormal by hatching: in L1s, they do not express SGP-specific markers, they do not grow in size and they do not undergo further cell divisions. A similar effect on SGP maturation is not observed in either *tra-1* or *ehn-3* single mutants. Therefore, either TRA-1 or EHN-3 is sufficient to promote SGP maturation.

How might TRA-1 and EHN-3 interact to control SGP maturation? The TRA-1/GLI zinc-finger proteins bind DNA and regulate transcription (Alexandre et al., 1996; Chen and Ellis, 2000; Conradt and Horvitz, 1999; Kinzler et al., 1988; Yi et al., 2000; Zarkower and Hodgkin, 1992); EHN-3 similarly possesses zinc fingers and a rescuing EHN-3::GFP reporter protein is predominantly nuclear, suggesting a role for EHN-3 in the nucleus. One simple idea is that TRA-1 and EHN-3 are both transcription factors, and that they are essentially interchangeable for control of SGP maturation and proliferation. Alternatively, TRA-1 and EHN-3 might cooperate to regulate transcription, as suggested for murine GLI3 and ZIC1 (Koyabu et al., 2001). Tests of these molecular mechanisms must await identification of TRA-1/EHN-3 target genes.

In a parallel study, we showed that TRA-1 is redundant with a forkhead transcription factor, FKH-6, for gonadal divisions in both XX and XO animals (Chang et al., 2004). In *fkh-6; tra-1* mutants, the SGPs express L1-specific SGP markers and divide, but their daughters are arrested in development and fail to divide further. This *fkh-6; tra-1* cell division arrest is strikingly similar to the *tra-1; ehn-3* SGP arrest, albeit one division later. These two parallel studies support the idea that TRA-1 regulates proliferation of the SGPs and their descendants. This *tra-1* control is masked by redundancy with *ehn-3* for SGP divisions and with *fkh-6* for later divisions. We do not yet know if the TRA-1 control of proliferation extends to other tissues, a possibility that may also be masked by tissue-specific redundant factors.

TRA-1 regulation of precursor cells may be ancient

The *tra-1* gene encodes the single Ci/GLI homolog in the *C. elegans* genome (Ruvkun and Hobert, 1998; Zarkower and Hodgkin, 1992). In flies and vertebrates, Ci/GLI transcription factors control embryonic patterning and cell proliferation in response to *hedgehog* signaling (Berman et al., 2003; Ingham and McMahon, 2001; Ruiz i Altaba, 1999; Taipale and Beachy, 2001; Thayer et al., 2003). In nematodes, Ci/GLI functions in patterning and proliferation were thought to be lost: instead, TRA-1 appears to have been co-opted for sex determination (Meyer, 1997; Pires-daSilva and Sommer, 2004; Ruvkun and Hobert, 1998). In this paper, we show that TRA-1 is also a key regulator of precursor cells, including SGP position, polarity, maturation and proliferation. These TRA-1 functions are strikingly reminiscent of Ci/GLI functions in embryonic patterning and proliferation in other organisms.

The findings reported in this paper, together with classical studies of *C. elegans* sex determination (Hodgkin and Brenner, 1977), demonstrate that TRA-1 has two major developmental functions. It promotes female development in most tissues, and it controls development of somatic precursor cells in the gonad. The TRA-1 control of SGP positions and polarity can be interpreted as imposing a female symmetry on male gonads, but its control of SGP maturation and division is more difficult to envision as a female character. Furthermore, other sex-determining genes (e.g. *tra-2* and *fem-3*) do not control SGP development, suggesting that the TRA-1 control of SGP development is distinct from its control of sex determination. We suggest that the TRA-1 regulation of precursor cells may be an ancient function. This speculation is based in part on the similarity between TRA-1 and Ci/GLI controls of patterning and proliferation (see above), and in part on the well-documented rapid evolution of sex determining mechanisms (Zarkower, 2001). Indeed, a crucial role for TRA-1 in development of the gonadal precursor cells may have positioned this well-conserved regulator for its evolution to become a sex-determination regulator.

The recruitment of common regulators into sex determination is not unprecedented. The primary *Drosophila* sex-determining regulator, *Sxl*, is a splicing regulator used in both sexes of closely related flies, and *Sry*, the primary sex-determining regulator in mice, appears to have evolved from a transcription factor used in both sexes (reviewed by Zarkower, 2001). The notion that the TRA-1/GLI sex-determining regulator was recruited in *C. elegans* from the *hedgehog* signaling pathway is not new (Ruvkun and Hobert, 1998). However, the idea that TRA-1/GLI may have retained ancient functions in patterning and proliferation departs from the classic view.

Our findings pose two major questions for future studies. First, how does TRA-1 control gonadal symmetry and SGP maturation in *C. elegans*? What are its target genes and how do their products regulate precursor maturation and primordium structure? Second, is the TRA-1-mediated control of SGP development an ancient function? Do nematode GLI homologs generally control SGP development? In light of the

redundancy between *tra-1* and *ehn-3*, the recently described *P. pacificus tra-1* mutations (Pires-daSilva and Sommer, 2004) do not rule out an additional role in SGP development. If the role of TRA-1 in controlling precursor cells is ancient, then analyses of how TRA-1/GLI regulates SGPs in *C. elegans* may provide insights into how GLI homologs control embryonic pattern and proliferation more generally in the animal kingdom.

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