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

Laura D. Mathies1,2, Mara Schvarzstein3, Kristin M. Morphy2, Robert Blelloch4, Andrew M. Spence3 and Judith Kimble1,*

1Department of Biochemistry and Howard Hughes Medical Institute, University of Wisconsin-Madison, Madison, WI 53706-1544, USA
2Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614, USA
3Department of Molecular and Medical Genetics, University of Toronto, Toronto M5S 1A8, Canada
4Whitehead Institute, Cambridge, MA 02142-1479, USA
*Author for correspondence (e-mail: jekimble@facstaff.wisc.edu)

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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 SGP daughter ablated. All ablations were validated. The fate of the remaining SGP daughter was assayed in L3-L4 using lag-2::GFP.

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.

**LGI:** ehn-1(q699) (Mathies et al., 2003), tra-2(ar221ts)

**LGI:** 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)

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

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

Dominant GFP balancers: hT2[qsl48] for LGI and LGIII, and nT1[qsl50] for LGIV and LGV.

Molecular markers: qsl47 [X-linked GFP], qsl55 [hnd-1(1)::GFP] (Mathies et al., 2003), qsl56 [lag-2::GFP] (Bieloch et al., 1999), qsl61 [pes-1::GFP] (Molin et al., 2000), qsl74 [GFP::POP-1] (Siegfried et al., 2004), qsl76 [tra-1::GFP], a transcriptional reporter of tra-1 expression (Chang et al., 2004), qsl77 [unc-122::GFP]

(Xin et al., 1999), and rdEx1 [GFP::TRA-1], a translational TRA-1 reporter with partial rescuing activity.

Phenotypic analysis and laser ablation

OX tra-1 males were identified as non-GFP animals from a cross of tra-1(e1099)/hT2[qsl48] females to tra-1(e1099)/hT2[qsl48]; qsl47 XO males. qsl47 is an X-linked GFP marker and hT2[qsl48] is a GFP balancer chromosome. tra-1(hT2[qsl48]) 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 (Photic 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; mdDf4 complemented and ndDf4 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 rescued 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-3. 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 transspliced to SL1; ehn-3(q766) 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 qbs68. 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 (BamHI to Apal) 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 BamHI 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 GFP::TRA-1 (pJK946) (from 18% to 1% defective, n=604) and tra-1; ehn-3(q766) double mutants (from 97% to 8% absent gonad, n=37).

**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 Z1 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). Therefore, 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.

**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 nucleic 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 similarly dimorphic difference
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.

**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 PGCs was examined by Nomarski imaging of an *ehn-3* *tra-1* double mutant, which revealed the probable cause – cells with morphology typical of SGPs were not seen (n=16).

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

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<td>282</td>
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<tr>
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<td></td>
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<td>41</td>
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*Gonadal morphology scored by DIC optics.
associated cells could not be ruled out, because of bright expression in adjacent ventral neurones. 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 remain associated with the germ cells (Fig. 4 E), whereas the male leader cell, called a linker cell (DTC), is crescent-shaped (Fig. 4A) and resides next to germ 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.

Following 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.

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-penetration 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-penetration 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.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gonadal morphology (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>tra-1(e1099)/+</td>
<td>100</td>
</tr>
<tr>
<td>ehn-3(q766)</td>
<td>97</td>
</tr>
<tr>
<td>tra-1(e1099)/+; ehn-3(q766)</td>
<td>86</td>
</tr>
<tr>
<td>ehn-3(RNAi)</td>
<td>90</td>
</tr>
<tr>
<td>tra-1(e1099)/+; ehn-3(RNAi)</td>
<td>40</td>
</tr>
<tr>
<td>ehn-3(q689)</td>
<td>82</td>
</tr>
<tr>
<td>tra-1(e1099)/+; ehn-3(q689)</td>
<td>35</td>
</tr>
<tr>
<td>ehn-3(q689) fem-3(e1996)/ehn-3(q689)</td>
<td>81</td>
</tr>
<tr>
<td>tra-2(e1996)/+; ehn-3(q689)</td>
<td>74</td>
</tr>
</tbody>
</table>

*Gonadal morphology scored by DIC optics.
†Includes single-armed, abnormal morphology, and absent gonads.
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 transgenic allele bears a frame shift mutation in a large intron and part of the first common exon, including coding sequence for the first 14 amino acids of EHN-3B1 and B2 (Fig. 5D), suggesting that its phenotypically more severe than ehn-3(q766)/ehn-3(q689) double mutants (see Materials and methods), suggesting that its

![Fig. 5. ehn-3 encodes a SGP-specific protein with paired zinc fingers. (A) The ehn-3 locus. CDNA analyses identify three transcripts, each predicted to encode a protein with paired zinc fingers (black boxes). All cDNAs are transspliced 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 1 of ehn-3A. ehn-3(q689) deletes a region including exon 1 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, hhl-2 PCR product was similar in wild-type and ehn-3 mutants. (Right) Ratio of ehn-3 to hhl-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 transgenic 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.

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 normally works, we constructed EHN-3::GFP reporter transgene in embryonic SGPs (see Materials and methods), suggesting that its

**Table 3. ehn-3 allelic series**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gonadal morphology (%)</th>
<th>Wild type</th>
<th>Abnormal†</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ehn-3(q766)</td>
<td>97</td>
<td>3</td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>ehn-3(q766)/nDf41</td>
<td>96</td>
<td>4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ehn-3(q766)/ehn-3(q689)</td>
<td>89</td>
<td>11</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>ehn-3(q689)</td>
<td>82</td>
<td>18</td>
<td>1031</td>
<td></td>
</tr>
<tr>
<td>ehn-3(q689)/nDf41</td>
<td>74</td>
<td>26</td>
<td>197</td>
<td></td>
</tr>
</tbody>
</table>

*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.

**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 gonadal-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,
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 genes 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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References

TRA-1/GLI controls SGP development