A forkhead protein controls sexual identity of the *C. elegans* male somatic gonad

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Summary

In sex determination, globally acting genes control a spectrum of tissue-specific regulators to coordinate the overall development of an animal into one sex or the other. In mammals, primary sex determination initially occurs in the gonad, with the sex of other tissues specified as a secondary event. In insects and nematodes, globally acting regulatory pathways have been elucidated, but the more tissue- and organ-specific downstream effectors of these pathways remain largely unknown. We focus on the control of sexual dimorphism in the *C. elegans* gonad. We find that the forkhead transcription factor FKH-6 promotes male gonadal cell fates in XO animals. Loss-of-function *fkh-6* mutant males have feminized gonads and often develop a vulva. In these mutant males, sex-specific cell divisions and migrations in the early gonad occur in the hermaphrodite mode, and hermaphrodite-specific gonadal markers are expressed. However, sexual transformation is not complete and the male gonad is malformed. By contrast, *fkh-6* mutant hermaphrodites exhibit no sign of sex reversal. Most *fkh-6* hermaphrodites form a two-armed symmetrical gonad resembling that of the wild type, but differentiation of the spermatheca and uterus is variably abnormal. The function of *fkh-6* appears to be restricted to the gonad: *fkh-6* mutants have no detectable defects in extra-gonadal tissues, and expression of a rescuing *fkh-6* reporter is gonad-specific. Genetic and molecular analyses place *fkh-6* downstream of *tra-1*, the terminal regulator of the global sex determination pathway, with respect to the first gonadal cell division. We conclude that *fkh-6* regulates gonadogenesis in both sexes, but is male specific in establishing sexual dimorphism in the early gonad.

Key words: Sex determination, Gonad, Gonadogenesis, *C. elegans*, Forkhead

Introduction

Sex determination is the process by which cells, tissues and indeed animals are directed to develop as one of two sexes. The gonad must be specialized to support different modes of gametogenesis in each sex, and development of other tissues and organs also is sexually dimorphic in most animals. For sexual reproduction to occur, not only must the gonad differentiate into a functional testis or ovary, but other sexually dimorphic tissues must also adopt the same mode as the gonad, either male or female. Two general strategies have evolved to ensure that sexual dimorphism of the gonad is coordinated with that of other tissues. In vertebrates, the gonad itself plays a dominant role: primary sex determination initially occurs in the gonad, which then secretes hormones that induce appropriate sexual differentiation of other tissues (reviewed by Nef and Parada, 2000). Consequently, master regulatory genes like the mammalian testis determining gene *Sry*, although expressed only in the gonad, initiate a process that masculinizes the entire body. A second strategy, which is used by insects and nematodes, relies on more direct control of target tissue differentiation. Although cell-cell interactions play a role (Hunter and Wood, 1992), master regulators such as *tra-1* of *C. elegans* or *Sex lethal* of *Drosophila* are expressed in all sexually dimorphic tissues, both gonadal and extra-gonadal, and cell-autonomously control their sexual differentiation (reviewed by Cline and Meyer, 1996). Despite differences in how sex determination is coordinated, some aspects of the process may be evolutionarily conserved between phyla: members of a protein family sharing the DM DNA-binding domain regulate sex determination in vertebrates, insects and nematodes (Erdman and Burtis, 1993; Lints and Emmons, 2002; Matsuda et al., 2002; Nanda et al., 2002; Raymond et al., 2000; Raymond et al., 1998).

The global regulators of sexual development presumably act via more tissue-restricted downstream genes. Indeed, several tissue-specific sexual regulators have been identified in worms and flies; for example, in the nematode *C. elegans* the DM domain protein MAB-3 controls male development of the intestine and parts of the nervous system (Shen and Hodgkin, 1988; Yi et al., 2000), the related protein MAB-23 regulates

male development of mating muscles, nervous system and posterior hypodermis (Lints and Emmons, 2002), and FOG-1 and FOG-3 act in the germline to promote spermatogenesis (Barton and Kimble, 1990; Ellis and Kimble, 1995). Similarly, in *Drosophila*, Fruitless controls male development of the CNS and musculature (Ito et al., 1996; Ryner et al., 1996), Takeout acts in fat cells in the head to promote male courtship behavior (Dauwalder et al., 2002) and Dissatisfaction regulates courtship behavior in the nervous system of both sexes (Finley et al., 1997). Although the action of such proteins begins to reveal how global regulators impose sexual dimorphism on individual tissues, the downstream regulators remain unknown in many tissues. In particular, no gonad-specific sex-determining gene has been identified in any invertebrate, despite the central role in sexual reproduction of the gonad and its highly dimorphic anatomy in most species.

This paper focuses on control of sexual dimorphism in the gonad of *C. elegans*. The two sexes of *C. elegans* are the self-fertilizing hermaphrodite (chromosomally XX) and the male (XO). Hermaphrodites can be considered females with sperm, a view underscored by the existence of closely related nematodes that reproduce as typical females and males. Therefore, *C. elegans* sex determination is likely to rely on a primal male/female control mechanism, with more recent regulatory modifications that permit hermaphroditism (Fitch and Thomas, 1997). Arguably the most crucial global regulator of sex determination in *C. elegans* is *tra-1*, which promotes female development in hermaphrodites and encodes TRA-1A, a zinc-finger protein of the Ci/GLI class of transcription factors (Hodgkin, 1987; Zarkower and Hodgkin, 1992). *tra-1* activity is essential for all aspects of female sex determination in somatic cells and also plays a crucial role in the germline (Hodgkin, 1987; Schedl et al., 1989). TRA-1A controls some downstream targets by direct transcriptional repression (Chen and Ellis, 2000; Conradt and Horvitz, 1999; Yi et al., 2000). The picture emerges, therefore, that TRA-1A promotes female cell fates by repressing the expression or activity of genes that otherwise would direct male development in specific tissues (reviewed by Zarkower, 2001).

Despite major differences in mature gonad morphology between the two sexes, most regulators of gonadogenesis identified so far play similar roles in both sexes (Hubbard and Greenstein, 2000; Friedman et al., 2000; Mathies et al., 2003; Miskowski et al., 2001; Siegfried and Kimble, 2002). Indeed, even *tra-1*, in addition to its essential role in directing hermaphrodite gonadal development, also has a minor role in male gonadogenesis (Hodgkin, 1987; Schedl et al., 1989) (L.M. et al., unpublished). Therefore, sexual dimorphism in the gonad may not arise by execution of unrelated parallel developmental programs in each sex, but rather by sex-specific modulation of an underlying common gonadogenesis program. In this respect, control of *C. elegans* gonadogenesis may resemble genital disc development in *Drosophila*, in which the *Doublesex* branch of the sex-determination pathway sex-specifically modulates the response to cell signaling pathways found in both sexes (reviewed by Christiansen et al., 2002).

In the work described here, we identify the forkhead transcription factor FKH-6 as a regulator of sexual dimorphism in the *C. elegans* gonad. The gonad of *fkh-6* mutant males is feminized: hermaphrodite-specific gonadal reporters are expressed; a vulva is frequently present; and early gonadogenesis resembles that of hermaphrodites. By contrast, the overall morphology of mutant hermaphrodite gonads is normal in most animals, although mutants are infertile, because of defects in the differentiation of the hermaphrodite somatic gonad. Extra-gonadal tissues in both sexes appear to be normal in *fkh-6* mutants, and *fkh-6* reporters are expressed only in the gonad. Genetic and molecular analyses suggest that *fkh-6* acts downstream of *tra-1* in the first division of the somatic gonadal precursor cells, which establishes gonadal sexual dimorphism. Collectively, these results indicate that FKH-6 is an organ-specific regulator of sexual dimorphism.

Materials and methods

Strains and genetic methods

C. elegans strains were cultured and genetically manipulated as described previously (Sulston and Hodgkin, 1988). Strains were maintained and experiments conducted at 20°C unless otherwise stated. Most strains included a *him-8(e1489)* or *him-5(e1490)* high incidence of male mutation. The following mutations were used in this study: LGII, *bli-2(e768); lin-31(n301); unc-4(e120); tra-2(e1095)*; LGIII, *tra-1(e1099)*; LGIV, *him-8(e1489)*; LGV, *him-5(e1490)*.

Integrated transgene arrays were as follows: *qIs56[lag-2::gfp, unc-119+]V; ayIs2[egl-15::gfp, dpy-20+]IV; ezIs1[K09C8.2::gfp, pRF4]X; bxIs13[egl-5::gfp, lin-15+]X*;

syIS50[cdh-3::gfp, dpy-20+]; tnIs5[lim-7::gfp, pRF4]; leIs8[pes-8::gfp; pRF4];

ezIs2[fkh-6(pro)::gfp, unc-119+]III;

and *qIs76[tra-1::gfp, pRF4]*.

Extrachromosomal transgene arrays were leEx780[*ZK813.3*::gfp, pRF4]; and ezEx133[*fkh-6*(FL)::gfp, pRF4].

fkh-6 mutations were maintained in trans to the balancer *mIn1[dpy-10(e128) mIs14]II*, where *mIs14* carries the integrated array *ccEx9747*. *ccEx9747* is an extrachromosomal array composed of three GFP constructs, one driven by a gut-specific enhancer and the others by *myo-2* and *pes-10* promoters (Edgley and Riddle, 2001). Heterozygotes were identified as animals of normal size with GFP expression in the pharynx and intestine, and mutants were identified as animals of normal size lacking GFP expression in these tissues.

Isolation, mapping, and genetic manipulation of *fkh-6* mutations

fkh-6(ez16) was isolated in an F2 EMS mutagenesis screen (Sulston and Hodgkin, 1988) of *him-8(e1489)* animals carrying the integrated array *ezIs1*, which contains the male seminal vesicle and vas deferens marker *K09C8.2::gfp*. K09C8.2 was identified in a cDNA microarray screen for sex-enriched L4 mRNAs, comparing XX wild-type (N2) hermaphrodites with XX pseudomales (*tra-2; xol-1)* (K.T., W. Yi, V. Reinke, and D.Z., unpublished). Mutant lines with abnormal or absent *K09C8.2::gfp* expression were identified using a dissecting microscope with fluorescence optics. The *fkh-6(q641)* allele was isolated in a F2 EMS mutagenesis screen of *him-5(e1490)* animals for gonadogenesis defects.

The *ez16* and *q641* mutations were mapped between *lin-31* and *let-172* on LGII by three-factor mapping, and further localized by deficiency mapping. The deficiencies *maDf4*, *nDf3* and *nDf4* complemented *ez16* and *q641*, whereas *ccDf4*, *ccDf7*, *ccDf5* and *ccDf1* failed to complement. Single nucleotide polymorphism (SNP) recombinant mapping (Wicks et al., 2001) placed *ez16* to the left of a polymorphism contained in the cosmid M03A1. A cosmid from this region (B0286) was injected with pRF4 [*rol-6(su1006sd)*] (Mello et al., 1991) into *ez16/mIn1* hermaphrodites and found to rescue the *ez16* mutation. Fertile *fkh-6* homozygous hermaphrodites and *fkh-6* males with normal gonadal morphology were scored as positive for rescue.

A PCR fragment containing 6.7 kb of *fkh-6* upstream of the predicted start codon and 2 kb downstream of the predicted stop codon also rescued *ez16* mutants. In addition, RNAi directed against *fkh-6* gave the same phenotypes as *ez16* and *q641*. RNAi directed against *fkh-6* was performed by feeding bacteria expressing double-stranded RNA corresponding to *fkh-6* (B0286.5; a gift from J. Ahringer) as described (Ashrafi et al., 2003; Kamath et al., 2001).

Molecular analysis of *fkh-6* alleles

Template DNA for sequencing was made by amplifying exons from genomic DNA prepared from N2 or *fkh-6(ez16, q641)* animals using the ExpandLong™ or Expand™ High Fidelity PCR System (Roche). Three independent PCR reactions were sequenced for each exon from each strain using Big-Dye Terminator Ready Reaction Mix (PE/Applied Biosystems). Sequencing of *ez16* DNA identified a mutation in the predicted first codon, changing ATG to ATA (methionine to isoleucine). Sequencing *q641* DNA identified a G to A change in the first position of intron 2.

To confirm that the *fkh-6(q641)* mutation affects splicing, RT-PCR was performed. RNA was isolated from N2 or *fkh-6(q641)* L1 and L4 XO and XX animals using Tri Reagent™ (Molecular Research Center), and cDNAs were prepared using Superscript II (Gibco). Sequencing of PCR products from two overlapping primer sets identified identical transcripts in wild-type XO and XX animals at L1 and L4. To predict the consequences of the *q641* insertion for the FKH-6 structure, the primary amino acid sequence of FKH-6 was aligned with residues in the Foxd3 (2HDC) solution structure (Jin et al., 1999) using Clustal-W (Thompson et al., 1994). The insertion caused by the *q641* mutation is in helix 2, which is immediately prior to two hydrophobic amino acids that are integral to the forkhead domain hydrophobic core (Phe-32 and Ile-33 in 2HDC; Phe-50 and Ile-51 in FKH-6). By altering the register of helix 2, the insertion is likely to disrupt formation of the hydrophobic core in FKH-6.

Lineage analysis and laser ablations

Cell divisions and migrations were followed by DIC microscopy using standard methods (Sulston and Horvitz, 1977). To determine whether Z1.a and Z4.p can divide, we laser ablated Z1.p and Z4.a (plus two of the four germ cells present at the time, to simplify the analysis). In wild-type males, this ablation does not affect the divisions of remaining cells (Kimble and White, 1981) (J. Kimble, unpublished). Ablations were performed as previously described (Bargmann and Avery, 1995) using a Micropoint Ablation Laser System (Photonics Instruments, Arlington, IL). L1 XO *fkh-6(q641)* and *fkh-6(ez16)* homozygotes were obtained as self-progeny from *fkh-6(q641)/mIn1; him-5(e1490)* or *fkh-6(ez16)/mIn1; him-8(e1489)* mothers. XO animals were identified based on the presence of an enlarged B blast cell. L1 *fkh-6(q641); tra-1(e1099)* double homozygotes were identified as self-progeny with a large B cell from *fkh-6(q641)/mIn1; tra-1(e1099)/+* mothers.

fkh-6 reporters

fkh-6(pro)::gfp (pWC1) was made by inserting a genomic DNA PCR fragment, extending from 6708 bp upstream of the predicted start codon to 239 bp downstream, into the pPD95.69 GFP vector (gift of A. Fire). The *fkh-6(FL)::gfp* (pWC2) rescuing GFP was made by inserting a genomic DNA PCR fragment, from 6708 upstream of the predicted start codon to 3402 downstream, into pPD95.67. Transgenic strains were created by microinjection as previously described (Mello and Fire, 1995). pWC1 was injected with pMM106b (*unc-119+*; a gift from D. Pilgrim) into *unc-119(ed3)* worms to generate *ezEx147*, and this strain was subsequently integrated to generate *ezIs2*. pWC2 was injected with pRF4 (Mello et al., 1991) into *fkh-6(ez16)* to generate the extrachromosomal array *ezEx133*.

pWC1 contains a single consensus TRA-1A binding site. pWC7, a derivative of this reporter with the consensus sequence changed from TTGGTGGTC to TTCTGCAGC was made by site-directed mutagenesis with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene).

Genetic mosaic analysis

fkh-6(ez16) mutant hermaphrodites carrying an extrachromosomal array of *fkh-6(FL)::gfp* and pRF4 were assayed for the ability to lay eggs, indicating rescue of the *fkh-6* somatic gonadal defects. Progeny of rescued animals were examined for viability and inheritance of pRF4 (which causes a dominant roller, or Rol, phenotype). Five hermaphrodites produced large broods of fully viable mutant progeny, all non-Rol. In these hermaphrodites, the extrachromosomal array must have been lost, prior to the embryonic cell division forming P4, the blastomere from which the germline precursors Z2 and Z3 derive. Male and hermaphrodite progeny of germline mosaic hermaphrodites had the same gonadal phenotypes as homozygous *fkh-6* progeny from *fkh-6* heterozygotes. This indicates that any maternal contribution of FKH-6 to the germline is not functionally significant.

tra-1 reporter

The *tra-1::gfp* reporter (pJK876) was generated by inserting a *Pst*I to *Bam*HI fragment from cosmid F56C2 into pPD96.04 (a gift from A. Fire). This construct contains 8138 bp upstream of the *tra-1* start codon and fuses the first six amino acids of TRA-1 to GFP and βGAL. pJK876 was injected with pRF4 to generate *qEx480*, which was integrated to make *qIs76*.

Results

A forkhead transcription factor required for male gonadal morphogenesis

To find regulators of sexual dimorphism of the *C. elegans* gonad, we screened for mutants with sex-specific gonadal defects. In these screens, we examined F2 progeny of mutagenized hermaphrodites for mutations that either disrupted expression of the male seminal vesicle and vas deferens marker *K09C8.2::gfp*, or altered the morphology of the male gonad. Among the mutations identified were the alleles *ez16* and *q641*, on which we focus here. Both mutations are recessive and map between *lin-31* and *let-172* on LGII; they also fail to complement, indicating that they are mutations in the same gene (see Materials and methods).

We cloned the gene defective in *ez16* and *q641* by a combination of fine genetic mapping, cosmid rescue and RNAi (Materials and methods). The gene identified was *fkh-6*, one of a previously described family of *C. elegans forkhead*-related genes (Hope et al., 2003). The FKH-6 protein contains 323 amino acids, including a 96 amino acid region with high similarity to the DNA-binding domain of the forkhead/winged-helix family of transcription factors (Hope et al., 2003; Weigel and Jackle, 1990) (Fig. 1A). Sequencing genomic DNA from *ez16* and *q641* homozygotes revealed their molecular lesions: *ez16* is a G-to-A transition predicted to alter the initiation codon from methionine to isoleucine, and *q641* is a G-to-A transition at the first nucleotide of intron 2, predicted to affect splicing between exons 2 and 3 (Fig. 1A). To confirm the effect of *q641* on splicing, we sequenced *fkh-6* cDNAs prepared by RT-PCR from wild-type and *fkh-6(q641)* L1 XO animals, and from mixed-stage *fkh-6(q641)* XX animals. *fkh-6(q641)* cDNA has a six nucleotide insertion at the junction of exons 2 and 3, owing to use of a downstream cryptic 5′ splice site. This is predicted to insert two amino acids into helix 2 of the forkhead domain. Alignment of FKH-6 to known forkhead domain structures (Materials and methods) suggests that the *q641*

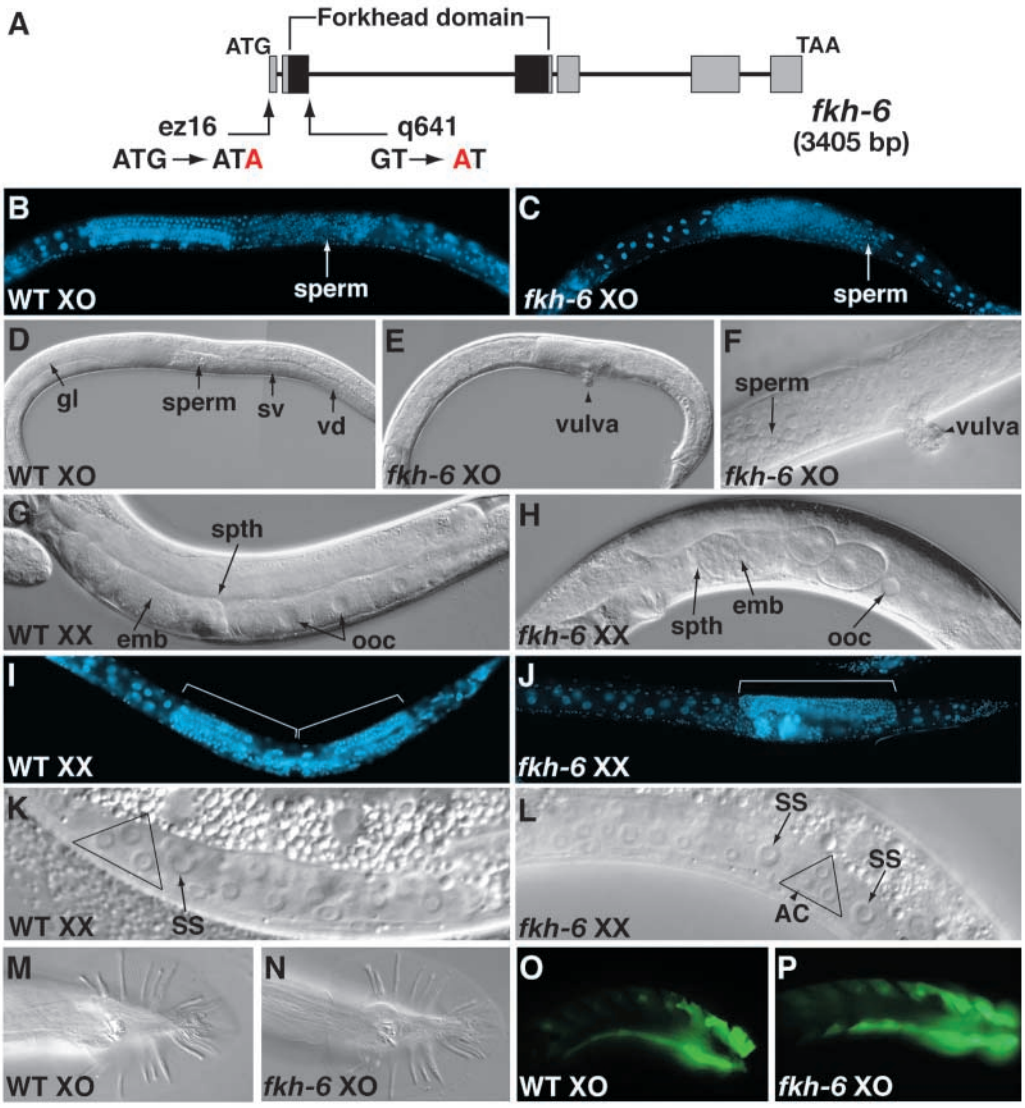


Fig. 1. *fkh-6* is required for gonadal development. (A) Molecular basis of *fkh-6* mutations. Boxes indicate exons; lines indicate introns. The forkhead domain (black) is encoded by two exons. Arrows indicate locations of mutations. The *ez16* and *q641* alleles are both single base changes, indicated in red: *ez16* affects the ATG start codon and *q641* affects the predicted splice donor site of the second intron. The *q641* transcript is spliced aberrantly, resulting in a two amino acid insertion in the forkhead domain (see text). (B,C) Adult XO males stained with DAPI to highlight gonadal nuclei. (B) Wild-type adult male gonad with elongate, J-shaped gonad. (C) *fkh-6(ez16)* adult male with disorganized gonad that has failed to elongate. Sperm are indicated by arrow. (D-F) Male gonads, differential interference contrast (DIC) optics. (D) Wild-type adult male with germline (gl), mature sperm, seminal vesicle (sv) and vas deferens (vd) indicated. (E) *fkh-6(ez16)* adult male with vulva indicated (arrowhead). (F) *fkh-6(ez16)* male at higher magnification, with mature sperm indicated (arrow). (G) Wild-type young adult XX hermaphrodite showing posterior gonad arm, with spermatheca (spth), oocytes (ooc) and embryo (emb) indicated. (H) Young adult *fkh-6(ez16)* hermaphrodite; embryo is lodged in spermatheca and fertilized eggs, and oocytes can be seen backing up behind embryo. (I,J) Adult hermaphrodites stained with DAPI. (I) Wild-type adult hermaphrodite with two gonad arms indicated by brackets. (J) *fkh-6* adult hermaphrodite showing the one-armed gonad phenotype that occurs in ~10% of XX mutants. (K,L) Early L3 wild-type and *fkh-6* hermaphrodites at ten-cell somatic gonad primordium (SPh) stage. Dorsal and ventral uterine precursor cells are indicated by triangles; larger sheath/spermathecal precursor cells (SS) are indicated by arrow. (K) Wild-type early L3 hermaphrodite. Three of the six dorsal and ventral uterine precursor cells are visible, as is one of the four SS cells (other SPh cells are out of focal plane or obscured by intestine). (L) *fkh-6* early L3 hermaphrodite showing normal position of SPh cells. Three of six dorsal and ventral uterine precursors are visible, including differentiating anchor cell (AC). Two of four SS cells are in focal plane. (M) Wild-type adult male tail, with sensory rays and other copulatory structures. (N) *fkh-6* adult male tail, showing normal morphology. (O) Ventrolateral view of wild-type adult male showing *egl-15::gfp* reporter expression in sex muscles in the tail (Harfe et al., 1998). (P) Ventral view of *fkh-6* adult male expressing *egl-15::gfp* in sex muscles with normal morphology.

insertion destabilizes the hydrophobic core of the forkhead domain by shifting the orientation of two conserved hydrophobic residues in helix 2 (Phe-50 and Ile-51). The two *fkh-6* alleles have essentially identical phenotypes, either as homozygotes or in trans to a deficiency, and closely resemble *fkh-6(RNAi)*; in addition, *fkh-6(RNAi)* of *fkh-6* homozygotes does not enhance the mutant phenotype (Table 1, and data not shown). Collectively, these results indicate that both *fkh-6* alleles are strong loss-of-function mutations and may be null.

The gonadal defects in *fkh-6* mutant males are severe. Whereas wild-type adult males have an elongate one-armed testis organized into distinct structures (Fig. 1B,D), *fkh-6* males have a gonad of variable size with no apparent elongation and with no obvious gonadal tissue organization (Fig. 1C,E,F). Spermatogenesis occurs in mutant males and mature sperm often develop (Fig. 1F), although the male germ line is disorganized, presumably owing to the severe dysgenesis of the somatic gonad. Strikingly, ~25% of *fkh-6* males have a vulva, and occasional animals (<1%) have two vulvae (Table 1, Fig. 1E,F).

The gonadal defects in *fkh-6* mutant hermaphrodites, by contrast, do not include sexual transformation and usually

Table 1. *fkh-6* adult gonad phenotypes

	Male gonad morphology (%)				Hermaphrodite gonad morphology (%)			
Genotype*	Wild type	Not elongated	Vulva	*n*	Two arms	One arm	No arm	*n*
Wild type	100	0	0	104	100	0	0	146
fkh-6(ez16)	0	100	26	211	90	9	1	312
fkh-6(q641)	0	100	25	198	92	7	1	326
fkh-6(ez16)/ccDf4	0	100	21	122	94	6	0	247
fkh-6(q641)/ccDf4	0	100	28	119	96	4	0	113

*All strains harbor the high incidence of male mutation *him-8(e1489)* or *him-5(e1490)*.

Table 2. Leader cells in *fkh-6* males and hermaphrodites (%)*

Genotype†	0 LC	1 LC	2 LC	0 DTC	1 DTC	2 DTC	3+ DTC	*n*
lag-2::gfp XO	1	98	1	0	4	96	0	70
fkh-6(q641); lag-2::gfp XO	94	6	0	5	14	33	48	64
lag-2::gfp XX	na	na	na	0	3	97	0	40
fkh-6(q641); lag-2::gfp XX	na	na	na	0	16	83	1	97

*Linker cell (LC) and distal tip cell (DTC) were scored by GFP fluorescence; LCs scored as intense expression with typical LC morphology. DTCs in hermaphrodite observed as bright cells at the anterior and posterior end. DTCs in males are observed as small, faint expression cells.
†All strains harbor the high incidence of male mutation *him-5(e1490)*.
na, not applicable.

do not affect overall gonadal morphology. Wild-type hermaphrodites have two symmetrical ovotestes with sheath, spermatheca and uterus (Fig. 1G,I; Table 1), and most *fkh-6* hermaphrodites have this same morphology (Fig. 1H; Table 1). In addition, sheath/spermathecal precursors are present at the normal time and position in early L3 (Fig. 1K,L; *n*=30) and form a spermatheca that expresses the spermathecal marker *leEx780* (*n*=50; data not shown). However, during L4, sheath/spermathecal daughters assume unusual and variable positions, and the adult spermatheca appears blocked, with embryos accumulating in the proximal sheath (Fig. 1H). A rarer hermaphrodite defect is the presence of only one of the two normal ovotestes (compare Fig. 1I with 1J; Table 1), which appears to result from the loss of a distal tip cell (see below). We conclude that *fkh-6* affects gonadogenesis in both sexes, but its affect on overall gonadal morphogenesis is largely male specific.

Gametogenesis appears normal in *fkh-6* hermaphrodites: spermatogenesis is followed by oogenesis and fertilization occurs (Fig. 1H). However, embryonic development arrests prior to hatching and all *fkh-6* hermaphrodites are infertile (*n*>500). This maternal effect lethality could reflect a requirement in the somatic gonad, the germline, or the early embryo. To distinguish between these possibilities, we analyzed germ line mosaics (Materials and methods). In these animals, a rescuing *fkh-6* array was present in the mother but was not transmitted to progeny, indicating loss of the array in the embryonic cell lineage leading to the germline (*n*=5). All germline mosaic hermaphrodites produced large broods of viable *fkh-6* mutant progeny, demonstrating that neither germ line nor zygotic expression of *fkh-6* is required for viability. Instead, we suggest that the embryonic lethality results from somatic gonadal defects, perhaps owing to insufficient support of developing oocytes by somatic cells.

The *fkh-6* defects appear to be restricted to the gonad. Hermaphrodites and males are of normal size and have a growth rate similar to that of wild type. In addition, we observed no obvious defects in male tail differentiation (Fig. 1M,N) or sex muscle morphology and function (Fig. 1O,P). Males also display normal mating behavior, and do not accumulate yolk, indicating that the nervous system and intestine, respectively, are not feminized (data not shown).

fkh-6 males lack gonadal leader cells

Gonadal morphogenesis is controlled by ‘leader’ cells, which guide elongation of the growing gonadal arms (Kimble and White, 1981). In males, the ‘linker’ cell has leader function, and two male distal tip cells (DTCs) control germline proliferation. The severe defects in elongation of *fkh-6* male gonads suggested that the linker cell might be missing or defective. To test this, we used *lag-2::gfp*, a marker intensely expressed in the large round linker cell and more faintly in the two small flat male distal tip cells (Siegfried and Kimble, 2002). Most *fkh-6(q641)* males lacked cells intensely expressing *lag-2::gfp* with linker cell morphology (94%, *n*=64), and many had abnormal numbers of DTCs (Table 2). Because the linker cell and male DTCs are generated during L1, these results suggest that *fkh-6* acts early in male gonadogenesis.

We also examined the generation of leader cells in hermaphrodites, again using *lag-2::gfp*. In hermaphrodites, one DTC resides at the distal end of each ovotestis. Hermaphrodite DTCs are bifunctional, controlling germline proliferation, as in males, and also guiding elongation of each growing gonadal arm (Kimble and White, 1981). Almost all wild-type hermaphrodites (97%, *n*=40, Table 2), and most *fkh-6* hermaphrodites (83%, *n*=97, Table 2) had two *lag-2::gfp*-expressing DTCs. However, some *fkh-6* hermaphrodites had only one *lag-2::gfp* expressing DTC (16%, *n*=97, Table 2), consistent with the percentage of one-armed *fkh-6* hermaphrodite gonads we observed. The DTC deficit was already apparent in L2/L3 larvae: of 53 L2/L3s followed, 48 had two DTCs and also had two arms as adults, whereas five had one DTC and subsequently had one arm as adults. Loss of

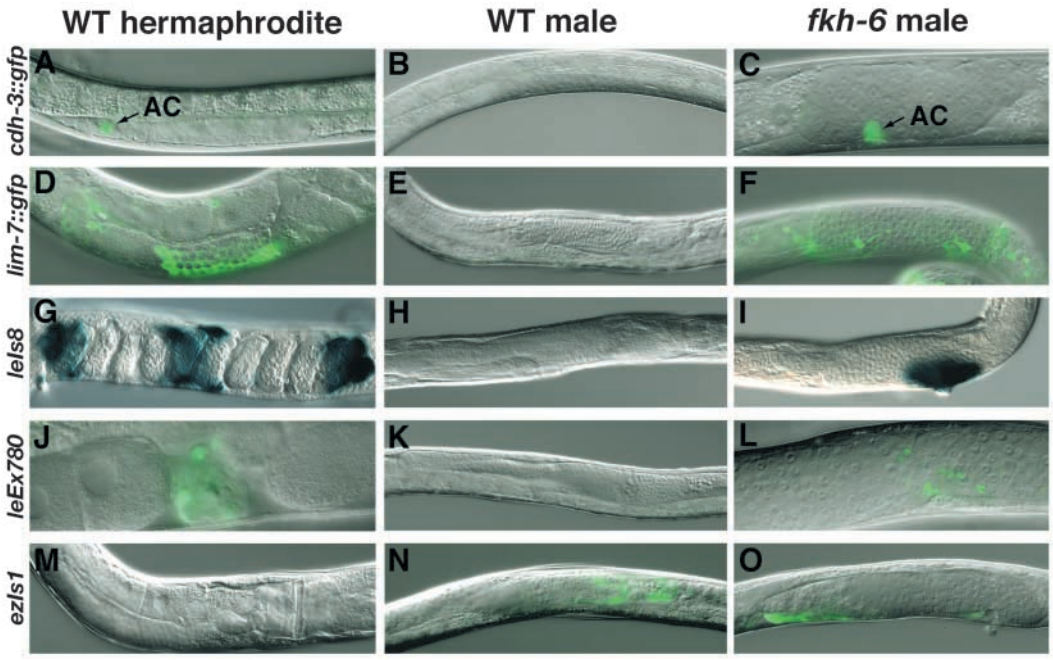


Fig. 2. Sex reversal of cell fates in the *fkh-6* male gonad. Left column (A,D,G,J,M): wild type XX hermaphrodites. Middle column (B,E,H,K,N): wild type XO males. Right column (C,F,I,L,O): *fkh-6(ez16)* mutant XO males. All panels are overlaid DIC and fluorescence images, except those showing *leIs8*, which are DIC images of β-gal stained animals. For each reporter, fluorescence images are identical exposures for all genotypes. Cell types expressing each reporter in wild-type hermaphrodites are as follows. *cdh-3::gfp* is expressed in the hermaphrodite anchor cell (AC, arrow), but not in wild-type males. *lim-7::gfp* is expressed in hermaphrodite sheath cells. *leIs8* is a *lacZ* reporter expressed in hermaphrodite spermathecal cells and some uterine cells. *leEx780* is a *gfp* reporter expressed in spermathecal cells. *ezIS1* is a *gfp* reporter not expressed in hermaphrodites, but expressed in male seminal vesicle and vas deferens (see Materials and methods).

the hermaphrodite DTC might result from an early lineage defect, the death of the DTC or its transformation to another cell type. Distinguishing these possibilities was impractical given the low penetrance. The *lag-2::gfp* transgene was a mild enhancer of one-armed gonads, an effect also seen in wild-type and with other gonadogenesis mutants (Siegfried and Kimble, 2002).

Feminization of cell fates in the *fkh-6* male gonad

In wild-type hermaphrodites, vulval development is induced by the anchor cell, a hermaphrodite-specific cell in the somatic gonad (Kimble, 1981). The vulvae present in *fkh-6* males suggested that the gonad might be feminized, possessing an anchor cell and possibly other hermaphrodite cell types. We tested this possibility using reporter transgenes specific for hermaphrodite gonadal cells. First, we asked whether *fkh-6* male gonads make an anchor cell. In wild-type animals, the *cdh-3::gfp* marker is expressed specifically in hermaphrodite anchor cells and is not expressed in the male gonad (Pettitt et al., 1996) (Fig. 2A,B). Remarkably, almost all *fkh-6(ez16)* males (97%, *n*=64), expressed *cdh-3::gfp* in at least one anchor cell (Fig. 2C), and most had more than one (83%), with an average of four. The anchor cells present in *fkh-6* XO gonads demonstrate that the *fkh-6* male gonad is feminized and are likely to be responsible for induction of male vulvae. *fkh-6* hermaphrodites do not have supernumerary anchor cells: all animals examined had a single *cdh-3::gfp*-expressing cell in the gonad (*n*=50). The frequency of vulvae in *fkh-6* males is lower than the frequency of *cdh-3::gfp*-expressing cells (25% versus 97%). This suggests that some of the *cdh-3::gfp*-expressing cells may not have full anchor cell function.

We next tested reporters for other hermaphrodite-specific somatic gonadal cell types. We used *lim-7::gfp* to mark production of hermaphrodite sheath cells (Hall et al., 1999), *leIs8* to mark spermatheca and uterine cells (Hope, 1991) and *leEx780* to mark spermathecae (gift of Ian Hope). Each marker was expressed in wild-type hermaphrodite gonadal cells and absent from wild-type male gonads (Fig. 2, left and center columns) but all were expressed in *fkh-6* male gonads [*lim-7::gfp:* 33%, *n*=51 (Fig. 2F), *leIs8*: 100%, *n*=35 (Fig. 2I), *leEx780*: 36%, *n*=20 (Fig. 2L)]. Expression of these hermaphrodite-specific markers was variable in *fkh-6* males with respect to both the number and the position of cells. The expression of multiple hermaphrodite-specific gonadal markers indicates that the *fkh-6* male gonad is extensively feminized.

To test whether the feminization of *fkh-6* male gonads is complete, we examined expression of two male-specific gonadal markers. We used *ezIs1* to score seminal vesicle and vas deferens cells. In wild type, *ezIs1* is not expressed in hermaphrodites ($n \gg 1000$) (Fig. 2M), but is expressed in all male gonads from L4 through adulthood ($n \gg 1000$) (Fig. 2N). In *fkh-6(ez16)* males, a few gonadal cells expressed *ezIs1* in 25% of animals (*n*=52), indicating the presence of seminal vesicle or vas deferens cells (Fig. 2). Similarly, *egl-5::gfp*, which is expressed in four seminal vesicle valve cells in wild-type adult males (Ferreira et al., 1999), was expressed in one cell in 21% of *fkh-6* mutant male gonads (*n*=30). Neither male marker was expressed in *fkh-6* hermaphrodites (not shown). We conclude that the feminization of *fkh-6* XO gonads, although extensive, is not complete, and that *fkh-6* XX gonads are not masculinized.

Feminization of early gonadogenesis in *fkh-6* males

We next asked whether early gonadogenesis during the first larval stage (L1) in *fkh-6* mutants resembles that of males or hermaphrodites. At hatching, the L1 gonadal primordium consists of four cells: two somatic gonadal precursor cells (Z1 and Z4) occupy the poles and flank two central germ line

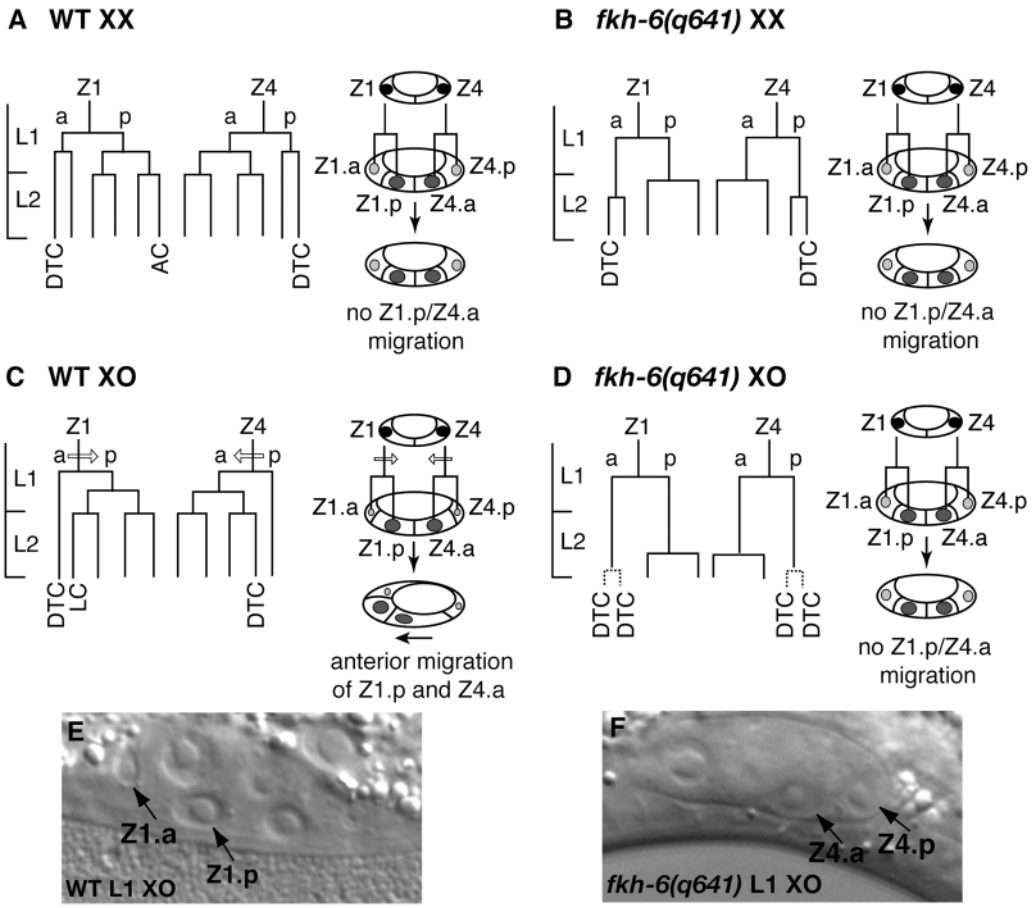


Fig. 3. Feminization of early cellular events in the *fkh-6* male gonad. (A-D) Left, Z1/Z4 cell lineages; right, schematics of early gonadogenesis; L1, first larval stage; L2, second larval stage; a, anterior daughter; p, posterior daughter; AC, anchor cell; LC, linker cell; DTC, distal tip cell. Open arrows, size asymmetry of Z1/Z4 divisions in the wild-type males. In all schematics, Z1 and Z4 are black, and their daughters are either light grey (Z1.a, Z4.p) or dark grey (Z1.p, Z4.a). (A) Wild-type hermaphrodite. Z1 and Z4 produce daughter cells of similar size. Z1.a and Z4.p generate DTCs, while either Z1.p or Z4.a generates an AC. (B) *fkh-6* hermaphrodite. Z1/Z4 early lineage appears normal, but is delayed relative to wild type (*n*=2). (C) Wild-type male. Z1 and Z4 divide asymmetrically to generate daughter cells with a pronounced size asymmetry (open arrows). Z1.p and Z4.a migrate anteriorly, and one or the other generates a LC. (D) *fkh-6* male. Z1 and Z4 do not produce daughters with a dramatic size difference (*n*=8), Z1.p and Z4.a do not migrate anteriorly (*n*=8), and Z1.a/Z4.p were able to divide (broken lines, 3/6 cells examined). In animals with Z1.a/Z4.p divisions, extra DTCs were observed. (E,F) DIC micrographs of L1 developing gonad. (E) Wild-type male. Z1.a is smaller than Z1.p. Z4.p (out of focal plane) is similarly smaller than Z4.a. (F) *fkh-6 (q641)* male. Z4.a and Z4.p are about the same size; Z1.a and Z1.p (out of focal plane) also are similar in size.

precursor cells (Z2 and Z3) (Hubbard and Greenstein, 2000). This four-cell primordium is morphologically indistinguishable in the two sexes; sexual dimorphism is established during and after the first Z1/Z4 division and involves three sex-specific events (Kimble and Hirsh, 1979) (Fig. 3A,C). First, Z1/Z4 daughter cells have a more pronounced size asymmetry in XO than XX gonads: in males, Z1.a and Z4.p are visibly smaller than their sisters Z1.p and Z4.a, while in hermaphrodites these sisters appear similar in size. Second, in males Z1.p and Z4.a migrate anteriorly, while in hermaphrodites they retain their central positions. Third, Z1.a and Z4.p do not divide in males, but do in hermaphrodites.

The four-cell gonadal primordium in *fkh-6* males was morphologically normal (*n*=8), but the subsequent L1 development of Z1 and Z4 resembled that of hermaphrodites rather than males (Fig. 3). Thus, in *fkh-6* XO gonads, the first Z1/Z4 division produced daughters of nearly equal size (8/8 cases, Fig. 3E versus 3F), Z1.p and Z4.a failed to migrate (8/8 cases, Fig. 3D), and Z1.a and Z4.p were able to divide (3/6 cases). After this stage the *fkh-6* male gonad lineage became variably abnormal and specific cell divisions were not followed further; observation in later larval stages (L2, L3, L4) revealed that the eight-cell somatic gonadal primordium typical of male gonadogenesis did not develop in *fkh-6* males. However, the 10-cell somatic gonadal primordium typical of hermaphrodites was not seen either; instead, the gonad became a disorganized mass composed of variable numbers of somatic gonadal and germ cells. To determine whether the extra division of Z1.a and Z4.p generates the extra DTCs seen in about half the *fkh-6(q641)* males (Table 2), we examined *fkh-6(q641)*; *lag-2::gfp* males. Indeed, the extra division of Z1.a or Z4.p could produce extra DTCs (2/4 did not divide and had no extra DTCs; 2/4 did divide and had extra DTCs, Fig. 3D). In summary, cellular events of L1 gonadogenesis are feminized in *fkh-6* mutant males, consistent with the feminization of *fkh-6* somatic gonadal cell fates we observed in later larval stages.

We also examined L1 gonadogenesis in *fkh-6* XX hermaphrodites. The four-cell gonadal primordium appeared normal (*n*=30), and the first divisions of Z1 and Z4 were typical of wild-type hermaphrodites (*n*=2). However, Z1/Z4 cell divisions were slower than wild-type in both XX and XO *fkh-6* mutants (Fig. 3B,D). By contrast, no delay occurred in the first two rounds of the B lineage (*n*=8), suggesting that *fkh-6* does not affect cell divisions generally.

fkh-6::gfp expression is gonad-specific

To study *fkh-6* expression, we made two GFP reporters. One, a transcriptional reporter, *fkh-6(pro)::gfp*, contains 6.7 kb

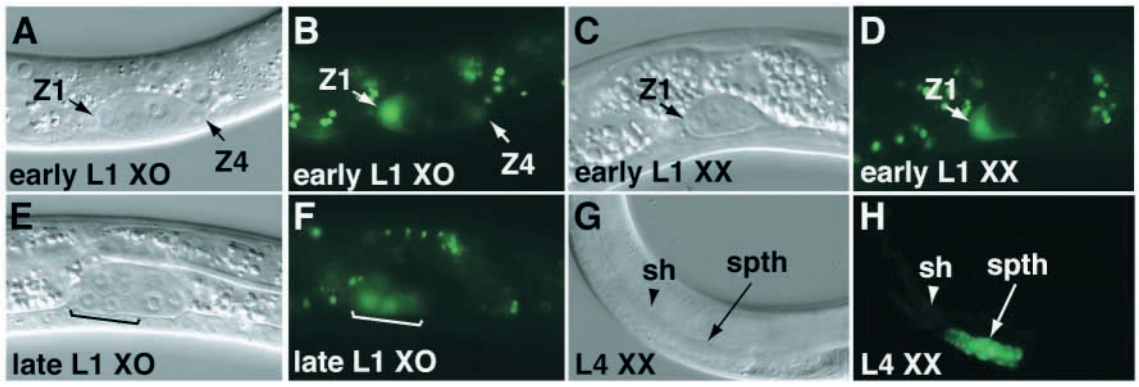


Fig. 4. *fkh-6::gfp* is expressed in the early somatic gonad and the spermatheca. (A-H) Expression of integrated *fkh-6(pro)::gfp* array. (A,B) DIC and fluorescence micrographs in four-cell gonad of wild-type L1 male, with *fkh-6::gfp* expression in Z1 and Z4 (arrows). (C,D) Four-cell gonad of wild-type L1 hermaphrodite showing expression in Z1 (arrow). Z4 is out of focal plane. (E,F) Wild-type male gonad in late L1, with expression of *fkh-6::gfp* reporter in four daughters of Z1 and Z4, which have moved to the anterior (bracket). Reporter expression is no longer detectable in hermaphrodites by this stage (not shown). (G,H) Wild-type L4 hermaphrodite expressing *fkh-6::gfp* reporter in spermatheca (spth) and weakly in sheath (sh). Fluorescence outside the gonad in these images is intestinal autofluorescence. Reporter expression is not detected in males at this stage (not shown).

upstream of the predicted initiation codon and fuses the *gfp*-coding region in frame at the beginning of exon 2. The other reporter, *fkh-6(FL)::gfp*, contains the same 6.7 kb of upstream sequences, followed by genomic sequences containing the complete *fkh-6* coding region, and the *gfp*-coding region fused in frame immediately 5′ to the FKH-6 stop codon. This reporter fully rescued *fkh-6* male gonadal defects and restored hermaphrodite self-fertility, suggesting appropriate spatial and temporal expression.

The two reporters have the same expression pattern. Both are expressed in Z1 and Z4 of XO (Fig. 4A,B) and XX (Fig. 4C,D) L1 larvae. However, later during L1, the timing of expression is sexually dimorphic: in XO larvae reporter expression persists until late L1 in Z1/Z4 descendants, whereas in XX larvae it decreases in mid L1 and is undetectable by late L1 (Fig. 4E,F, not shown). Later expression also is sexually dimorphic. In L3 hermaphrodites, the *fkh-6* reporters resume expression in sheath-spermathecal precursor cells, continuing through adulthood in spermatheca and weakly in proximal sheath (Fig. 4G,H; data not shown). By contrast, no expression was observed in XO animals past the L1 stage. Reporter expression is consistent with *fkh-6* mutant defects in L1 male gonadal morphogenesis and in hermaphrodite gonadal differentiation during L3 and L4. No expression was detectable outside the gonad in larvae or adults of either sex.

fkh-6 acts downstream of *tra-1* and *tra-2* to control early gonadogenesis

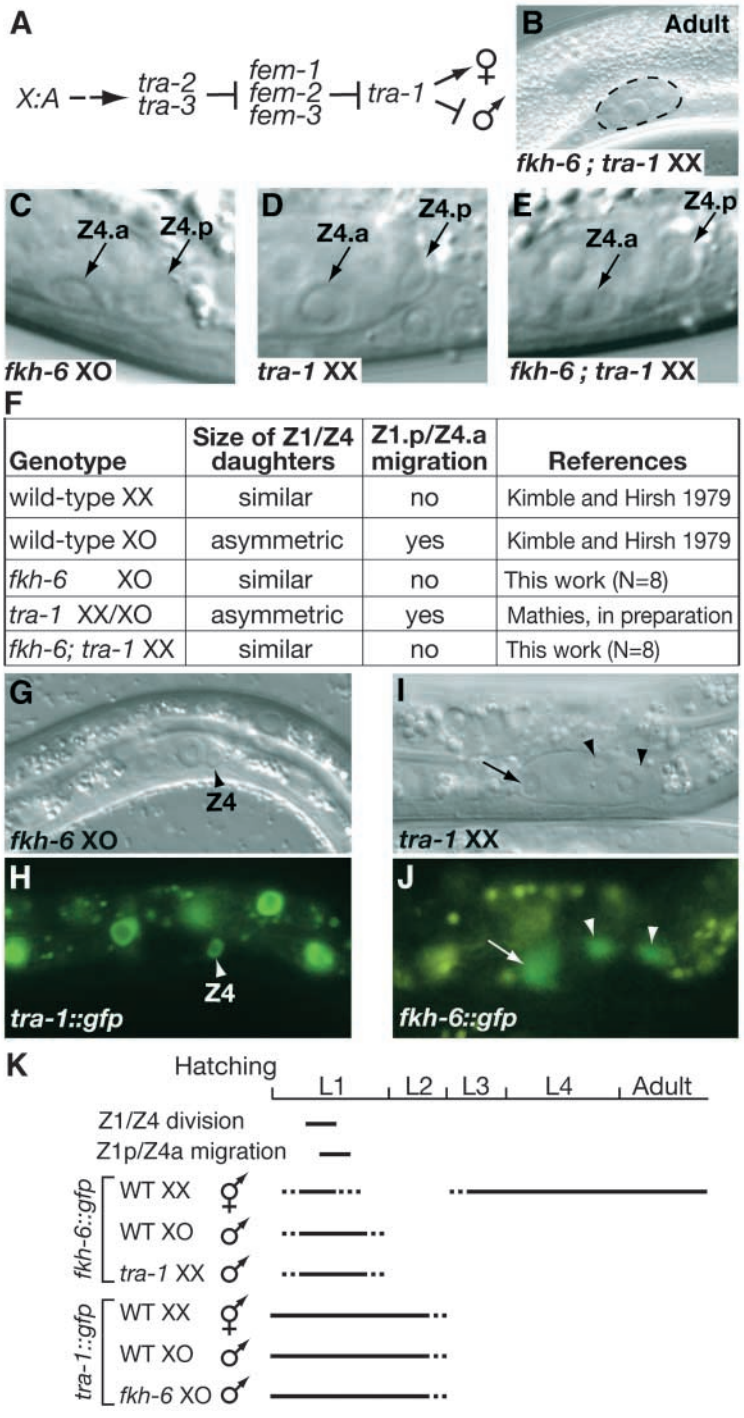
The gonad-specific sexual transformation of *fkh-6* mutants indicates that *fkh-6* is a tissue-specific sex-determining gene. We therefore investigated the relationship of *fkh-6* to two key genes of the global sex determination pathway (Fig. 5A). The *tra-1* and *tra-2* genes normally promote female development: XX *tra-1* or *tra-2* null mutants are strongly masculinized (Hodgkin and Brenner, 1977). Two simple models could account for the role of *fkh-6* in promoting male development of the gonadal primordium. First, *fkh-6* could block the feminizing activities of *tra-1* or *tra-2* in males. Conversely, *tra-1* or *tra-2* could block the masculinizing activity of *fkh-6* in hermaphrodites. To distinguish between these alternatives, we examined *fkh-6; tra-1* and *fkh-6 tra-2* double mutants. The gonads of *fkh-6 tra-2* double mutants closely resembled those of *fkh-6* single mutants (no elongation, disorganized, frequent vulval induction), indicating that *fkh-6* is epistatic to *tra-2* and is likely to act downstream of *tra-2* to promote male development (Table 3).

The genetic relationship between *fkh-6* and *tra-1* is more

Table 3. *fkh-6* is epistatic to the global sexual regulators *tra-2* and *tra-1*

	Gonad phenotype (%)*					
Genotype†	Elongated wild type	Elongated abnormal	Not elongated	Four- to six-cell gonad	Vulva	*n*
fkh-6(q641) XO‡	0	0	100	0	25	198
tra-2(e1095) XX	100	0	0	0	0	97
fkh-6(q641) tra-2(e1095) XX	0	0	100	0	28	128
tra-1(e1099) XX	31	51	18	0	0	51
fkh-6(q641)/+; tra-1(e1099) XX	17	57	20	6	0	69
fkh-6(q641); tra-1(e1099) XX	0	0	10	90	0	42

*Gonad morphology was scored by DIC optics.
†XO animals all harbor the high incidence of male mutation *him-5(e1490).*
‡*fkh-6(q641)* data are also presented in Table 1.



Genotype	Size of Z1/Z4 daughters	Z1.p/Z4.a migration	References
wild-type XX	similar	no	Kimble and Hirsh 1979
wild-type XO	asymmetric	yes	Kimble and Hirsh 1979
fkh-6 XO	similar	no	This work (N=8)
tra-1 XX/XO	asymmetric	yes	Mathies, in preparation
fkh-6; tra-1 XX	similar	no	This work (N=8)

Fig. 5. *fkh-6* acts downstream of *tra-1* in the early gonad. (A) Simplified version of *C. elegans* sex-determination pathway (Hodgkin et al., 1986). The ratio of X chromosomes to autosomes (Madl and Herman, 1979) signals via a regulatory cascade and regulates *tra-1*, such that high *tra-1* activity promotes female somatic development and represses male development. Early genes in the pathway and some genetic interactions are not shown. (B) Gonad is severely underdeveloped in *fkh-6;tra-1* XX adult (broken line indicates gonadal boundary). (C-E) Comparison of Z4 daughters in *fkh-6*, *tra-1*, and *fkh-6; tra-1* mutants. Z4.a and Z4.p are similar in size in *fkh-6(q641)* XO L1 male, whereas Z4.a is larger than Z4.p in *tra-1(e1099)* XX L1 pseudomale. In *fkh-6; tra-1* double mutant XX L1 pseudomales, Z4.a and Z4.p are similar in size. Z1 daughters (out of focal plane) have the same size polarities. (F) Summary of early events of gonadogenesis in *fkh-6* and *tra-1* single mutants and *fkh-6; tra-1* double mutants. Relative sizes of Z1 and Z4 daughters and male-specific anterior migration of Z1.p and Z4.a were scored. Eight Z1/Z4 divisions were scored for each mutant class (*fkh-6* XO, *tra-1* XX, and *fkh-6; tra-1* XX). (G,H) DIC and fluorescence images showing *tra-1::gfp* expression in the gonadal primordium of *fkh-6(q641)* XO L1 male. Arrowhead indicates Z4. Reporter also is expressed in Z1, which is out of focal plane. Other brightly expressing nuclei are intestinal. (I,J) DIC and fluorescence images showing *fkh-6(pro)::gfp* expression in the gonadal primordium of *tra-1(e1099)* XX L1 pseudomale. Z1 (arrow) and Z4 daughters (arrowheads) express *fkh-6::gfp*. Z4 daughters are displaced to the dorsal side of the gonad, a common *tra-1* defect (L.M. and J.K., unpublished). (K) Summary of *fkh-6::gfp* and *tra-1::gfp* expression. Wild-type timing of Z1/Z4 division and anterior migration of Z1.p/Z4.a is indicated at top (unbroken lines). Periods of GFP reporter expression in wild-type and mutant transgenic animals are indicated (broken-ended lines indicate imprecision in timing of expression).

complex. The terminal phenotype of *fkh-6; tra-1* double mutants indicates a synergistic effect on gonadogenesis. Specifically, the entire gonad of most adult *fkh-6; tra-1* XX double mutants possessed only ~10 total cells (somatic plus germline) and no vulva, and in *fkh-6; tra-1* double mutants observed continuously, gonadal development arrested with only four to six total Z1/Z4 descendants and a similar number of germ cells (*n*=8) (Fig. 5B, Table 3). Development of non-gonadal tissues was not similarly arrested, and these tissues were typical of *tra-1* single mutants. This type of very early gonadal arrest has not been seen in either *fkh-6* or *tra-1* single mutants (Hodgkin, 1987; Schedl et al., 1989). We suggest that the two genes may have a partially overlapping function in gonadal cell proliferation.

We next examined the relationship of *tra-1* and *fkh-6* in controlling Z1/Z4 divisions. We found that Z1/Z4 divisions in *fkh-6; tra-1* mutants resemble those in *fkh-6* single mutants rather than those in *tra-1* single mutants (Fig. 5C-F). The detailed cellular analysis of *tra-1* single mutants will be described elsewhere (L.M., unpublished). The Z1/Z4 cell division and movements of their daughters in *tra-1* mutants have characteristics typical of wild-type XO males (e.g. asymmetric division, anterior migration) (Fig. 5D,F). By contrast, in both *fkh-6* single mutants (Fig. 5C) and in *fkh-6; tra-1* double mutants (Fig. 5E), the Z1 and Z4 divisions are not markedly asymmetric, and none of the daughters move to the anterior (summarized in Fig. 5F). Thus, for these early events of gonadogenesis, *fkh-6* is epistatic to *tra-1*, suggesting that *fkh-6* acts downstream of *tra-1* to specify a male-specific division of Z1 and Z4 and the male-specific anterior migration of Z1/Z4 daughters.

To investigate the relationship of *tra-1* and *fkh-6* at the molecular level, we employed GFP reporters for each gene (Fig. 5G-J; summarized in Fig. 5K). A *tra-1::gfp* transgene is expressed in Z1 and Z4 and their descendents, both in wild-

type XX and XO L1 gonads (L.M. and J.K., unpublished) and in *fkh-6* mutant L1 gonads, with no obvious change in pattern or timing (Fig. 5G,H). This lack of effect is consistent with the genetic epistasis result placing *tra-1* upstream of *fkh-6* at this early stage of gonadogenesis. By contrast, *fkh-6::gfp* expression in the L1 XX gonad was extended by removal of *tra-1* activity. Thus, whereas *fkh-6::gfp* in wild-type XX animals was undetectable by late L1, its expression continued into late L1 in *tra-1(null)* XX pseudomales (Fig. 5I,J); likewise, the L3 to adult expression of *fkh-6::gfp* observed in wild-type XX hermaphrodites was absent in *tra-1* XX pseudomales (not shown). This dependence of *fkh-6::gfp* expression on *tra-1* but not vice versa (summarized in Fig. 5K) is consistent with the genetic epistasis observed in early L1 gonads of *fkh-6; tra-1* double mutants. To ask whether TRA-1A might directly repress *fkh-6* transcription, we examined the 5′ flanking region of *fkh-6* present in the GFP reporters, and identified one close match to the TRA-1A DNA-binding consensus (TTGGTGGTC from –6523 to –6531 relative to the initiation codon). However, we did not find this site in the related nematode *C. briggsae*, and mutating it to TTCTGCAGC, a change that should eliminate regulation by TRA-1A in vivo (Yi et al., 2000), did not affect the level or timing of *fkh-6::gfp* expression in either sex (data not shown). We conclude that *tra-1* affects the timing of early *fkh-6* expression, but probably not by direct transcriptional repression (see Discussion).

Discussion

fkh-6 and gonadal sex determination

In this paper, we show that *fkh-6* is a gonad-specific sexual regulator that promotes male development: in *fkh-6* mutant males, early cellular events of gonadogenesis occur in the hermaphrodite mode and later, when gonadal cells differentiate, they express hermaphrodite markers. The sexual transformations evident in *fkh-6* males indicate that *fkh-6* is not only required for male differentiation, but also determines male identity of gonadal cells. By contrast, overall gonadal morphogenesis in *fkh-6* hermaphrodites is largely normal and sexual identity is unaffected. The function of *fkh-6* seems to be limited to the gonad: non-gonadal aspects of male development and behavior appear normal, and *fkh-6* reporter genes are expressed only in the gonad. Genetic epistasis experiments indicate that *fkh-6* acts downstream of *tra-2*, and also downstream of *tra-1*, at least to control early male-specific events of gonadogenesis (male-specific Z1/Z4 division and male-specific migration of Z1/Z4 daughters). Therefore, we conclude that *fkh-6* is a gonad-specific sex-determining gene that acts downstream of the global sex determination pathway.

fkh-6 controls multiple aspects of male gonadogenesis

The L1 gonad in *fkh-6* mutant males is sexually transformed in several respects: the Z1/Z4 divisions lack male-specific size asymmetry, and their proximal daughters do not undergo their male-specific anterior migration. Furthermore, Z1.a and Z4.p, which do not divide in wild-type males, do divide in *fkh-6* XO gonads. The expression of hermaphrodite gonadal markers from L3 through adult stages in *fkh-6* mutant males confirms the sexual transformation. In these *fkh-6* males, the number and position of cells expressing each gonadal marker gene varies. This is likely to reflect variability in the extent of feminization of the mutant gonad, resulting in a variably intersexual cell lineage. In wild-type males, *fkh-6* reporters are expressed only during L1, suggesting that it is FKH-6 activity during this stage that determines the later male differentiation of gonadal cells.

We have considered two models to account for the range of defects in *fkh-6* male gonadogenesis. First, FKH-6 might independently control male-specific asymmetric division, cellular movement and cellular differentiation, canalizing each into the male mode. Alternatively, the initial division of Z1 and Z4 might determine whether subsequent events of gonadogenesis occur in the male mode, and FKH-6 might control the entire process by regulating only this first step. In this latter model, FKH-6 could, for example, regulate a male-specific determinant that is partitioned during the first Z1 and Z4 divisions, leading to male-specific cell migration and differentiation. There are a number of precedents for FKH transcription factors controlling asymmetry and cell polarity. For example, in *C. elegans*, UNC-130 acts in neuronal precursors to regulate their asymmetric division and confer distinct identities to daughter cells (Nash et al., 2000; Sarafi-Reinach and Sengupta, 2000). Similarly, in *Drosophila* the Jumeaux protein is required for proper localization and segregation of Numb in neuronal precursor cells (Cheah et al., 2000).

fkh-6 affects hermaphrodite and male gonadogenesis differently

The pattern of early gonadal cell divisions, migration and differentiation appear largely normal in *fkh-6* mutant hermaphrodites until early L3, in sharp contrast with the situation in mutant males. Most importantly, there is no evidence of sex reversal in *fkh-6* hermaphrodites. Normal hermaphrodite structures (sheath, spermatheca, uterus) are present, but malformations become apparent from L3 onwards, and *fkh-6* hermaphrodites are infertile. The onset of defective gonadogenesis correlates with the hermaphrodite-specific expression of *fkh-6::gfp* reporters from L3 onwards. Germline mosaic analysis demonstrates that embryonic viability requires maternal *fkh-6* activity in somatic cells, but not in the maternal germ line or in zygotic cells.

Thus, while *fkh-6* affects gonadogenesis in both sexes, its functions are mostly distinct in hermaphrodites and males. An exception is that early divisions of somatic gonadal cells are slower than normal in both sexes. One possibility is that *fkh-6* controls an early cellular process that not only is intrinsic to the rate of cell division in gonads of both sexes, but also is essential for asymmetric division in the male gonad.

FKH-6 is required in the early XO gonad for male gonadogenesis and cell fate determination, and *fkh-6* reporters are expressed longer in the L1 gonad in males than in hermaphrodites. Is persistent FKH-6 expression sufficient to direct male gonadal fates in hermaphrodites? To address this question, we overexpressed FKH-6 using either of two promoters (W.C. and D.Z., unpublished). Overexpression using a heatshock promoter was highly toxic even with very mild heatshock conditions. Overexpression using the *lag-2* promoter to drive expression of FKH-6::GFP specifically in Z1/Z4 lineages showed no clear signs of masculinization.

In summary, *fkh-6* has largely distinct functions in the two sexes. In XO animals it specifies male-specific cellular events in the early gonad and is required for commitment to male rather than female cellular differentiation, whereas in XX animals it controls later gonadal differentiation. In both sexes it affects the rate of gonadal cell division.

fkh-6 links global and organ-specific sex determination

The *tra-1* gene encodes the terminal regulator in the global somatic sex-determination pathway (Hodgkin, 1987). We suggest that *fkh-6* acts downstream of *tra-1* to establish sexual dimorphism during the division of Z1/Z4 and in their daughters. This suggestion is based on two lines of evidence. First, *fkh-6* defects in the early L1 gonad are epistatic to those of *tra-1*; and second, *fkh-6::gfp* expression in XX animals is extended during L1 by a *tra-1(null)* mutation. As *tra-1* promotes female development and *fkh-6* promotes male development, *tra-1* activity is likely to inhibit *fkh-6* in the early XX gonad, allowing Z1 and Z4 to divide in the hermaphrodite mode. How does this occur? Mutating the only consensus TRA-1A binding site in a *fkh-6::gfp* reporter had no apparent effect on reporter expression (Fig. 5; data not shown). The simplest conclusion is that the inhibition of *fkh-6* expression by TRA-1A is indirect; however, it remains possible that TRA-1A regulates *fkh-6* transcription through a binding site we did not recognize or redundantly with another factor.

The roles of *fkh-6* and *tra-1* are not limited to sex determination. *fkh-6* mutants exhibit delayed cell divisions in both sexes, and *fkh-6;tra-1* double mutant males have a synthetic phenotype: shortly after the first division of Z1 and Z4, gonadogenesis arrests. By contrast, *fkh-6 tra-2* double mutant gonads do not arrest, and they resemble the *fkh-6* single mutant throughout gonadal development. This difference suggests that *tra-1* and *fkh-6* share a function distinct from that of the global sex determination pathway. A simple model is that both genes regulate proliferation of somatic gonadal cells, and are partially redundant for this function. This idea helps reconcile previous observations that *tra-1* null mutant gonads, while strongly masculinized, often are smaller than normal and disorganized (Hodgkin, 1987; Schedl et al., 1989), whereas null mutants in *tra-2* and other upstream genes develop gonads of normal size and morphology.

Is *fkh-6* regulation of gonadal sexual development conserved?

fkh-6 is a gonad-specific regulator of sex determination. Is this role unique to *C. elegans*, or might forkhead genes be conserved regulators of gonadal sex determination? At least one forkhead gene, *Foxl2*, is expressed in developing gonads of a variety of vertebrates (Loffler et al., 2003), and is required in the mammalian gonad (Crisponi et al., 2001; De Baere et al., 2001). Like *fkh-6*, *Foxl2* is required in the female gonad in somatic cells that support the germline. However, unlike *fkh-6*, *Foxl2* is not required in the male gonad and *Foxl2* mutations have not been shown to cause sexual transformation. It is difficult to assess whether gonadal regulation by *fkh-6* and *Foxl2* in different phyla reflects evolutionary conservation or is an example of convergent evolution. *C. briggsae* has an unambiguous *fkh-6* homolog (Hope et al., 2003), but sequence conservation of the forkhead domain is insufficient for robust phylogenetic analysis in more distant species, and FKH-6 lacks other conserved sequence motifs that might aid in identifying orthologs.

fkh-6 provides an entry point for elucidating the process of gonadal sex determination in *C. elegans*. Once functionally equivalent genes are found in other phyla and more components of the *fkh-6* regulatory pathway are discovered, the evolutionary history of gonadal sex determination should become much clearer.

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