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# *fear of intimacy* encodes a novel transmembrane protein required for gonad morphogenesis in *Drosophila*

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## SUMMARY

**Gonad formation requires specific interactions between germ cells and specialized somatic cells, along with the elaborate morphogenetic movements of these cells to create an ovary or testis. We have identified mutations in the *fear of intimacy* (*foi*) gene that cause defects in the formation of the embryonic gonad in *Drosophila*. *foi* is of particular interest because it affects gonad formation without affecting gonad cell identity, and is therefore specifically required for the morphogenesis of this organ. *foi* is also required for tracheal branch fusion during tracheal development. *E-cadherin/shotgun* is similarly required for both gonad coalescence and tracheal branch fusion, suggesting that E-cadherin and FOI cooperate to mediate these processes. *foi* encodes a member of a novel family of transmembrane proteins that includes the closely related human protein LIV1. Our findings that FOI is a cell-surface protein required in the mesoderm for gonad morphogenesis shed light on the function of this new family of proteins and on the molecular mechanisms of organogenesis.**

Key words: *Drosophila*, Germ cells, Cell-cell interaction, E-cadherin, Morphogenesis, Gonad coalescence, Tracheal development, LIV1

## INTRODUCTION

Germ cells follow a complex developmental program in order to form the gametes and give rise to the next generation of a species. In animals, much of germ cell development takes place in the gonads, where specialized somatic cells create the unique environment necessary for germ cell differentiation. Thus, proper gonad formation is crucial for germ cell development and reproductive health. Gonad formation is also an excellent system for studying basic questions of morphogenesis: how different cell types recognize one another and undergo the cellular movements required to form properly patterned tissues and organs. There are at least two different types of cellular movements that are required for gonad formation. The first is individual cell migration: as the primordial germ cells migrate from their site of origin to make contacts with the cells of the somatic gonad. The second is coordinated tissue morphogenesis: where the germ cells and somatic gonadal cells together coalesce to form the embryonic gonad. Little is known about how such cellular movements combine to produce the gonad, or, indeed, any organ.

In *Drosophila*, the germ cells initially form as the pole cells at the posterior end of the embryo. The movements of gastrulation bring these cells into the interior of the embryo where they are contained in the posterior endoderm. From this location, the germ cells actively migrate out of the endoderm and into the mesoderm, and make contacts with specific mesodermal derivatives that will give rise to the somatic gonad or gonadal mesoderm [see Starz-Gaiano and Lehmann (Starz-Gaiano and Lehmann, 2001) for a review of germ cell migration]. The gonadal mesoderm forms from three clusters of mesodermal cells on each side of the embryo (Boyle et al., 1997). These cells are specified in the *eve* domain of the dorsolateral mesoderm, and form only in parasegments (PS) 10-12 because of the action of the homeotic gene *abdA* (Cumberledge et al., 1992; Brookman et al., 1992; Boyle and DiNardo, 1995; Moore et al., 1998; Riechmann et al., 1998). Approximately 10 cells form in each cluster, and are recognizable by their expression of the nuclear proteins EYES ABSENT (EYA) and ZFH1 (Boyle et al., 1997; Broihier et al., 1998). The three clusters of gonadal mesoderm join to form a single band of cells across PS10-12 at the same time the germ cells complete their migration and specifically associate with these cells.

In the next step of gonad formation, the germ cells and gonadal mesoderm cells undergo a dramatic rearrangement to coalesce in PS10 and form a spherically shaped embryonic gonad. Although this process has not previously been studied

in detail, early work suggests that some gonadal mesoderm cells form a sheath around the germ cells, while other mesodermal cells remain intermingled with them (Poulson, 1950). It has also been shown that the gonadal mesoderm does not require the germ cells for gonad formation, and a properly patterned gonad can form in embryos that completely lack germ cells (Geigy, 1931; Brookman et al., 1992). Thus, the gonadal mesoderm cells can independently undergo the morphogenetic movements of gonad coalescence, suggesting that they play an active role in this process, while the germ cells may be more passive. Although the gonadal mesoderm is specified from PS10-12, the gonad forms in PS10. Thus, it appears that gonadal mesoderm cells move with the germ cells from more posterior segments to PS10 to form the embryonic gonad (Boyle and DiNardo, 1995).

Although we know a considerable amount about how gonadal mesoderm cell identity is established, we know little about how this identity is translated into the cell-cell interactions and cellular movements required for gonad morphogenesis. Here we present the phenotypic and molecular characterization of a gene, *fear of intimacy* (*foi*), that is required for gonad coalescence but not for gonad cell identity. Thus, the FOI protein may play a specific role in gonad morphogenesis. FOI is a transmembrane protein localized to the cell surface and is a member of a new family of proteins that have been well-conserved evolutionarily. Our analysis of *foi* provides insight both into the molecular mechanisms controlling gonad morphogenesis and into the function of this new family of transmembrane proteins.

## MATERIALS AND METHODS

### Fly stocks

Ethylmethane sulfonate (EMS)-induced alleles of *foi* were identified previously (Moore et al., 1998) and are designated $foi^{20.71}$, $foi^{16.33}$ *and* $foi^{38.66}$. P-element-induced alleles of *foi*, *l(3)neo13* and *l(3)j8e8*, were identified by complementation testing and obtained from the Bloomington Stock Collection. Additional mutant alleles include $shg^{IH}$, $osk^{301}$, $osk^{CE4}$ and $esg^{G66B}$, and are as indicated in FlyBase (FlyBase, 1999). The UAS-CD8-GFP flies were a gift from L. Luo (Lee and Luo, 1999).

### In situ hybridization and antibody staining of embryos

In situ hybridization and antibody staining was conducted as described (Moore et al., 1998), except that in Fig. 6C the embryos were devitellinized by hand. The following plasmids were used for generating antisense riboprobes: pSK2.4#3 (412) (Brookman et al., 1992), pGemlacZ (*lacZ*) and pKS2.4Z (*foi*). Antibodies (dilutions) were as follows: anti-VASA (1:10,000), anti-EYA (1:25) (Bonini et al., 1993), 2A12 (1:5) (Samakovlis et al., 1996a), anti-β-GAL (1:20,000, Capel), anti-GFP (1:2000, Torrey Pines Biolabs), anti-ZFH1 (1:5000), anti-DLG (1:100) (Parnas et al., 2001) and anti-HA (Boehringer Mannheim, 4.0 μg/ml). Antibodies were localized using either a biotin labeled secondary antibody (Jackson) as described (Moore et al., 1998), or with Alexa-fluor conjugated secondary antibodies (Molecular Probes). Homozygous mutant embryos were identified by the loss of *lacZ* or β-GAL expression in crosses using *lacZ* expressing balancer chromosomes. Embryos were visualized using either conventional DIC microscopy (light micrographs), deconvolution microscopy or laser scanning confocal microscopy (as indicated for fluorescence images).

For examination of the tracheal cell bodies in *foi* mutants, a CD8-GFP fusion protein was expressed in tracheal cells using the pUAS-CD8-GFP (Lee and Luo, 1999) crossed to breathless-Gal4 (Shiga et al., 1996), all in a $foi^{20.71}$ mutant background. Expression of *esg* in the tracheal fusion tip cells in $foi^{20.71}$ mutant embryos was analyzed using an *escargot* enhancer trap ($esg^{G66B}$). Tracheal images shown are *z*-series of confocal images through lateral trunk branches combined into a single image.

### Molecular identification of *foi*

Once P-element alleles of *foi* were identified and verified [*l(3)neo13* and *l(3)j8e8*], the flanking genomic DNA from both was obtained by plasmid rescue. P1 clones in this region obtained from the Berkeley Drosophila Genome Project were probed using this flanking DNA and clone DS04044 was chosen as a source of genomic DNA in the region, mapped and subcloned. Northern blot analysis using probes from *l(3)neo13* flanking genomic DNA revealed a single 4 kb transcript from embryo RNA. Probing of the Nick Brown (Brown and Kafatos, 1988) and Kai Zinn (Zinn et al., 1988) cDNA libraries identified several classes of cDNA with the same 5′ region, but further northern analysis found evidence for the embryonic expression of only one of these classes and only this class contains a large open reading frame. A representative cDNA (2.4Z) from the 9-12 hour λgt11 cDNA library (Zinn et al., 1988) was subcloned into pBluescript KS using *Eco*RI (pKS2.4Z) and entirely sequenced using a random sonication shearing/shotgun sequencing approach. The entire 2.4Z cDNA is 3.84 kb, in close agreement with the transcript size estimate from northern analysis. Comparison of cDNA sequence with DNA flanking the P-element insertions indicated that both transposons had inserted into the 5′ end of this transcription unit. To verify further that this was the *foi* transcription unit, we sequenced genomic DNA from the three *foi* EMS alleles. Genomic DNA was prepared from homozygous *foi*-mutant embryos and the region corresponding to the identified transcription unit was PCR amplified and directly sequenced. Single base-pair mutations were identified in the single long open reading frame contained within this transcription unit in each of the three EMS alleles. All of these produced nonsense mutations which were located at amino acid positions 353, 630 and 635 ($foi^{20.71}$, $foi^{38.66}$ and $foi^{16.33}$, respectively) (see Fig. 4). Sequence comparisons of FOI and related family members were conducted using the BLAST algorithm at NCBI comparing individual FOI domains.

### Subcellular localization of FOI

FOI was expressed in Schneider S2 cells using the pUAST vector (Brand and Perrimon, 1993) driven by co-transfection with an actin-Gal4 plasmid (a gift from K. Howard). A PCR-based strategy was used to place a 3× hemagglutinin epitope (HA) tag in three positions within the FOI protein: the N terminus (after amino acid 189), between TM1-3 and TM4-6 (after amino acid 531) and the C terminus (after amino acid 704). These constructs were then transferred to the multiple cloning site of the pUAST vector (all cloning details available upon request). Tissue culture transfection was performed using the cationic liposome reagent Cellfectin (Invitrogen) as described by the manufacturer for suspension cells. Protein localization by immunofluorescence was essentially as described by Kast and Gros (Kast and Gros, 1997) except that 5% normal goat serum was used in blocking and cells were collected by centrifugation during washes rather than adhering to a substrate. Primary antibodies used (dilution) included: anti-HA (Boehringer Mannheim, 0.8 μg/ml), anti-GFP (Torrey Pines Biolabs, 1:200) and anti-CD8 (Caltag, 1:100). Alexa-fluor-conjugated secondary antibodies (Molecular Probes) were used at a 1:500 dilution after reconstitution according to manufacturer's instructions. Nuclei were stained with DAPI (2 μg/ml) and cells were mounted in 70% glycerol including DABCO (Sigma), and analyzed by deconvolution microscopy.

For examination of HA-FOI expression in embryos, the above UAS-HA-FOI constructs were transformed into the *Drosophila* genome (Rubin and Spradling, 1982) and crossed to the following Gal4 expressing lines: germ cell Gal4 [nosGal4VP16 (Van Doren et

al., 1998a)], mesoderm Gal4 [a combination of twist-Gal4 (Greig and Akam, 1993) and 24B-Gal4 (Brand and Perrimon, 1993) or 24B-Gal4 alone] or a tracheal Gal4 [breathless-Gal4 (Shiga et al., 1996)]. Embryos were immunostained as described above and analyzed by deconvolution microscopy or laser scanning microscopy as indicated.

### Tissue-specific rescue of *foi* mutants

A Gal4-dependent *foi* transgene (pUAS-2.4Z) was generated by using *Eco*RI to subclone the 2.4Z cDNA into the pUAST vector (Brand and Perrimon, 1993), and was transformed into the *Drosophila* genome (Rubin and Spradling, 1982). Independent UAS-FOI transgenes located on the third chromosome were recombined with the $foi^{20.71}$ allele. Relevant Gal4-expressing lines ('drivers'), also inserted on the third chromosome, were similarly recombined with $foi^{20.71}$. These stocks were balanced over *lacZ*-expressing balancer chromosomes and lines carrying the UAS-FOI transgene were crossed to lines carrying the Gal4 drivers to generate offspring that were homozygous mutant for *foi* at the endogenous locus, but which expressed the *foi* cDNA in a tissue-specific manner. Controls include crosses where only the Gal4 drivers or the UAS-FOI transgenes are present in a *foi*-mutant background. Gal4 drivers used included those expressing Gal4 in the germ cells (nosGal4VP16) (Van Doren et al., 1998a), mesoderm (twist-Gal4) (Greig and Akam, 1993) and trachea (breathless-Gal4) (Shiga et al., 1996). Embryos of the correct genotype (~40 hemi-embryos/genotype) were scored based on the phenotype of the gonad (judged by anti-VASA staining) and the trachea (judged by 2A12 staining). A similar approach was taken using UAS-HA-FOI transgenes. Although rescue of the tracheal and gonad phenotypes was achieved using different Gal4 drivers, we were unable to rescue the lethality of *foi* trans-heterozygotes using general UAS-FOI expression.

## RESULTS

### *fear of intimacy* is required for gonad coalescence but not gonadal cell identity

Previously, we conducted a large-scale screen for mutations affecting gonad formation in *Drosophila* (Moore et al., 1998). One class of mutations identified in this screen affects the coalescence of the embryonic gonad (Fig. 1). Germ cell migration occurs normally in these mutants, and the germ cells are able to correctly associate with the gonadal mesoderm (Fig. 1C). However, the germ cells fail to form the tight cluster typically found in a properly coalesced gonad, and instead remain only loosely aligned (compare Fig. 1A with 1B). We identified three such mutant lines in our screen which exhibit similar phenotypes in the gonad and trachea (below), and which all form a single complementation group. Because the germ cells fail to become intimately associated with one another in the gonad in these mutants, we call the gene represented by this complementation group *fear of intimacy* (*foi*).

We examined the gonadal mesoderm in *foi* mutants and found that this tissue is defective in its ability to undergo the morphogenetic movements of gonad coalescence. In what we interpret to be the strongest mutant phenotype, the cells of the gonadal mesoderm do not coalesce with the germ cells, and instead can be seen extending into the other tissues of the embryo (Fig. 2B). In embryos exhibiting a weaker phenotype, the gonadal mesoderm appears to partially coalesce but this process is incomplete, resulting in misshapen gonads (Fig. 2C). To determine if gonadal mesoderm coalescence was being blocked because the germ cells were in some way defective, we examined the morphogenesis of the gonadal mesoderm in embryos lacking germ cells. *oskar* (*osk*) is required for germ cell formation and specific alleles of *osk* cause complete germ cell loss without affecting other aspects of embryonic development (Lehmann and Nüsslein-Volhard, 1986). In such mutants, the gonadal mesoderm coalesces normally, even though these embryos lack germ cells (Brookman et al., 1992) (Fig. 2D). However, in *osk foi* double mutants, the gonadal mesoderm fails to coalesce (Fig. 2E), as is the case in *foi* mutants where germ cells are present (Fig. 2B). Thus, *foi* mutants are clearly defective in gonadal mesoderm morphogenesis independent of the germ cells (though this experiment does not exclude the possibility of an additional role for *foi* in the germ cells themselves).

A crucial question is whether *foi* affects gonadal mesoderm coalescence by altering the identity of these cells or by affecting their ability to carry out the appropriate morphogenetic program. To address this, we examined the expression of a number of molecular markers for the gonadal mesoderm, including the 412 retrotransposon RNA (Fig. 2) (Brookman et al., 1992), *Wnt2* (Kozopas et al., 1998), *Hmgcr* (Van Doren et al., 1998b), ZFH1 (Broihier et al., 1998) and EYA (Fig. 1C) (Boyle et al., 1997). In all cases, the expression of these markers in the gonadal mesoderm is unchanged in *foi* mutants, indicating that *foi* does not affect the identity of these cells (data not shown except for 412 and EYA). Furthermore, the cells of the gonadal mesoderm exhibit their normal behavior prior to coalescence. In *foi* mutants, these cells still form in three independent clusters that then join to form a

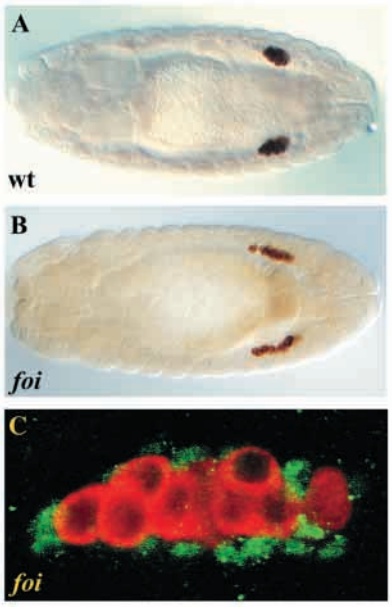


**Fig. 1.** *foi* mutants are blocked in gonad coalescence. (A,B) Stage 14 embryos immunostained with an anti-VASA antibody (brown) that labels the germ cells. Dorsal view, anterior left. (A) Wild-type embryo. Note that the germ cells have properly coalesced into the embryonic gonad. (B) *foi* mutant embryo. The germ cells have aligned on either side of the embryo, but have failed to coalesce into the gonad. (C) Confocal microscopy image showing the gonad region of a stage 14 *foi* mutant embryo double immunolabeled to reveal the germ cells (anti-VASA, red) and the gonadal mesoderm (anti-EYA, green). Note that although gonad coalescence is blocked, the germ cells have migrated to, and are correctly associating with, the gonadal mesoderm.

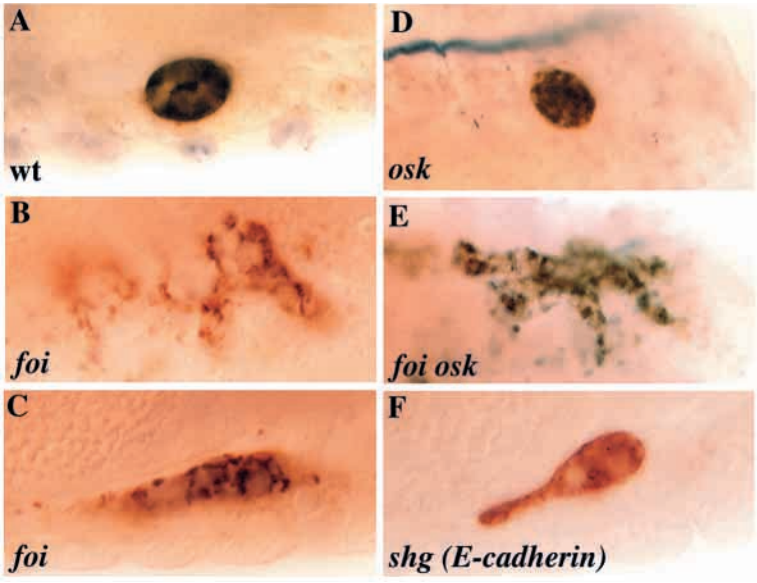


**Fig. 2.** Gonadal mesoderm phenotype of *foi* and *E-cadherin/shg.* Stage 14-15 embryos labeled by in situ hybridization to reveal expression of the 412 retrotransposon (brown), a gonadal mesoderm marker. (A) Wild type. The gonadal mesoderm has coalesced with the germ cells to form the embryonic gonad. (B,C) $foi^{16.33}$ mutant embryos showing examples of the severe (B) or moderate (C) gonad coalescence defects. Note that in B, the gonadal mesoderm cells fail to coalesce and, instead, extend into the neighboring mesoderm. In C, the gonadal mesoderm partially coalesces with the germ cells, but the gonad does not attain the compact, spherical appearance observed in wild type. (D,E) Embryos from *osk* mutant mothers ($foi^{20.71}$ $osk^{CE4}/osk^{301}$) aged at 18°C that lack germ cells but otherwise exhibit normal embryonic patterning. Embryo in E is also zygotically mutant for *foi* ($foi^{20.71}$ $osk^{CE4}/foi^{16.33}$). Note that the gonadal mesoderm coalesces normally in the absence of germ cells (D), but that the *foi* mutant gonad phenotype is still readily apparent under these conditions (E). (F) Embryo zygotically mutant for $shg^{IH}$ (*E-cadherin*). Note that the gonadal mesoderm fails to fully coalesce into an embryonic gonad.

single band of cells (Fig. 1C, Fig. 2B,E), similar to wild type. They also act as the target cells for germ cell migration, specifically associating with germ cells at embryonic stages 12-13 and remaining associated at later stages (Fig. 1C). This is in contrast to mutations in genes such as *eya* that affect gonadal mesoderm identity; the germ cells do not remain associated with the gonadal mesoderm in these mutants (Boyle et al., 1997). We conclude that *foi* does not affect the identity of the gonadal mesoderm and, instead, affects gonad coalescence by interfering with the process of morphogenesis downstream of cellular identity. We have also examined molecular markers for germ cell identity [*vasa* (*vas* – FlyBase)RNA (Van Doren et al., 1998a) and *Iswi* (Elfring et al., 1994)] and have found that the identity of the germ cells is similarly unaffected in *foi* mutants (data not shown).

### *foi* and E-cadherin share phenotypes in the gonad and trachea

*foi* mutants die at the end of embryogenesis, but no general defects were observed in the development of a variety of different tissues analyzed, including the nervous system, midgut, musculature and embryonic cuticle pattern (data not shown). We did, however, find one other tissue that exhibits defects in *foi* mutants: the developing tracheal system. The tracheal network develops from individual groups of cells that form tracheal branches within different segments of the embryo (Manning and Krasnow, 1993). Some of these branches must fuse with branches from neighboring segments to make a continuous network of tubules throughout the embryo (Fig. 3A). This process is controlled by the terminal cell in the fusion branches, termed the fusion tip cell. During tracheal branch fusion, fusion tip cells from neighboring branches specifically adhere to one another and form a lumen between them (Samakovlis et al., 1996b).

In *foi* mutants, tracheal cells appear to differentiate and form tracheal branches normally, but lateral trunk (LT) tracheal branch fusion is disrupted (Fig. 3). By labeling the tracheal cells with a cell surface marker (CD8-GFP), we observe that the fusion tip cells from some neighboring branches fail to meet properly and form a lumen between them (Fig. 3C,D). This phenotype is highly penetrant [94% of *foi*-mutant hemi-embryos had at least one LT fusion defect and 77% had two or more ($n$=66)] but is incompletely expressive [an average of 3.3 out of 9 LT branches/hemi-embryo were affected ($n$=66)] as judged by a lumen marker. Thin cellular extensions are sometimes observed between fusion tip cells of failed fusions (Fig. 3D), suggesting that these cells retain the ability to recognize one another. However, the main cell bodies are clearly displaced from one another and have not become closely associated as is observed in wild type (Fig. 3B). Rather than fusing and forming a lumen with the appropriate partners, the defective branches appear to remain independent, but are still capable of extending ganglionic branches (GBs) ventrally, which is the appropriate behavior for the properly fused LT branch. Thus, we see no evidence that the tracheal branches are defective in cell migration or branch extension in *foi* mutants, and the defect appears more specific to the process of branch fusion. To address further whether the fusion tip cells retained their proper identity, we analyzed the expression of the fusion tip cell marker *escargot* (*esg*) (Samakovlis et al., 1996b) in *foi* mutants using an enhancer trap in this gene. This marker is still expressed in *foi* mutants (Fig. 3E), even in fusion tip cells from branches that fail to fuse. Thus, as was observed for the gonad, *foi* mutants show defects in tracheal morphogenesis but not in cell identity.

The homotypic cell adhesion molecule E-cadherin has also been shown to be essential for the process of tracheal branch fusion (Uemura et al., 1996). The similarity of the *foi* and *E-cadherin* [known as *shotgun* (*shg*) in *Drosophila*] mutant phenotypes in the trachea prompted us to examine the role of E-cadherin in gonad morphogenesis. Interestingly, we find that *E-cadherin/shg* mutant embryos do indeed exhibit defects in gonad coalescence (Fig. 2F). In mutant embryos, the gonadal mesoderm begins to coalesce with the germ cells, but the gonads are misshapen and coalescence often does not proceed to completion. This phenotype closely resembles the ‘weak’ phenotype observed in *foi* mutants (Fig. 2C). One possible explanation for *E-cadherin/shg* exhibiting a weaker gonad

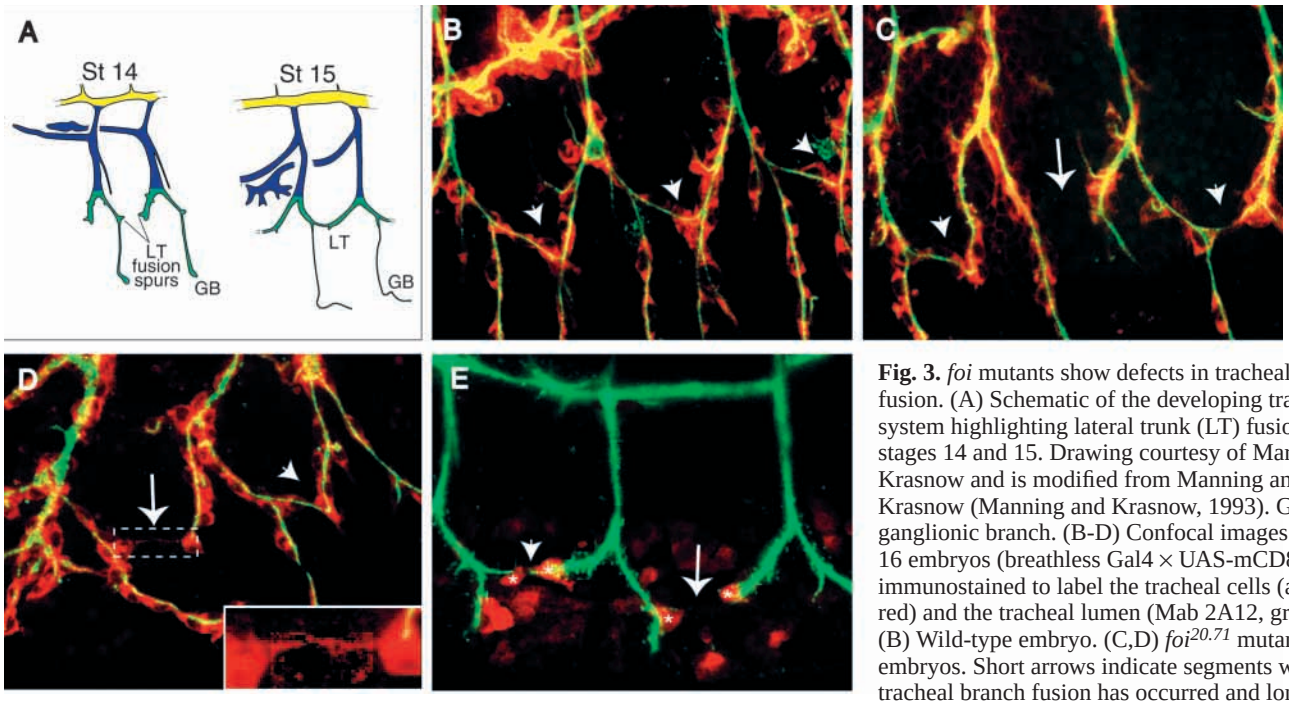


**Fig. 3.** *foi* mutants show defects in tracheal branch fusion. (A) Schematic of the developing tracheal system highlighting lateral trunk (LT) fusion during stages 14 and 15. Drawing courtesy of Mark Krasnow and is modified from Manning and Krasnow (Manning and Krasnow, 1993). GB, ganglionic branch. (B-D) Confocal images of stage 16 embryos (breathless Gal4 × UAS-mCD8-GFP) immunostained to label the tracheal cells (anti-GFP, red) and the tracheal lumen (Mab 2A12, green). (B) Wild-type embryo. (C,D) $foi^{20.71}$ mutant embryos. Short arrows indicate segments where tracheal branch fusion has occurred and long arrows indicate segments where fusion has failed to occur. Note that the ganglionic branches still extend ventrally (down) from segments with failed fusion. Inset in D highlights protrusions between fusion tip cells in a segment where fusion failed to occur. (E) Confocal image of a stage 15 $foi^{20.71}$ mutant embryo containing the $escargot^{G66B}$ enhancer trap, immunostained to reveal enhancer trap expression (anti-β-gal, red) and the tracheal lumen (Mab 2A12, green). Asterisks indicate fusion tip cells expressing the *esg* enhancer trap. Note that the fusion tip cells from the segment that has failed to fuse still express the enhancer trap. Short arrows indicate segments where tracheal branch fusion has occurred and long arrows indicate segments where fusion has failed to occur.

phenotype than *foi* is the substantial maternal contribution of *E-cadherin/shg*. As *E-cadherin/shg* is required for oogenesis, we were unable to analyze embryos where this maternal contribution was removed (Tepass et al., 1996; Uemura et al., 1996).

### *foi* is predicted to encode a member of a new family of transmembrane proteins

The specific manner in which *foi* affects both gonad and tracheal morphogenesis prompted us to pursue a molecular analysis of this gene. Recombination mapping indicated that *foi* was located on the left arm of chromosome 3 (3-25.2) and complementation tests identified two transposon insertion lines that failed to complement the phenotype of independent *foi* alleles. Experiments were performed to excise one of these transposon insertions, which demonstrated that the transposon was responsible for the *foi* phenotype in this line. Molecular analysis revealed that both transposons had inserted into the 5′ untranslated region of the same transcription unit which produces a 4 kb RNA as judged by northern blot (data not shown). We were able to identify several cDNAs corresponding to this transcript and the longest of these, which was 3.85 kb, was completely sequenced. Sequence analysis of genomic DNA from our three independent ethylmethane sulfonate-induced *foi* alleles revealed that all have nonsense mutations in the large open reading frame present in this cDNA (Fig. 4). These data, combined with the ability of this cDNA to rescue the *foi* mutant phenotype in transgenic animals (see below), led us to conclude that we have identified the *foi* transcription unit.

The FOI protein (Fig. 4) is predicted to be 706 amino acids in length and to have an N-terminal signal sequence and at least six transmembrane domains (TM1-3 and TM4-6). The highly conserved ‘HELP domain’ (see below) is weakly predicted by some algorithms to contain an additional 1-2 TM spans, and so the mature FOI protein is likely to have 6-8 TM spans in total. FOI also contains a histidine rich N-terminal domain and a short C-terminal tail.

Homology searches with FOI reveal that it is part of a larger family of proteins that are conserved from yeast to humans. Although only one FOI-related sequence currently appears in the genome databases of the fungi *S. cerevisae* and *S. pombe* and the plant *A. thaliana*, multiple family members are found in the genomes of animals such as *Drosophila* (four members), *C. elegans* (eight members), and humans (six members). In animals, this family can be divided into two subgroups, one more closely related to FOI and a second that is more related to another *Drosophila* protein CATSUP (Stathakis et al., 1999) (Fig. 4). For example, *Drosophila* FOI is more closely related to human LIV1 (Manning et al., 1988) than it is to *Drosophila* CATSUP. Likewise, *Drosophila* CATSUP is more closely related to human KE4 (Ando et al., 1996) than it is to *Drosophila* FOI. Thus, this family seems to have split into two subgroups prior to the divergence of protostome and deuterostome metazoans. As the founding members of this family include FOI, IAR1 (*Arabidopsis*) (Lasswell et al., 2000), CATSUP and LIV1, we will refer to this family of proteins as the FICL family.

Members of the FICL family share several regions of sequence homology as well as an overall similar domain

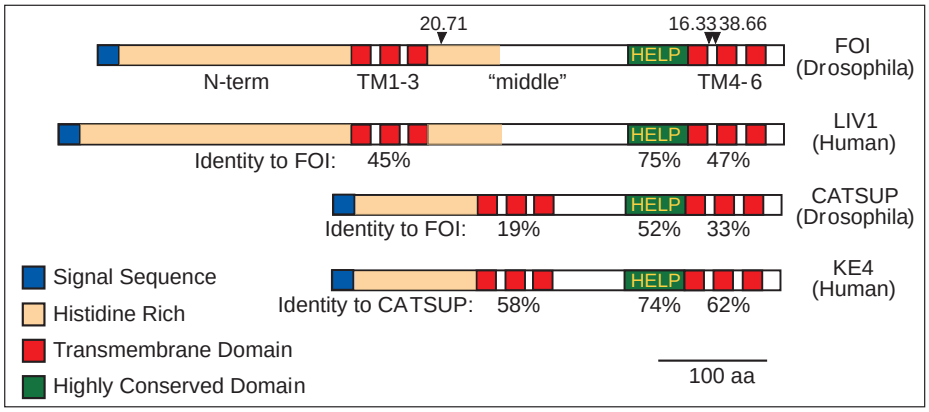


**Fig. 4.** FOI protein and selected FICL family members. Protein domains are as indicated in the key. Arrowheads above FOI show the position of nonsense mutations found in the three EMS-induced *foi* mutants. Percentages reflect the percent identical amino acids within the designated domains when compared to FOI or CATSUP as indicated.

structure and predicted membrane topology (Fig. 4). They each have a long N terminus that is histidine rich and contains several putative glycosylation sites, but does not show sequence homology. The TM domains, however, share considerable sequence homology. This homology appears to be more extensive than would be necessary to maintain their transmembrane character, and there are a number of invariant residues in these domains. Thus, these sequences may play a more specific role in FICL protein function in addition to their structural role in forming TM spans.

The most highly conserved region of the FICL family is the 'HELP' domain (named after a conserved amino acid cluster usually found in this domain). This domain is the region that shows the highest sequence identity throughout the family, and is also part of a larger domain family found in prokaryotes (ProDom analysis). This domain is 75% identical (90% similar) in *Drosophila* FOI and human LIV1, and 33% identical (47% similar) in FOI and the prokaryotic *M. xanthus* GufA protein (McGowan et al., 1993). Although the FICL family has clearly been well conserved across a broad evolutionary spectrum, little is known about how these proteins function at the molecular level.

### *foi* expression and subcellular localization

To begin to address how FOI might act in gonad coalescence, we first examined the expression pattern of the *foi* transcript. *foi* RNA that is likely to be maternal in origin is found throughout the early embryo with a higher concentration present at the posterior pole. This posteriorly localized RNA is taken up by the pole cells (future germ cells) as they form (Fig. 5A), while the remaining maternal transcript is degraded. Although the localization of *foi* transcript to the germ cells is intriguing, we have not found any function for this maternal RNA. Offspring that lack maternal *foi* activity (produced from homozygous germline clones in mosaic females) show no developmental defects and grow up to be fertile adults. In addition, the gonad and tracheal phenotypes associated with removing *foi* activity both maternally and zygotically are not more severe than the zygotic phenotypes alone (data not shown).

*foi* shows zygotic expression in a number of tissues, including general expression during gastrulation that is stronger in the invaginating mesoderm (Fig. 5B). Slightly later, the general expression is reduced, while high-level expression appears in the anterior and posterior endoderm primordia (stage 9, Fig. 5C). During the time of gonad coalescence (stage 14), broad expression is observed in the embryo, including the mesoderm, whereas *foi* is less highly-expressed in the epidermis (Fig. 5D).

As the FOI protein is predicted to contain multiple transmembrane spans, it should be localized either to the cell surface, or to a membrane bound cellular compartment. Although we have not yet been successful in raising antisera

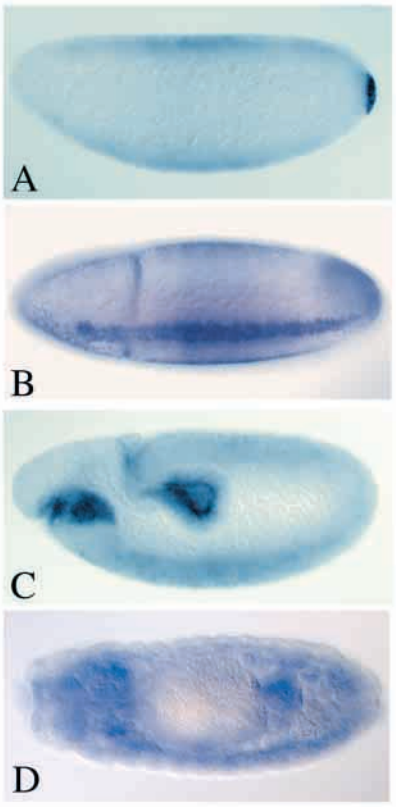


**Fig. 5.** *foi* RNA expression pattern. Embryos labeled by in situ hybridization revealing the *foi* RNA expression pattern (purple). (A) Stage 3 embryo. The maternal *foi* RNA has almost completely disappeared except for in the pole cells (posterior, right). (B) Stage 6 embryo. *foi* is expressed generally but is enriched in the invaginating mesoderm. (C) Stage 9 embryo. Note the high levels of *foi* RNA in the anterior and posterior midgut primordia. (D) Stage 14 embryo. *foi* is broadly expressed, but only low levels are found in the epidermis.

that recognize the endogenous FOI protein, we have examined the subcellular localization of FOI using epitope-tagged versions of the protein. We generated three versions of FOI where the hemagglutinin (HA) epitope tag (Wilson et al., 1984) was placed either in the N-terminal domain, the domain between TM1-3 and TM4-6, or the C-terminal domain. Constructs expressing these proteins were then transfected into *Drosophila* tissue culture cells (Schneider S2) and the subcellular localization of FOI was determined by immunofluorescence using anti-HA antibodies (Fig. 6A). We observed that FOI is localized to the cell surface, and very little staining was observed intracellularly. FOI co-localized with a control plasma membrane protein (CD8-GFP) (Lee and Luo, 1999), confirming its cell surface localization (data not shown). It is unlikely that the cell surface localization is due to overwhelming a system for localizing FOI to a subcellular compartment, because we detect little FOI protein intracellularly and even weakly expressing cells show FOI on the cell surface. Identical results were obtained for all three HA-tagged versions of FOI, making it also unlikely that the epitope tag is interfering with the normal subcellular localization of FOI. Finally, both the N-terminal and C-terminal epitope tagged versions of FOI are able to rescue the *foi*-mutant phenotype in a transgenic rescue assay (e.g. Fig. 7D), indicating that these proteins retain wild-type activity.

To observe the subcellular localization of FOI in the embryo, we generated transgenic lines expressing HA-FOI from a Gal4-responsive promoter (UAS) (Brand and Perrimon, 1993), and used this to express HA-FOI in specific tissues in the embryo. We first examined HA-FOI expression in the germ cells, as the large size and spherical shape of these cells allows for a more accurate assessment of subcellular localization (Fig. 6B). HA-FOI is clearly localized to the surface of these cells. To examine HA-FOI in the somatic cells of the gonad, we expressed it throughout the embryonic mesoderm (Fig. 6C). Although the small size of these cells makes subcellular localization difficult to assess, we observe a restricted staining pattern that is consistent with HA-FOI being at the cell surface. HA immunoreactivity is found along the borders between the gonadal mesoderm and the germ cells (arrowheads), and around each germ cell. We have found that the gonadal mesoderm cells extend cellular projections that ensheath each individual germ cell (A. Jenkins and M.V.D., unpublished). HA-FOI appears to localize to these cellular projections. HA-FOI shows significant co-localization with the membrane associated protein Discs Large (DLG) (inset, Fig. 6C), further indicating that FOI is present at the cell surface in the gonadal mesoderm. Finally, we analyzed HA-FOI expression in the trachea (Fig. 6D). HA-FOI is preferentially localized to the cell periphery, and can also be observed on cellular extensions between the fusion tip cells during tracheal branch fusion. Thus, an epitope-tagged version of FOI that is competent to rescue FOI activity exhibits cell surface localization in both tissue culture cells and the embryo. Although FOI is likely to also be present in the secretory pathway on its way to the plasma membrane, the localization of FOI suggests that it normally functions as a cell-surface protein.

### *foi* rescue and tissue-specific function

The *foi* RNA expression pattern does not specifically indicate in which tissues *foi* might be acting. To further address this issue, and to verify that we have correctly identified the *foi* transcription unit, we attempted to rescue the *foi*-mutant phenotype using tissue-specific FOI expression. These experiments were done largely with non-HA-tagged versions of FOI (Fig. 7A,B), but similar rescue is observed with N- and C-terminally HA-tagged versions of FOI (e.g. Fig. 7D). As shown in Fig. 7A, expression of UAS-FOI in the mesoderm of a *foi* mutant is sufficient to rescue the gonad coalescence defect. Expression of UAS-FOI in the germ cells is unable to rescue this phenotype. Thus, *foi* is required within the mesoderm for gonad coalescence. Expression of UAS-FOI

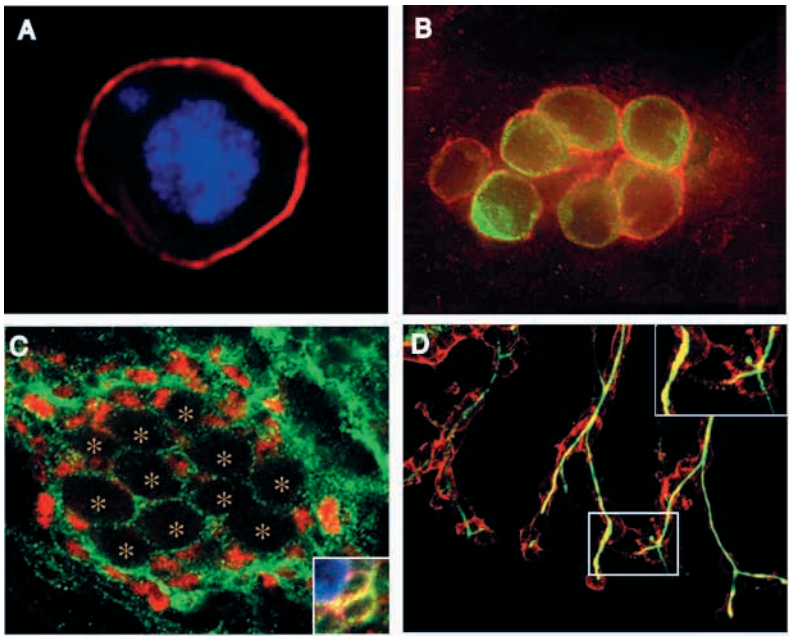


**Fig. 6.** Subcellular localization of HA-FOI. (A) Deconvolution microscopy image of a Schneider S2 cell expressing HA-FOI immunolabeled with an anti-HA antibody (red) and stained with DAPI to reveal the nucleus (DNA, blue). Note that the FOI protein is localized to the plasma membrane.
(B) Deconvolution microscopy image of a stage 14 embryonic gonad expressing HA-FOI in the germ cells, immunostained for a germ cell marker (anti-VASA, green) and HA-FOI (red). HA-FOI is predominantly localized to the germ cell surface. (C) Confocal image of a stage 16 embryonic gonad expressing HA-FOI in the mesoderm (expression driven by twist 24B gal4), including gonadal mesoderm, immunostained to label the gonadal mesoderm (anti-ZFH-1, red) and HA-FOI (green). Note that HA-FOI is observed surrounding each germ cell (asterisks), although the germ cells are not expressing HA-FOI in this experiment. HA-FOI partially co-localizes with DLG at the surface of gonadal mesoderm cells (inset) in a stage 16 embryo immunostained to label DLG (red), HA-FOI (green, expression driven by 24B Gal4) and germ cells (anti-vasa, blue). (D) Confocal image of a stage 15 embryo expressing HA-FOI in the trachea, immunostained to reveal the tracheal lumen (Mab 2A12, green) and HA-FOI (red). Lateral trunk branches are shown. HA-FOI appears to localize to the cell periphery and also to extensions between fusion tip cells during branch fusion (inset). In all panels shown, the HA-tag is in the N-terminal region of FOI.

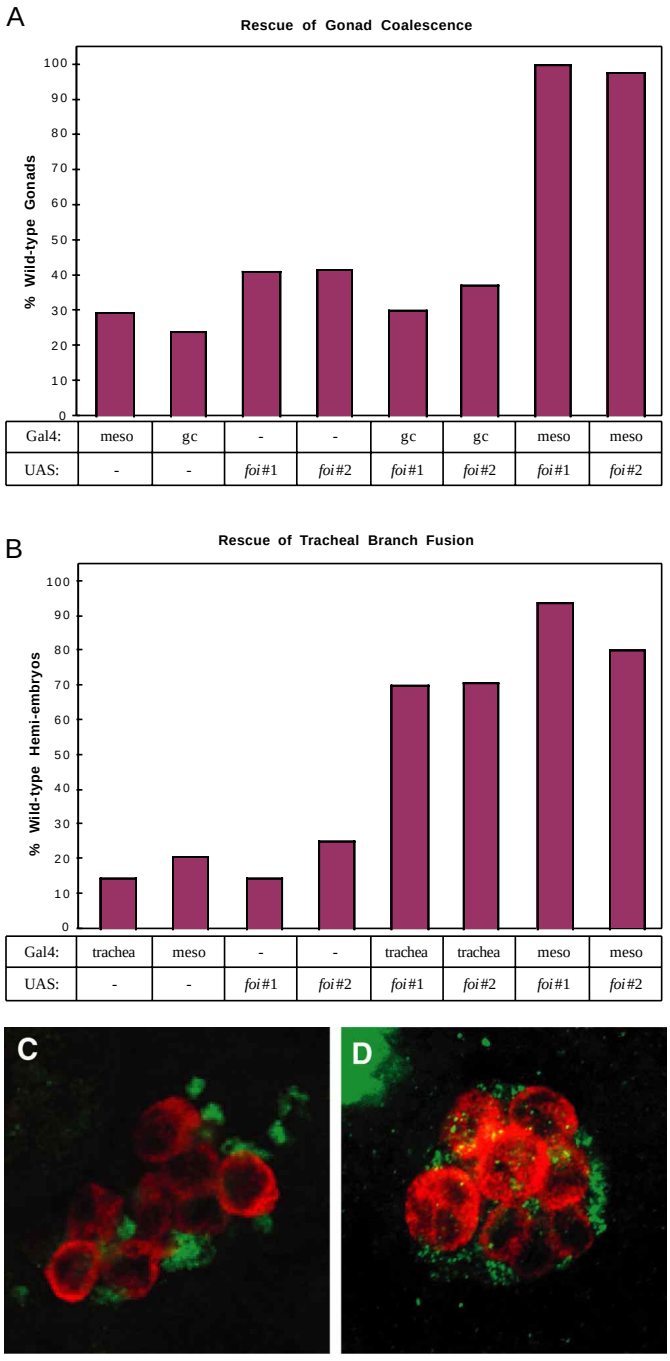


**Fig. 7.** Tissue-specific rescue of the *foi* mutant phenotypes. In all cases, embryos are mutant for the endogenous *foi* gene ($foi^{20.71}$) and rescue experiments were conducted as described in the Materials and Methods. Gal4 indicates the tissue expressing the Gal4 transcriptional activator (meso, mesoderm; gc, germ cells). UAS indicates whether a UAS-*foi* expression transgene was present and which one. (A) Rescue of the *foi* gonad phenotype. Expression of *foi* in the mesoderm fully rescues the *foi* mutant gonad defect, while expression of *foi* in the germ cells shows no rescue. (B) Rescue of the *foi* tracheal phenotype. Expression of *foi* in either the trachea or mesoderm rescues the tracheal fusion defect. (C,D) Stage 15 gonads immunostained to reveal the germ cells (anti-VASA, red) and the gonadal mesoderm (anti-EYA, green). (C) *foi* mutant showing defective gonad coalescence. (D) *foi* mutant rescued by expression of HA-FOI (C-terminal tag) in the mesoderm. Coalescence is normal.

within tracheal cells is able to rescue the tracheal fusion defect of *foi* mutants (Fig. 7B); however, mesodermal expression is also able to rescue. Whether rescue of the tracheal phenotype by the mesoderm Gal4 represents a non-autonomous role for *foi*, or is due to low-level expression of this driver in the trachea, can be addressed in the future with more traditional genetic mosaic analysis.

## DISCUSSION

We have identified a new protein, FOI, that is essential for gonad morphogenesis in *Drosophila*, and that is part of a conserved family of transmembrane proteins found in diverse species. The *foi* loss-of-function phenotype is unique in that gonad coalescence is blocked, but development of the germ cells and gonadal mesoderm is completely normal prior to this event. FOI is also required for proper tracheal branch fusion, but not for other aspects of tracheal cell behavior or fusion tip cell marker expression. This indicates that *foi* does not affect cell identity, but is instead required for cells to translate their identity into the proper form and pattern of the embryonic tissues. Thus, FOI may act during a critical window of organogenesis, where discrete cell types undergo coordinated morphogenesis to create the final architecture of a tissue.

### Role of FOI in gonad morphogenesis

*foi* mutants exhibit a highly-specific gonad phenotype. Not only are molecular markers for the germ cells and somatic cells of the gonad still expressed, but these cells undergo the initial morphogenic movements required for gonad formation, including the proper association of the germ cells and gonadal mesoderm. What is defective is the ability of these cells together to undergo the transition from a loosely associated tissue to the tightly compacted and patterned embryonic gonad. There are several morphogenetic processes that could contribute to such a transition in tissue architecture. FOI does not appear to be affecting cell death or cell division as we have not observed dramatic changes in cell number between wild-type and *foi*-mutant gonads. Instead, it is likely that FOI is affecting changes in cell-cell contact or cell shape that may be required for gonad coalescence. Coalescence of the gonad does not require the presence of the germ cells, indicating that the gonadal mesoderm may be 'driving' this process, and we have found that *foi* is required in the mesoderm. Thus, our current hypothesis is that FOI is essential for changes in cell-cell contact or cell shape within the gonadal mesoderm that mediate the transition of this tissue from an uncoalesced to a coalesced gonad.

### Molecular mechanism of action of FOI and the FICL family

Our sequence database analysis indicates that the FICL family of transmembrane proteins is ancient in origin, yet has expanded in animals to include multiple family members and independent subgroups that are likely to have diverged functions. Although members of the FICL family are well represented in the databases, little is known about the function of any family member. Loss-of-function mutations in bacterial (*M. xanthus*) and yeast (*S. cerevisae*) family members are viable with no growth defects on rich medium (McGowan et al., 1993) (Saccharomyces Genome Database), but have

apparently not been further analyzed. In *Arabidopsis*, mutations in *IAR1* confer resistance to high levels of conjugated auxins (Lasswell et al., 2000), and IAR1 is therefore likely to be important for the uptake or metabolism of these hormone derivatives. In *Drosophila*, mutations in *Catsup* lead to elevated catecholamine levels due to increased activity of the rate-limiting enzyme in this pathway, tyrosine hydroxylase (TH) (Stathakis et al., 1999). Thus, the CATSUP protein may act as a negative regulator of TH activity. Virtually nothing is known about how this family of proteins functions at the molecular level to control such apparently different cellular processes.

Our data indicate that FOI is a cell-surface protein and is required in the mesoderm for gonad coalescence. This suggests several models for how FOI might be acting at the molecular level. First of all, FOI might act in cell adhesion, either directly via its extracellular domains or by regulating the activity of a cell adhesion molecule such as E-cadherin. The lack of clear sequence homology within the putative extracellular N-terminal domain in the FICL family suggests that either this domain is not acting in protein-protein interaction, or that different FICL family members have very different binding partners. FOI might also be involved in contacting and regulating the cytoskeleton, which is likely to mediate the changes in cellular morphology observed during gonad coalescence. Such a role might include affecting cytoskeletal changes in response to signals or providing contact between the cytoskeleton and the cell-surface or cell-cell junctions. Finally, FOI might act in sending or receiving a signal that is required for the onset of gonad coalescence. In this capacity, FOI might act non-autonomously in the surrounding non-gonadal mesoderm to produce a signal to the gonad, or autonomously within the gonadal mesoderm to respond to this signal and initiate gonad morphogenesis.

Recently, epitope-tagged versions of two other FICL family members, ermelin and KE4, have been reported to localize to the endoplasmic reticulum when expressed in tissue culture (Suzuki and Endo, 2002). Our data in both tissue culture and in embryos with functional HA-FOI transgenes indicates that FOI is localized to the cell surface. Thus, different FICL family members may have distinct subcellular localizations.

As FICL family members are predicted to have multiple transmembrane domains, an interesting possibility is that these proteins act as channels, either alone or as homo- or heteromultimers. For example, gonad morphogenesis might be initiated or coordinated by an intercellular signal that involves membrane transport by FOI or cell adhesion might be regulated by transport of a required ion or small molecule effector. In support of the channel model, the TM domains of FOI show sequence homology with other FICL family members. This homology appears to be more extensive than would be necessary to simply retain TM character, and suggests that the primary sequence of these domains is critical for some aspect of FOI function, such as the formation of a transmembrane channel. Sequence comparisons have revealed some homology between the ZIP family of metal transporters and members of the FICL family (Eng et al., 1998). However, there are many regions of homology that discriminate between the ZIP and FICL families, and there are several 'true' ZIP family members in both the human and *Drosophila* genome databases. Thus, the ZIP and FICL families may be evolutionarily related in a more distant manner, but this does not necessarily indicate that the FICL proteins will also be metal transporters. Whether FICL family members act as channels at all, and what their substrates might be, are interesting questions for future analysis.

### FOI and E-cadherin

*foi* and *E-cadherin* share similar mutant phenotypes in gonad coalescence and tracheal branch fusion. This suggests that there is a common molecular mechanism at work in both gonad and tracheal morphogenesis, and that E-cadherin and FOI may be cooperating to mediate this common mechanism. In the gonad, E-cadherin-based cell adhesion might act to promote proper cell-cell contacts required for coalescence and gonad organization. An important aspect of the mechanism of action of FOI may be to somehow modulate E-cadherin based cell adhesion. In support of this, we have found that E-cadherin expression increases in the gonadal mesoderm at the time that coalescence begins, and that E-cadherin expression in the gonad is drastically reduced in *foi* mutants (A. Jenkins and M.V.D, unpublished).

The relationship between FOI and E-cadherin is particularly interesting as the closest homolog of FOI in humans, LIV1, was identified as an estrogen-responsive gene in breast cancer cells (Manning et al., 1988). LIV1 expression has been correlated with mammary tumor metastasis (Manning et al., 1994). E-cadherin is also known to play an important role in regulating metastatic potential in a variety of human cancers, with downregulation being correlated with increased metastasis (reviewed by Wheelock et al., 2001) and upregulation being found at the site of secondary tumor formation (Bukholm et al., 2000). Our analysis of FOI in *Drosophila* suggests that LIV1 and E-cadherin may be working together during breast cancer progression.

### Gonad formation

Gonad formation and gametogenesis are essential for the fundamental process of sexual reproduction, and are therefore likely to be evolutionarily conserved. There are many parallels between gonad formation in mammals and in *Drosophila*, and these parallels may well extend to the molecular level. Formation of the mouse gonad, for example, involves very similar stages of germ cell migration, association between germ cells and gonadal mesoderm, and gonad coalescence as we see in *Drosophila*. Furthermore, it has recently been shown that E-cadherin has a role in mouse gonad formation, and appears to function in the germ cells for their proper coalescence into the developing gonad (Bendel-Stenzel et al., 2000). We have also demonstrated a role for E-cadherin in *Drosophila* gonad coalescence, although our evidence points to roles for E-cadherin in both the germ cells and the gonadal mesoderm (A. Jenkins and M.V.D., unpublished). It is intriguing to speculate that a *foi* homolog may also function with E-cadherin in mouse gonad formation. Thus, as has been true for other developmental processes, understanding the mechanisms of gonad formation in *Drosophila* may provide a molecular picture of how this process works in other species.

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