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Drosophila E-cadherin is essential for proper germ cell-soma interaction during gonad morphogenesis

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SUMMARY

In most animal species, germ cells require intimate contact with specialized somatic cells in the gonad for their proper development. We have analyzed the establishment of germ cell-soma interaction during embryonic gonad formation in *Drosophila melanogaster*, and find that somatic cells undergo dramatic changes in cell shape and individually ensheath germ cells as the gonad coalesces. Germ cell ensheathment is independent of other aspects of gonad formation, indicating that separate morphogenic processes are at work during gonadogenesis. The cell-cell adhesion molecule *Drosophila* E-cadherin is essential both for germ cell ensheathment and gonad compaction, and is upregulated in the somatic gonad at the time of gonad formation. Our data indicate that differential cell adhesion contributes to cell sorting and the formation of proper gonad architecture. In addition, we find that Fear of Intimacy, a novel transmembrane protein, is also required for both germ cell ensheathment and gonad compaction. E-cadherin expression in the gonad is dramatically decreased in *fear of intimacy* mutants, indicating that Fear of Intimacy may be a regulator of E-cadherin expression or function.

Key words: *Drosophila*, E-cadherin, *fear of intimacy*, Gonad coalescence, Organogenesis, Germline-soma interaction

INTRODUCTION

During development, complex organs are formed from individual cells and simple tissues. Organogenesis is characterized by many cellular behaviors, including cell migration, modification of cell contacts, changes in cell shape, cell proliferation and cell death. Extensive communication between cells ensures that different cell types act together in a coordinated manner to form a properly patterned, functional organ. Gonad formation provides an excellent model for studying organogenesis. The germ cells and specialized somatic cells that make up the gonad are initially located far apart from one another, and must undergo a series of morphogenic movements to come together and create the proper gonad architecture. This process is crucial for continued germ cell development, gametogenesis and the propagation of a species.

In Drosophila, germ cells are formed at the posterior pole during the syncytial blastoderm stage of embryogenesis. Gastrulation brings the germ cells to the interior of the embryo, after which they actively migrate through the midgut epithelium and into the mesoderm (reviewed by Starz-Gaiano and Lehmann, 2001). They then make contact with specialized mesodermal cells with which they form the gonad (Sonnenblick, 1950), known as somatic gonadal precursors (SGPs) (Boyle et al., 1997). SGPs are specified in bilateral clusters within parasegments (PS) 10, 11 and 12 (Brookman et al., 1992), and can be identified by their expression of the nuclear proteins Eyes Absent (EYA) (Boyle et al., 1997) and ZFH-1 (Broihier et al., 1998). They arise within the *eve* domain of the dorsolateral mesoderm, where groups of cells are selected to become either SGPs or fat body. The repressive effects of *serpent* and the positive regulatory effects of *abdA* limit the SGPs to PS10, 11 and 12 (Riechmann et al., 1998; Moore et al., 1998a; Hayes et al., 2001). During gonad formation, the three clusters of SGPs come together to form a band of cells on each side of the embryo as the germ cells end their migration, and the two cell types associate along PS10-12. Germ cells and SGPs then undergo gonad coalescence to form a rounded structure in PS10.

In addition to SGPs that are specified in PS10, 11 and 12, there is an additional cluster of somatic mesoderm cells, called msSGPs (for male-specific SGPs), that arises in PS13 (DeFalco et al., 2003). msSGPs can be distinguished from the SGPs by co-expression of EYA and the nuclear protein Sox100B (DeFalco et al., 2003). As the germ cells and SGPs coalesce to form the gonad, the msSGPs move anteriorly and join the posterior of the gonad specifically in males (DeFalco et al., 2003).

Gonad coalescence involves the concerted movements of germ cells and SGPs as they transition from a broad association of cells into a condensed and organized gonad. Coalescence is

complete by the start of embryonic stage 15, with the gonad assuming a compact, spherical shape. Mutations in *iab4*, a *cis*-regulatory region of *abdA*, or *eya* specifically block gonad coalescence. In these mutants, initial specification of the SGPs is normal, and the germ cells successfully migrate to them. However, instead of coming together into a rounded organ, the SGPs arrest in PS10-PS11 and the germ cells scatter throughout the embryo (Cumberledge et al., 1992; Boyle and DiNardo, 1995; Boyle et al., 1997). In both *iab4* and *eya* mutants, this phenotype is attributed to a failure in late SGP differentiation.

abdA and *eya* are examples of genes that specify SGP identity. The downstream genes that coordinate the morphogenic movements of gonad formation are still unknown. One candidate is the *Drosophila* homolog of E-cadherin, a transmembrane cell adhesion molecule that plays a major role in tissue morphogenesis (reviewed by Tepass, 1999). E-cadherin typically acts in homophilic cell adhesion, binding to E-cadherin molecules on opposing cells and connecting to the cytoskeleton via its partner proteins α- and β-catenin (reviewed by Yap et al., 1997). *Drosophila* E-cadherin is encoded by the *shotgun* (*shg*) locus (Tepass et al., 1996; Uemura et al., 1996). Mutations in *shg* cause defects in gonad coalescence (Van Doren et al., 2003), but the role that E-cadherin plays in embryonic gonad morphogenesis in *Drosophila* has not yet been analyzed. Another gene thought to control gonad morphogenesis downstream of gonad cell identity is *fear of intimacy* (*foi*). FOI is a member of a novel, conserved family of transmembrane proteins of unknown function. *foi* mutants exhibit defects in both gonad coalescence and tracheal branch fusion, as is also observed in *shg* mutants (Tanaka-Matakatsu et al., 1996; Van Doren et al., 2003). The similarities of the *shg* and *foi* mutant phenotypes suggest that they may cooperate in the same or related pathways to control morphogenic events.

We present work that furthers our understanding of both the cellular and molecular events that control gonad formation. Through a detailed analysis of gonad coalescence, we have found that germ cells and SGPs interact intimately from the moment they associate, with SGPs undergoing dramatic changes in cellular morphology as they individually ensheath each germ cell in the gonad. Furthermore, we show that *Drosophila* E-cadherin is upregulated in the gonad at the time of gonad coalescence, and its function is crucial for several aspects of gonad morphogenesis, including the ensheathment of germ cells by SGPs. E-cadherin expression is dependent on *eya*, providing a molecular link between cell identity and the morphogenic movements of gonad coalescence. Finally, we show that FOI is required for proper E-cadherin protein expression in the gonad, suggesting that improper regulation of E-cadherin is the basis for the *foi* mutant gonad phenotype.

MATERIALS AND METHODS

Fly stocks

w^{1118} and *ru st fafl e ca* flies (Moore et al., 1998b) carrying a *fat facets-lacZ* transgene on the third chromosome (Fischer-Vize et al., 1992) were used as wild-type stocks. *Df(1)JA27/Fm7, Kruppel-Gal4, UAS-GFP* flies (Bloomington) were used to determine embryonic sex for TEM analysis (see below). *24B-Gal4* (Brand and Perrimon, 1993) and *twist-Gal4* (Baylies and Bate, 1996) are mesoderm-specific drivers, while *nos 3′UTR-VP16-Gal4* (nos-Gal4) (Van Doren et al., 1998) drives gene expression in the germ cells. *UAS-mCD8::GFP* flies were provided by L. Luo (Lee and Luo, 1999). *UAS-DE-cadherin*^{5,9} transgenic flies, which are homozygous for two independent insertions of a *UAS-DE-cadherin* transgene, were provided by J. P. Vincent (Sanson et al., 1996). *shg* alleles and *df(2R)E2* were provided by V. Hartenstein and are described elsewhere (Tepass et al., 1996). *shg*^{IH} is a strong allele, and *shg*^{G317} appears to be stronger than a zygotic null, and may act in a dominant-negative manner to counteract the maternal contribution of *shg*. *foi* alleles *foi*$^{16.33}$ and *foi*$^{20.71}$ were used for phenotypic analysis (Moore et al., 1998b). *eya*$^{Cli\text{-}IID}$ flies were obtained from the Bloomington Stock Center. The P-element insertion line *68-77* (Simon et al., 1990), which expresses *lacZ* in the gonadal mesoderm, was provided by D. Godt. *osk*^{301}/*osk*^{CE4} female flies (Lehmann and Nüsslein-Volhard, 1986) were mated at 18°C to wild-type males to produce agametic embryos. *esg*^{G66B}/CyO flies (Whiteley et al., 1992) were mated with w^{1118} flies to mark the anterior of the gonad with β-galactosidase in Fig. 3D. All mutations are maintained over balancer chromosomes carrying *lacZ* or GFP transgenes to identify homozygous mutant embryos.

Immunohistochemistry

The following antibodies (dilutions, sources) were used: rabbit or mouse anti-β-galactosidase (1:10000, Capel and Promega), rabbit anti-VASA (1:5000, R. Lehmann), rabbit anti-ZFH-1 (1:5000, R. Lehmann), chick anti-VASA (1:5000, K. Howard), rat anti-*Drosophila* E-cadherin (DCAD2 and DCAD1, 1:20, T. Uemura), rabbit anti-GFP (1:1000, Torrey Pines Biolabs) and rabbit anti-Sox100B (1:2000, S. Russell). Mouse anti-EYA 10H6 (1:25, S. Benzer and N. Bonini), mouse anti-ARM N2 7A1 (1:100, E. Wieschaus) and mouse anti-NRT BP106 (1:10, C. Goodman) were provided by the Developmental Studies Hybridoma Bank. Fluorescently conjugated secondary antibodies were used at 1:500 (Molecular Probes, Rockland, and Amersham Pharmacia Biotech).

Tissue fixation for anti-DCAD2 and anti-DCAD1 was as described (Rothwell and Sullivan, 2000) with the following modifications. Embryos were dechorionated in 50% bleach, washed with 1× PBS + 0.1% Triton (PBTx), fixed in 4% formaldehyde in 1.75 ml PCM (100 mM PIPES pH 6.9, 1 mM $CaCl_2$, 2 mM $MgSO_4$) and 8 ml heptane for 20 minutes at room temperature and transferred to 3MM Whatman paper to allow the heptane to evaporate. Embryos were then transferred to double-sided tape and covered with PBTx. Vitelline membranes were removed by hand under a dissecting scope with a 25-gauge needle and transferred in PBTx to a 2 ml screw cap vial. All subsequent incubations were done on an upright shaker in BBTx (1× PBS, 1% BSA, 0.3% Triton). Embryo fixation for all other antibodies and the immunolabeling protocol were as described (Moore et al., 1998b). For most genotypes, embryos were staged by gut development according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). Because *shg* mutants display defects in gut formation, *shg* embryos were aged for 11.5 hours at 25°C after egg laying (AEL) to yield collections of embryos stage 15 and older. Embryos were mounted in 70% glycerol containing 2.5% DABCO (Sigma) and viewed on a Leica NT or Zeiss 510 Meta confocal microscope. Occasionally two Z-sections through a stage 13 gonad were stacked together in order to display the gonad in one image. Image brightness and contrast were adjusted with Adobe Photoshop 6.0. Germ cell ensheathment was quantitated using single confocal sections through embryos expressing *UAS-mCD8-GFP* and the mesoderm-specific *twist*-Gal4. Percentage values, in 25% increments, were assigned to each germ cell to represent the amount of its surface surrounded by GFP signal.

Electron microscopy

Four-hour embryo collections from the mating of *fafl* females and *Fm7, Kruppel-Gal4, UAS-GFP/Y* males were aged for 20 hours at

18°C. Female offspring were distinguished by the presence of the Kruppel-GFP X chromosome. Embryos were dechorionated in 50% bleach and sorted under a fluorescence dissecting scope. Male and female embryos were fixed separately in 10 ml of heptane (previously saturated with 25% glutaraldehyde and 2% acrolein contained in a 100 mM cacodylate buffer, pH 7.4) for 15 minutes at room temperature. The heptane was removed, and the embryos were transferred to double-sided tape and covered with 3% formaldehyde, 2% glutaraldehyde, 0.5% DMSO in 100 mM cacodylate buffer, pH 7.4. After 15 minutes, the embryos were hand-devitellinized with a 25-gauge needle and fixed for an additional 2 hours at room temperature. Embryos were post-fixed in 1% OsO_4 containing 0.1% potassium ferrocyanide, 100 mM cacodylate and 5 mM $CaCl_2$, pH 6.8 for 30 minutes at room temperature, then washed in H_2O four times over 10 minutes. They were transferred to 1% thiocarbohydrazide contained in H_2O for 5 minutes, washed four times in H_2O over 10 minutes, and transferred to 1% OsO_4/1% potassium ferrocyanide in cacodylate buffer, pH 6.8, for 5 minutes at room temperature. Embryos were then placed into Kellenberger's uranyl acetate overnight at room temperature, dehydrated through a graded series of ethanol and subsequently embedded in Spurr resin. Sagittal or transverse sections were cut on a Leica UCT ultramicrotome, placed onto monohole formvar/carbon coated grids, stained in 2% uranyl acetate and lead citrate, and observed on a Philips EM 420 or 410 TEM. Images were recorded using Kodak SO-163 electron image film or a Megaview III digital camera. The cells in Fig. 2 were traced and colored using plasma membranes as a guide with Adobe Photoshop 6.0.

RESULTS

SGPs individually ensheath germ cells during gonad formation

It is likely that the dramatic morphogenic movements of gonad coalescence require equally dramatic changes in individual cell shape and cell-cell interactions. To examine these changes, we used an exogenous marker that would allow us to visualize the cell surface of either germ cells or SGPs independently. The marker, mCD8-GFP (Lee and Luo, 1999), contains the mouse CD8 extracellular and transmembrane domains fused with GFP, and is expressed from a Gal4-responsive UAS element (Brand and Perrimon, 1993). In our hands, this marker labels the cell surface but also exhibits considerable intracellular staining (presumably the secretory pathway). We first expressed mCD8-GFP in the germ cells to examine their morphology during gonad formation (Fig. 1A,B). At stage 13, just prior to gonad coalescence, germ cells are round in appearance and lack cellular extensions (Fig. 1A). They maintain this rounded shape during and after coalescence (e.g. stage 15, Fig. 1B). This is in contrast to the migratory phase of germ cell development, when they clearly extend protrusions as they move toward the SGPs (Jaglarz and Howard, 1995) (Fig. 1B inset). Thus, during gonad coalescence germ cells do not exhibit an 'active' morphology, nor does this process appear to involve germ cell migration.

When mCD8-GFP is expressed throughout the mesoderm, and SGPs are specifically identified using an anti-EYA antibody, we find that SGPs undergo striking shape changes as the gonad coalesces. mCD8-GFP expression is observed around each germ cell, indicating that the SGPs ensheath the germ cells with thin extensions of cellular material (Fig. 1C-E). To ensure that the observed mCD8-GFP labeling does not represent expression within the germ cells themselves, similar experiments were conducted with a nuclear-localized GFP, and no germ cell expression was observed (data not shown). Therefore any GFP expression around the germ cells is derived from SGPs. Germ cell ensheathment is apparent at stage 13, just after the germ cells have arrived at the site of the forming gonad (Fig. 1C). This is before the germ cells and SGPs begin compacting from PS10-12 into a tight cluster in PS10. Germ cell ensheathment persists throughout all stages examined (stage 17), and the extent of ensheathment remains relatively constant during this time [average % ensheathment/germ cell (*n*): st13=80% (76), st14=80% (66), st15=78% (191), st16/17=81% (134)].

The images shown in Fig. 1C,D are male embryos, but we have also observed germ cell ensheathment in female gonads (data not shown). This indicates that germ cell ensheathment is not a sex-specific phenomenon, though there may be a small difference in the extent to which male and female germ cells

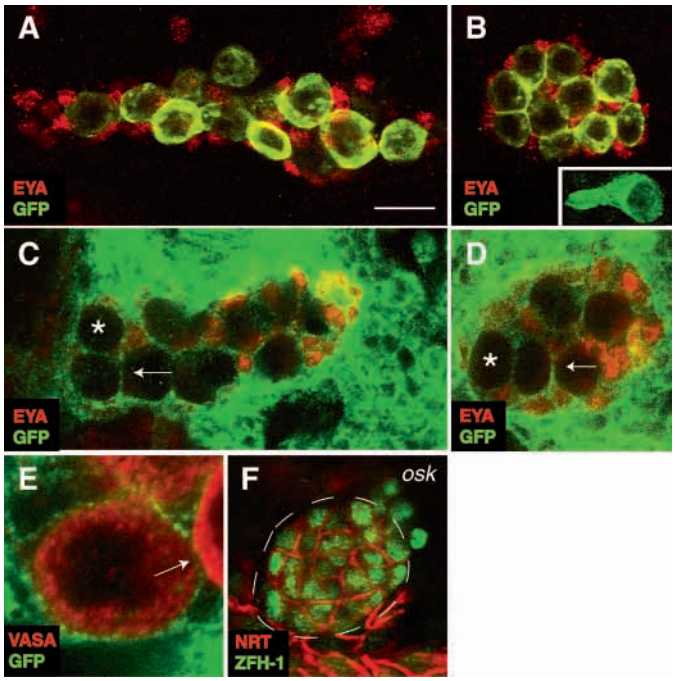

Fig. 1. SGPs individually ensheath germ cells during gonad formation. Anterior is leftwards. (A-E) UAS-mCD8-GFP expressed in the germ cells (nos-Gal4; A,B) or the mesoderm (24B-Gal4; C-E) of stage 13 (A,C), stage 15 (B,D) or stage 16 (E) embryos. (A,B) Anti-GFP labels the surface of germ cells, SGPs are labeled with anti-EYA. Note that the germ cells are round during gonad coalescence. (B, inset) Migrating germ cell (stage 11). (C,D) Somatic cell surfaces labeled with anti-GFP and SGP nuclei labeled with anti-EYA. Embryos are male, identified by the presence of EYA-positive msSGPs at the posterior of the gonad. Germ cells (e.g. asterisk) are surrounded by thin, SGP-derived cellular extensions in the uncoalesced (C) and coalesced (D) gonad. (E) Somatic cell surfaces labeled with anti-GFP and germ cells labeled with anti-Vasa. Closely apposed germ cells remain extensively ensheathed in late stages of embryogenesis. (F) Agametic stage 15 embryo (*osk*). SGPs labeled with anti-ZFH-1; anti-Neurotactin (NRT) labels the surface of many cell types, including the SGPs. Broken line indicates the boundary of the gonad. Arrows indicate SGP extensions around germ cells. Scale bar in A: 10 μm.

are ensheathed [average % ensheathment/germ cell (*n*): female st15=70% (96); male st15=86% (95)]. Interestingly, some germ cells begin to undergo cell division at stage 15 (Sonnenblick, 1950; Asaoka-Taguchi et al., 1999; Deshpande et al., 1999) (A.B.J. and M.V.D., unpublished), yet the extent of germ cell ensheathment does not decrease in older embryos. This indicates that ensheathment remains an active process as SGPs are able to establish contact with the newly formed surface between daughter germ cells. Finally, although germ cell ensheathment has already occurred as the gonad forms, it is not required for the compaction of the SGPs into PS10. Compaction occurs normally in gonads that completely lack germ cells, such as in embryos derived from mothers with weak mutations in *oskar* (Brookman et al., 1992) (Fig. 1F).

To extend our analysis of gonad coalescence, we analyzed stage 14 wild-type embryonic gonads by transmission electron microscopy (TEM). Electron micrographs of the coalesced gonad confirm that somatic cells wrap around and between germ cells (Fig. 2). SGPs display long processes and a variety of shapes as they extend in different directions to contact germ cells (Fig. 2A). Germ cells, however, are always very rounded in shape and lack processes or extensions. There is also a high degree of soma-soma contact within the gonad and SGPs often overlap each other as they surround germ cells, with a cellular process from one SGP juxtaposed with a process from a neighboring SGP (Fig. 2B). Previous work suggested that there are two populations of SGPs: the interstitial cells that associate with the germ cells inside the gonad and a separate group of cells surrounding the perimeter of the gonad (Poulson, 1950). However, we have not observed a distinct population of somatic cells surrounding the gonad, and we find that many of the SGPs contribute to both the interior and the exterior of the gonad (e.g. purple cell in Fig. 2A).

E-cadherin is expressed in both the germ cells and the SGPs

Our analysis of gonad coalescence reveals that specific and extensive cell-cell contacts are made within the gonad as it forms. To determine how these contacts are mediated and maintained, we turned our attention to the cell adhesion molecule E-cadherin. Previous work had reported that *shg* (*Drosophila* E-cadherin) mRNA is expressed within the gonad (Tepass et al., 1996), and we had observed a defect in gonad coalescence in *shg* mutants (Van Doren et. al., 2003). To further investigate the role of E-cadherin in gonad coalescence, we first examined its expression within the gonad using antibodies specific for *Drosophila* E-cadherin (αDCAD2, αDCAD1) (Oda et al., 1994). Both antibodies give similar staining patterns, and αDCAD2 immunoreactivity is severely reduced in embryos homozygous for a deletion of *shg* (data not shown), indicating that these antibodies reflect the localization of E-cadherin protein within the gonad.

At stage 12, SGPs exist as three distinct clusters of cells (Fig. 3A). E-cadherin within the SGPs is indistinguishable from the background levels throughout the mesoderm at this time, in contrast to nearby tracheal tissue (Fig. 3A). By stage 13, when germ cells and SGPs are associated across PS10-12, E-cadherin is now clearly observed around germ cells (closed arrowhead, Fig. 3B) in the same regions where we observe ensheathment of germ cells by SGPs. E-cadherin is also observed between SGPs (open arrowhead, Fig. 3B). The pattern of E-cadherin remains similar as the gonad coalesces (stage 14, Fig. 3C). Additionally, as msSGPs approach and join the posterior of the male gonad, they too display clear E-cadherin staining (large arrow, Fig. 3C,F), similar to what is observed in the main body of the gonad.

At later stages, E-cadherin expression becomes highly concentrated at the anterior end of the gonad (stage 17, Fig. 3D), which may be the result of increased protein expression or a high density of somatic cells. This pattern appears to be male-specific, and is likely to reflect E-cadherin expression in the developing proximal testis structure known as the hub, since these cells co-express *escargot* (A.B.J. and M.V.D., unpublished) and both *escargot* and E-cadherin mark the hub in the adult testis (Kiger et al., 2000; Tazuke et al., 2002).

In addition to E-cadherin, other components of classical cadherin complexes are present in the gonad. Double labeling experiments with antibodies against E-cadherin and Armadillo (ARM), the *Drosophila* homolog of β-catenin, reveal that localization of the two proteins overlaps almost completely within the gonad (Fig. 3E). An antibody specific for α-catenin also presents a staining pattern similar to that of αDCAD2 in the gonad (data not shown). We also observed small ring-like structures labeled with the DCAD2 antibody interspersed throughout the gonad at all stages of development (small arrow in Fig. 3B,C). However, these ring structures do not stain with αARM antibodies and their αDCAD2 immunoreactivity is unchanged in embryos homozygous for a deletion that removes *shg*/E-cadherin (in contrast to other *Drosophila* E-cadherin staining in the gonad, which is greatly reduced). Thus, either these structures represent highly stable complexes of maternal E-cadherin, or they represent artifactual staining of the αDCAD2 antibody.

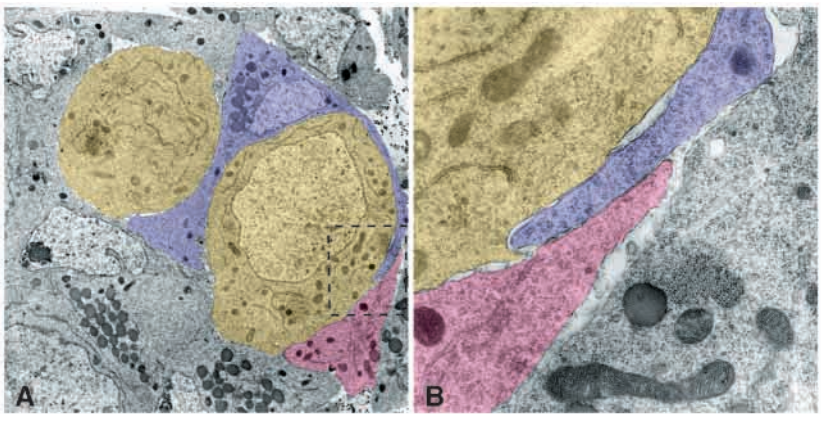

Fig. 2. TEM analysis of the coalesced gonad. (A,B) Stage 14 male wild type gonad, transverse section. Two germ cells are highlighted in yellow, two SGPs highlighted in pink and purple. (A) SGPs ensheath germ cells with thin cellular extensions. Note the unusual morphology of the purple SGP as it extends processes to ensheath the neighboring germ cell. (B) Higher magnification of region indicated in A. Two SGPs overlap each other to completely surround a germ cell.

As E-cadherin is localized to sites of germ cell-soma contact, it may act as a homophilic cell adhesion molecule to promote this interaction. In this model, E-cadherin should be present on the plasma membranes of both germ cells and SGPs. To test this, we expressed mCD8-GFP in either germ cells or SGPs and looked for colocalization with E-cadherin. We find that αDCAD2 colocalizes with αGFP at the cell surface of germ cells expressing mCD8-GFP (Fig. 3F). αDCAD2 also labels migrating germ cells before they reach the site of the gonad (Fig. 3G), providing additional evidence that E-cadherin is present in the germ cells. When mCD8-GFP is expressed in SGPs (Fig. 3H), we also see colocalization with E-cadherin. Furthermore, E-cadherin staining is observed in gonads that lack germ cells (Fig. 3I), confirming that it is expressed in SGPs. Thus, E-cadherin appears to be on the cell surface of both germ cells and SGPs, which is consistent with a role for this protein in promoting cell-cell adhesion between these cell types. Interestingly, E-cadherin does not appear to be localized to regions where germ cells are contacting other germ cells (black arrowhead, Fig. 3F) instead of SGPs. Thus, it appears to be preferentially engaged in cell contacts between germ cells and SGPs.

To investigate whether E-cadherin expression in the somatic gonad is dependent upon genes that specify SGP identity, we examined its localization in *eya* mutant embryos. Immunostaining in *eya* mutants reveals a lack of somatic E-cadherin in the gonad region, although germ cell expression is still clearly present (Fig. 3J). Antibodies against the SGP

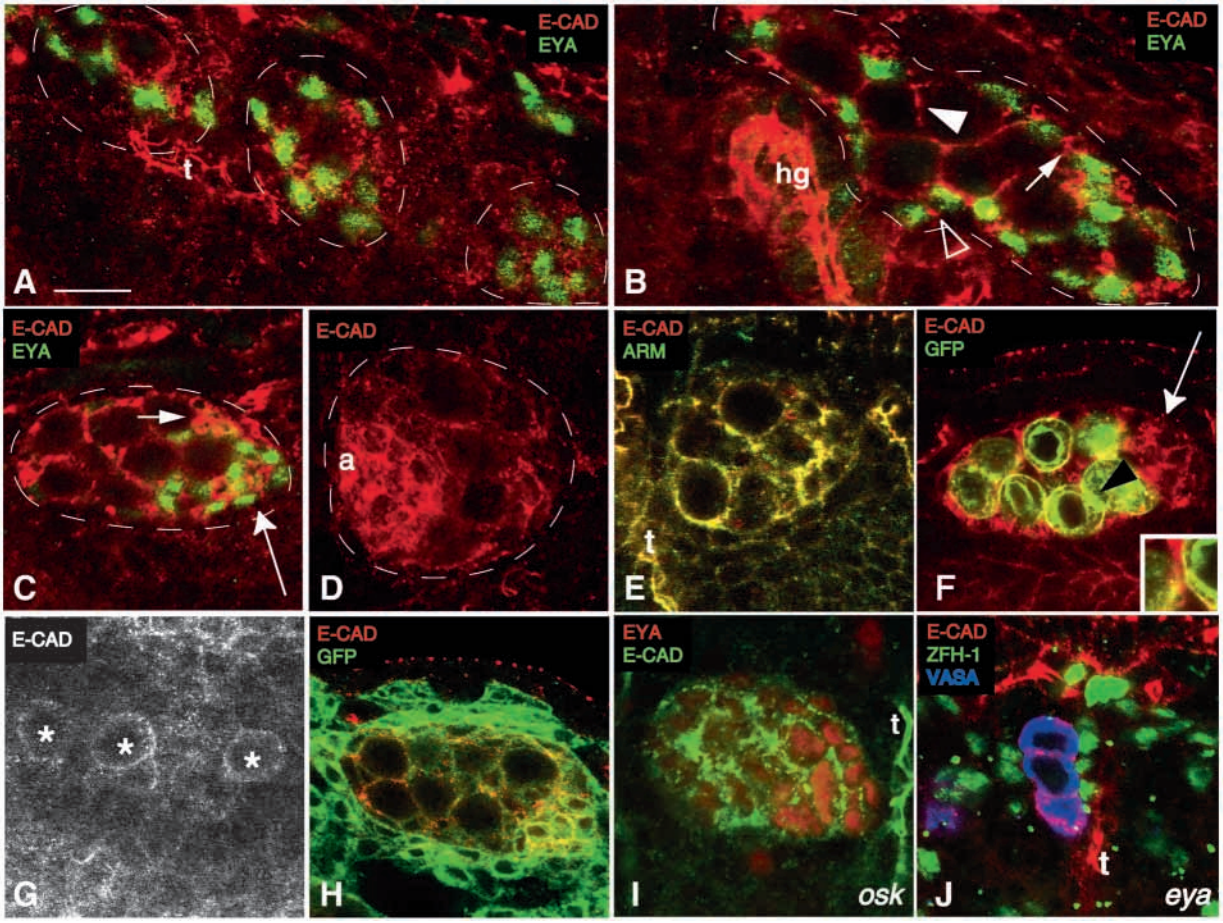

Fig. 3. *Drosophila* E-cadherin is expressed in germ cells and SGPs during gonad coalescence. Anterior is leftwards. All embryos are stained for *Drosophila* E-cadherin. (A-D) Expression of E-cadherin in stage 12 (A), stage 13 (B), stage 14 (C) and stage 17 (D) gonads. (C,D) Male embryos. SGPs and msSGPs (large arrow in C) are labeled with anti-EYA in A-C. Gonad boundaries (SGPs) indicated with broken lines. E-cadherin expression in the SGPs appears between stages 12 (A) and 13 (B), correlating with the onset of coalescence. E-cadherin expression is maintained in the gonad throughout embryogenesis and is increased at the anterior of the gonad during late stages (a, anterior; D). See text for further discussion of symbols. (E) Stage 15 embryo double labeled for E-cadherin and Armadillo/β-catenin. Note the extensive co-localization of these proteins. (F) Stage 15 male. UAS-mCD8-GFP expressed in germ cells (nos-Gal4) co-localizes with E-cadherin, indicated by yellow, except in regions of germ cell-germ cell contact (black arrowhead). msSGPs (arrow) also exhibit E-cadherin expression. Higher magnification view of two germ cells co-expressing mCD8-GFP and E-cadherin on their cell surfaces. There is also a region of E-cadherin alone between these two cells that is probably an ensheathing SGP. (G) Stage 11. E-cadherin is present at the surface of migrating germ cells (asterisks). Germ cells were identified by co-expression of mCD8-GFP crossed to nanos-Gal4 (not shown). (H) Stage 15. mCD8-GFP expressed in the mesoderm (24B-Gal4) co-localizes with E-cadherin. (I) Stage 15 agametic (*osk*) embryo, EYA labels SGPs. Note that E-cadherin is expressed even though no germ cells are present. (J) Stage 14 $eya^{Cli\text{-}IID}$ mutant. Germ cells labeled with Vasa clearly express E-cadherin. Surrounding ZFH-1-positive cells, potentially SGPs or other mesodermal cells, do not display E-cadherin staining. t, trachea; hg, hindgut. Scale bar in A: 10 μm.

marker ZFH-1 show a decrease of ZFH-1-positive cells in the gonad region as expected. Some of the remaining ZFH-1 positive cells associate with germ cells, suggesting they are residual SGPs. Although a few of these remaining cells express some E-cadherin (not shown), most show a lack of E-cadherin expression (Fig. 3J). Thus, the SGP identity gene *eya* is required for proper E-cadherin expression in SGPs.

E-cadherin is required for gonad compaction and germ cell ensheathment

We examined gonad formation in *shg*/E-cadherin mutants to determine what aspects of gonad formation require E-cadherin. Interestingly, we find that *shg* is required for both gonad compaction and germ cell ensheathment. In *shg* mutants, gonad compaction is sometimes initiated, but often does not proceed to completion (Fig. 4A,B). The three clusters of SGPs from PS10-12 are able to associate correctly with one another, and with germ cells, to form a cohesive group. However, these cells often remain loosely associated and spread over more than one parasegment, rather than compacting tightly in PS10. In the most severe cases, compaction from PS10-12 to PS10 appears completely blocked (Fig. 4A). In weaker examples, compaction is initiated but not completed, resulting in partially compacted and misshapen gonads (Fig. 4B). Phenotypes are stronger with the shg^{G317} allele (22% severe, 41% weak, n=49) than with shg^{1H} (3% severe, 50% weak, n=36), consistent with previous observations on the relative strengths of these alleles (Tepass et al., 1996). *shg*/E-cadherin also has a strong maternal contribution, which cannot be removed because it is required for oogenesis (González-Reyes and St Johnston, 1998; Godt and Tepass, 1998). It is likely that the loss of both maternal and zygotic E-cadherin would result in an increased penetrance of the more severe phenotype.

msSGPs also show defects in *shg* mutants, and often fail to join the posterior of the male gonad (Fig. 4D, 42% of shg^{G317} male embryos, n=49). This defect cannot be attributed to a general failure in gonad coalescence, as msSGPs join the gonad normally in *foi* mutant embryos where coalescence is blocked (DeFalco et al., 2003) (see below). Thus, E-cadherin is specifically required for the migration and/or fusion of msSGPs with the gonad, an early step in the establishment of gonad sexual dimorphism.

To analyze the role of E-cadherin in germ cell ensheathment, we expressed mCD8-GFP in the mesoderm of *shg* mutants. Germ cell ensheathment is clearly defective in these embryos (Fig. 4E). *shg* mutant gonads exhibit a dramatic reduction in the extent to which germ cells are surrounded by SGP-derived mCD8-GFP, and we often saw gonads where germ cells showed little or no mCD8-GFP extending around them (Fig. 4E, compare with Fig. 1D). Again, the germ cell ensheathment defect was stronger in shg^{G317} than in shg^{1H}. Interestingly, the severity of the germ cell ensheathment defect did not correlate with the severity of the gonad compaction defect.

Finally, we have observed that *shg* mutants also display defects in germ cell migration. Although part of this defect is likely to be to be due to a zygotic requirement for E-cadherin in tissues through which the germ cells move, our data indicate that there is also a requirement for E-cadherin in the germ cells themselves for proper migration to the gonad. First, we clearly see E-cadherin expression within the germ cells while they are migrating (Fig. 3H). Second, shg^{G317} exhibits a dominant, maternal effect on germ cell migration (Table 1). Offspring from heterozygous shg^{G317}/+ females have a clear germ cell migration defect (Fig. 4F), independent of the zygotic genotype. Offspring from the reciprocal cross using shg^{G317}/+ males show no germ cell migration defect. As the offspring from heterozygous females are viable, the dominant maternal effect is unlikely to be causing a global disruption of the embryonic tissues through which the germ cells are moving. Instead, it is likely to reflect a role for maternal E-cadherin within the germ cells themselves for proper migration.

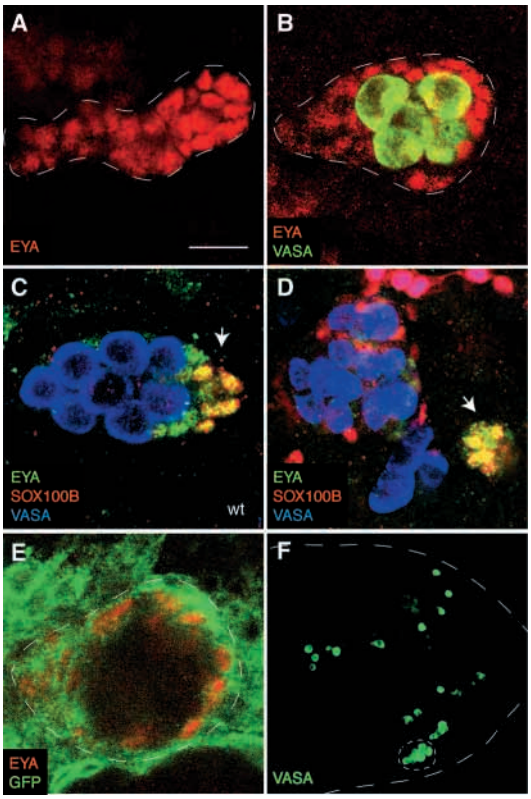

Fig. 4. *shg*/E-cadherin mutants have defects in gonad compaction, germ cell ensheathment and germ cell migration. Anterior is leftwards, embryos are stage 15 or later. Vasa labels the germ cells in B-D,F. (A,B) shg^{G317} homozygous embryos. (A) SGPs labeled with EYA. Example of a strong gonad compaction defect. *shg* mutants also display defects in germ cell migration, and the gonad in A lacks germ cells completely (Vasa channel not shown). (B) EYA marks the SGPs. Example of a weak gonad compaction defect. (C) Stage 15 wild-type male. msSGPs (arrow) express EYA and Sox100B, while SGPs express only EYA. msSGPs join the posterior of male gonads. (D) shg^{G317} homozygous male embryo. msSGPs have failed to join the gonad, remaining in a tight cluster posterior to the gonad (arrow). (E) shg^{G317} homozygous embryo. UAS-mCD8-GFP expressed in the mesoderm (24B-Gal4) labeled with anti-GFP and anti-EYA. The lack of GFP-labeled SGP extensions between the germ cells indicates a failure of germ cell ensheathment. (F) Stacked *z*-series through an embryo from a cross of shg^{G317}/CyOftz-*lacZ* females and w^{1118} males that did not inherit the shg^{G317} chromosome zygotically. Anti-EYA (not shown) was used to identify the normal position of the one gonad visible in this image (circle). Many lost germ cells are observed, indicating a dominant maternal effect of shg^{G317} on germ cell migration. Scale bar in A: 10 μm.

Table 1. Germ cell migration is affected by maternal E-cadherin

Generation	Genotype	Average number of lost germ cells/embryo (*n*)
P	w^{1118} female × shg^{G317}/CyO male	
F1	shg^{G317}/+	1.5±1.3 (40)
	+/CyO	0.8±1.2 (34)
P	shg^{G317}/CyO female × w^{1118} male	
F1	shg^{G317}/+	12.4±7.4 (30)
	+/CyO	7.1±3.8 (36)

A balance of E-cadherin levels is critical for normal gonad formation

In classic cell-sorting experiments, it has been shown that cadherin-mediated cell contacts depend upon the amount of cadherin a cell expresses (reviewed by Tepass et al., 2002). Cells with high levels of E-cadherin will sort out from cells with lower levels of E-cadherin, associating with each other in a homotypic fashion (Steinberg and Tacheichi, 1994). To test whether proper germ cell-SGP contacts within the gonad are dependent on a balance of E-cadherin expression within these cell types, we overexpressed wild-type E-cadherin using the UAS promoter (Sanson et al., 1996). When E-cadherin is ectopically expressed in the mesoderm, we see a decrease in the number of SGPs, most probably owing to dominant-negative effects of E-cadherin on *armadillo* and the *wingless* signaling pathway, which plays a role in SGP specification (Warrior, 1994; Boyle et al., 1997). Thus, we could not analyze the effects of increased E-cadherin in the mesoderm on gonad formation. However, when E-cadherin is overexpressed in the germ cells, we see a clear cell-sorting defect in the gonad (Fig. 5B,B′). As we were already using the Gal4 system to express E-cadherin in the germ cells, we were unable to assess germ cell ensheathment by expressing mCD8-GFP in the SGPs. Instead, we analyzed ensheathment using two additional markers, the 68-77 enhancer trap that expresses β-galactosidase in the SGP cytoplasm (Simon et al., 1990), and antibodies to the cell-surface protein Neurotactin (de la Escalera et al., 1990). These markers clearly label the SGP extensions around the germ cells of wild-type gonads (Fig. 5A,A′). When E-cadherin is overexpressed in the germ cells, both the cell bodies and ensheathing cytoplasmic extensions of the SGPs are excluded from the cluster of germ cells (Fig. 5B,B′). Thus, a balance of E-cadherin-based adhesion is required to promote the proper tissue architecture of the coalesced gonad.

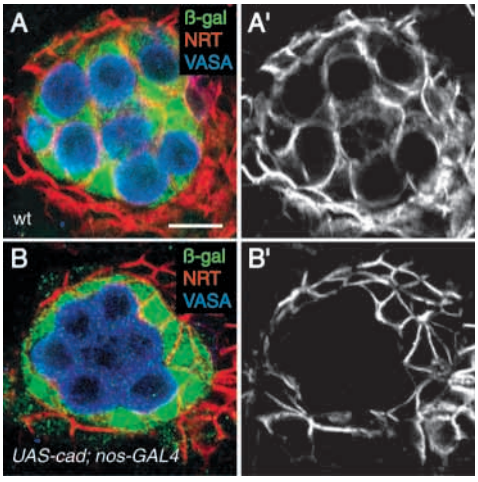

Fig. 5. A balance of E-cadherin is crucial for normal gonad formation. Anterior is leftwards. Stage 15 embryos carrying an enhancer trap (*68-77*) that expresses β-GAL in SGPs. Embryos are labeled to reveal germ cells (anti-Vasa), the enhancer trap (anti-β-GAL) and the cell surface of SGPs (anti-NRT). (A,A′) Wild type. Germ cells are completely ensheathed by SGPs. Note that both β-GAL and NRT staining is observed between germ cells. (A′) NRT channel alone. (B,B′) Embryo expressing two copies of a UAS-*D*E-cadherin transgene in the germ cells (nos-Gal4). Germ cells are tightly clustered inside the gonad, with no β-GAL or NRT labeling between them. (B′) NRT channel alone. Scale bar in A: 10 μm.

foi is required for proper levels of E-cadherin in the gonad

We have previously characterized another gene, *fear of intimacy* (*foi*), that is similar to *shg*/E-cadherin in that mutations in the gene affect both gonad coalescence and lateral trunk tracheal branch fusion (Van Doren et al., 2003). The similarity of the *foi* and *shg* mutant phenotypes suggests that these genes might be acting through a common mechanism or pathway to regulate

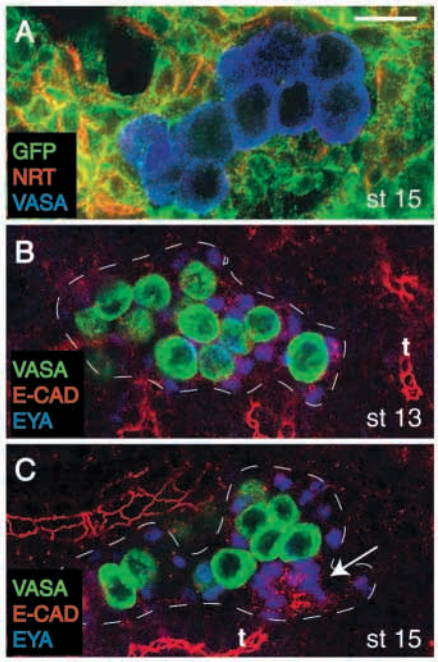

Fig. 6. *foi* is required for proper *Drosophila* E-cadherin levels in the gonad. Anterior is leftwards. (A) Stage 15 $foi^{20.71}$/$foi^{16.33}$ mutant. UAS-mCD8-GFP expressed in the mesoderm (twist-Gal4) labeled with anti-Vasa, anti-GFP and anti-NRT. The absence of SGP-derived GFP and NRT labeling around the germ cells indicates a failure in germ cell ensheathment. (B,C) $foi^{20.71}$ homozygous mutants. Embryos labeled with anti-Vasa, anti-EYA and anti-*Drosophila* E-cadherin. Gonad boundaries indicated with broken line. (B) *Drosophila* E-cadherin staining is reduced in the SGPs of *foi* mutants at stage 13 compared with wild type (Fig. 3). (C) Stage 15 male. The SGPs and germ cells have not compacted into a coalesced gonad but the msSGPs (C, arrow) have joined the gonad. *Drosophila* E-cadherin expression remains low in the SGPs and germ cells, but is high in the msSGPs (arrow). Nearby tissues (t, trachea) are not affected. Scale bar in A: 10 μm.

these processes. To investigate this possibility further, we analyzed the coalescence defect in *foi* mutants to determine whether both gonad compaction and germ cell ensheathment were affected. *foi* mutants are clearly defective in gonad compaction (Moore et al., 1998b) (Fig. 6). SGPs and germ cells can associate in *foi* mutants, but the compaction of these cells from PS10-12 to PS10 is blocked (Fig. 6A,C). We examined germ cell ensheathment by expressing mCD8-GFP in the mesoderm of *foi* mutants and observed a dramatic decrease in mCD8-GFP staining around germ cells in these embryos (compare Fig. 6A with Fig. 1D). A similar result was observed when germ cell ensheathment was assessed by immunolabeling with anti-Neurotactin (compare Fig. 6A with 5A). Thus, both gonad compaction and germ cell ensheathment are defective in *foi* mutants, and these phenotypes bear a striking resemblance to the phenotypes observed in *shg* mutants.

To further explore the functional relationship between *foi* and E-cadherin, we examined the expression of E-cadherin in *foi* mutant gonads. We find that E-cadherin immunoreactivity is greatly diminished in *foi* mutants when compared with wild-type embryos (compare Fig. 6B,C with Fig. 3B,C). Small, punctate amounts of protein remain in the gonad, but the clear cell surface labeling present in wild-type embryos is absent. Unlike mutations in *eya* that affect E-cadherin expression in SGPs but not germ cells, we observe very little E-cadherin within germ cells of *foi* mutants. This effect on DCAD2 immunoreactivity is gonad specific, and surrounding tissues like the ectoderm (not shown) and the main body of the trachea (Fig. 6B,C) show normal E-cadherin expression. The reciprocal experiment, examining FOI expression in *shg* mutants, was not possible as no FOI antibodies are currently available.

Interestingly, *foi* does not affect E-cadherin staining in the msSGPs (Fig. 6C). As they join the main body of the gonad, msSGPs in *foi* mutants (Fig. 6C, arrow) display E-cadherin immunoreactivity similar to that seen in msSGPs of wild-type embryos (Fig. 3C,F, large arrow). Double-staining for Sox100B and E-cadherin confirm the expression of E-cadherin within msSGPs (data not shown). msSGPs represent an aspect of gonad development that differs in its requirement for *shg*/E-cadherin versus *foi*: these cells fail to associate correctly with the gonad in *shg* mutants (Fig. 4F), but they behave normally in *foi* mutants (DeFalco et al., 2003). This suggests that the basis for the *foi* mutant phenotype may be due to the effects of *foi* on E-cadherin.

DISCUSSION

Gonad coalescence in *Drosophila* is the rearrangement of germ cells and SGPs from a broad association stretching across three segments of the embryo to a tight cluster of cells located in PS10. In this process, germ cells become enclosed in the environment that will nurture them as they adopt stem cell fates and begin gametogenesis. We have found that germ cell-soma contact in the embryonic gonad is already extensive, with each germ cell becoming surrounded by somatic cell membrane. Furthermore, we have found that the homophilic cell adhesion molecule E-cadherin plays a key role in this and other aspects of gonad formation. Finally, we have shown that E-cadherin may be regulated by the novel, multipass transmembrane protein Fear of Intimacy.

E-cadherin and gonad morphogenesis

Our detailed analysis of gonad coalescence has shown that it can be subdivided into two processes: gonad compaction and germ cell ensheathment. In gonad compaction, SGPs and germ cells physically condense together to create a rounded organ. Germ cell ensheathment is characterized by the dramatic shape changes of SGPs that produce thin cellular extensions that surround the germ cells. Germ cells lack cellular extensions during gonad compaction, and need not be present for compaction to occur. This suggests that SGPs provide the ‘driving force’ behind the movements of compaction and germ cells play a more passive role.

Several pieces of data indicate that gonad compaction and germ cell ensheathment are distinct, separable events. Germ cell ensheathment is already apparent at stage 13, prior to the onset of compaction. In addition, compaction proceeds normally in agametic embryos, despite a lack of germ cell ensheathment. Furthermore, in mutants that affect gonad coalescence (*shg*, *foi*), we have observed examples of gonads with no ensheathment but a high degree of compaction, and also gonads with good ensheathment but little compaction (data not shown). Thus, gonad compaction and germ cell ensheathment are independent processes that together contribute to the proper architecture of the coalesced embryonic gonad. Both of these processes require the adhesion molecule E-cadherin.

How might *Drosophila* E-cadherin be functioning to promote gonad morphogenesis? Differential cell adhesion mediated by E-cadherin has been shown to govern cell sorting in vitro (Steinberg and Takeichi, 1994) and in at least one in vivo situation (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998). It is possible to explain our observations of gonad morphogenesis with a similar model of differential cell adhesion. In this model, gonad compaction results from an increased affinity of SGPs for one another relative to the surrounding mesoderm. Compaction would occur as SGPs maximize their contacts with one another and minimize their contacts with the surrounding mesoderm, hence forming a sphere. Contacts between SGPs and germ cells might also play a role in compaction, but SGP-SGP affinity would be sufficient to allow this process to occur in the absence of germ cells. Consistent with this hypothesis, E-cadherin expression becomes more apparent in SGPs relative to the surrounding mesoderm at the time that compaction is initiated (Fig. 3). This is likely to reflect an increase in E-cadherin expression or stability, but could also conceivably result from a change in subcellular localization. Upregulation of E-cadherin in the SGPs may contribute to an increase in SGP-SGP adhesion during gonad compaction.

The process of germ cell ensheathment may also be controlled by differential cell adhesion, but between SGPs and germ cells. Ensheathment would occur as a result of SGPs maximizing their contacts with germ cells. This model requires that SGPs and germ cells have a higher affinity for each other than for their own cell type. A prediction of this model is that ensheathment would be blocked if germ cell-germ cell adhesion were increased, which is exactly what we observe (Fig. 5).

What role might E-cadherin, traditionally a homophilic cell adhesion molecule, play in mediating the heterotypic

interactions between SGPs and germ cells during ensheathment? One possibility is the presence of additional heterophilic adhesion molecules that promote specific adhesion between these cell types. A candidate member of such a heterophilic adhesion system is Neurotactin, which is present on SGPs and has been shown to promote heterotypic cell adhesion (Barthalay et al., 1990). In this case, E-cadherin could provide additional 'glue' that is required for ensheathment once the heterotypic specificity between SGPs and germ cells is established. Alternatively, E-cadherin might somehow be biased to act in a heterophilic manner. E-cadherin could interact with a heterophilic binding partner (e.g. Cepek et al., 1994), or could be biased by a modification or co-factor to bind preferentially to E-cadherin molecules on heterotypic cells (e.g. a modified form of E-cadherin might interact only with an unmodified form).

Gonad coalescence may represent an elegant example of organogenesis based on differential cell adhesion. A hierarchy of cell affinity (SGP-germ cell>SGP-SGP>SGP-surrounding mesoderm) can account for much of the observed gonad organization. This model requires cell movement for proper execution of cell sorting. Although the morphology of the germ cells suggests that they may not be highly motile at this time, further work is needed to determine the extent to which SGP versus germ cell movement contributes to this process. In addition, other mechanisms, such as cytoskeletal-derived contractile and protrusive forces, may also be important for compaction and ensheathment. The contribution of these different factors to the overall architecture of the gonad can now be further tested using our more detailed understanding of gonad coalescence.

E-cadherin and *fear of intimacy*

Embryos with mutations in the *fear of intimacy* gene share several gonad defects with *shg* mutant embryos, including defects in gonad compaction and germ cell ensheathment. Both genes are also required for tracheal branch fusion (Tanaka-Matakatsu et al., 1996; Van Doren et al., 2003), suggesting that *Drosophila* E-cadherin and FOI may work together to promote all of these processes. Consistent with this, we show that E-cadherin protein levels are severely reduced within the gonads of *foi* mutants. E-cadherin expression is reduced in SGPs, which display defective behaviors in *foi* mutants, but not msSGPs, which appear to behave normally (Fig. 5). Thus, gonad defects in *foi* mutants correlate strongly with the cells in which E-cadherin expression is most affected, suggesting that this may be the cause of the *foi* mutant phenotype.

There are several possible models for how FOI, a cell surface, multipass transmembrane protein, might be affecting the levels of E-cadherin protein. First, FOI could act as a receptor or channel that signals the beginning of coalescence. Upregulation of E-cadherin in the SGPs could require such a signal. Or, FOI might act to localize E-cadherin complexes to sites of germ cell-soma and soma-soma contact within the gonad. As such, FOI could act during the export of E-cadherin to the cell surface, or to localize E-cadherin to specific sites of cell-cell contact. Alternatively, FOI might affect E-cadherin levels by affecting its function as a cell adhesion molecule. It has been suggested that the stability of E-cadherin is tightly linked to its function in adhesion complexes, with reduced E-cadherin function leading to a faster turnover of the protein (Tepass et al., 1996). FOI might modulate E-cadherin function by acting as a co-factor itself on the cell surface, or by acting as a transporter to alter the concentration of a small molecule modulator of E-cadherin adhesion, such as Ca^{2+}.

Germline-soma interactions in gonad development

Germ cell ensheathment in the *Drosophila* embryonic gonad is an example of a recurring theme in germ cell development; germ cells require close contact with specialized somatic cells for their proper differentiation. Germ cell-soma interaction has been shown to be essential for many phases of germ cell development in diverse species. The proper sexual identity of the germline is controlled by the soma in both the mouse and the fly (Steinmann-Zwicky et al., 1989; Adams and McLaren, 2002). In addition, germ cells often exist as stem cells in the adult gonad, dividing to produce one daughter that enters gametogenesis while the other retains stem cell identity. Interaction between germline stem cells and their somatic niche is essential for regulating cell division and stem cell maintenance (reviewed by Spradling et al., 2001). Finally, during gametogenesis, differentiating germ cells remain in close association with somatic cells that regulate their development into sperm or egg.

Adhesive contacts and cell-cell junctions are crucial for soma-germline signaling. Some somatic signals require specific cellular junctions, such as gap junctions (Tazuke et al., 2002; Kidder and Mhawi, 2002). Even secreted signals, such as those governing the regulation of germline stem cell maintanence, require the proper adhesion and orientation between germline and soma (Song et al., 2002). E-cadherin has been shown to play a crucial role in several examples of germ cell-soma interaction, including in the stem cell niche and developing egg chamber in *Drosophila* (Song et al., 2002; Godt and Tepass, 1998; González-Reyes and St Johnston, 1998; Niewiadomska et al., 1999; Geisbrecht and Montell, 2002).

Regulation of germ cell development by the soma may begin as soon as the gonad forms. There is evidence that the soma regulates sex determination and the cell cycle in the mouse germline (Adams and McLaren, 2002) and the pattern of germ cell gene expression in *Drosophila* (Mukai et al., 1999) at very early stages. Thus, regulation by the soma is crucial for every stage of germ cell development. We hypothesize that the E-cadherin-dependent germ cell ensheathment we have observed in embryonic gonads creates a nascent niche that allows the SGPs to regulate germ cell development and the transition to germline stem cells.

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