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Zinc transport activity of Fear of Intimacy is essential for proper gonad morphogenesis and DE-cadherin expression

Wendy R. Mathews , Daniel Ong, Allison B. Milutinovich and Mark Van Doren*

Embryonic gonad formation involves intimate contact between germ cells and specialized somatic cells along with the complex morphogenetic movements necessary to create proper gonad architecture. Previously, we have shown that gonad formation in *Drosophila* requires the homophilic cell-adhesion molecule *Drosophila* E-cadherin (DE-cadherin), and also Fear of Intimacy (FOI), which is required for stable accumulation of DE-cadherin protein in the gonad. Here, we present an in vivo structure-function analysis of FOI that strongly indicates that zinc transport activity of FOI is essential for gonad development. Mutant forms of FOI that are defective for zinc transport also fail to rescue morphogenesis and DE-cadherin expression in the gonad. We further show that expression of DE-cadherin in the gonad is regulated post-transcriptionally and that *foi* affects this post-transcriptional control. Expression of DE-cadherin from a ubiquitous (*tubulin*) promoter still results in gonad-specific accumulation of DE-cadherin, which is strongly reduced in *foi* mutants. This work indicates that zinc is a crucial regulator of developmental processes and can affect DE-cadherin expression on multiple levels.

KEY WORDS: ***fear of intimacy, shotgun,*** **DE-cadherin, Zinc transport, ZIP proteins, Post-transcriptional gene regulation,** ***Drosophila***

INTRODUCTION

The formation of complex organs from individual tissues is an essential developmental process that requires coordination of many different types of cellular events. Extensive cell-cell contact and communication ensures that these events are well orchestrated. The *Drosophila* gonad is an ideal model for studying organogenesis. Formation of this organ requires that germ cells and somatic gonadal precursors (SGPs), which are initially specified far apart from each other, undergo a complex morphogenetic program to form a properly patterned embryonic gonad. This organ then further differentiates into either an ovary or testis.

Germ cells are initially formed at the posterior pole of *Drosophila* embryos during the syncytial blastoderm stage. They are carried inside the embryo during gastrulation, where they migrate through the midgut epithelium into the mesoderm to contact SGPs (reviewed by Starz-Gaiano and Lehmann, 2001). SGPs are specified in bilateral clusters within three parasegments of the embryo (PS10-12) (Brookman et al., 1992) and are initially identified by their expression of Eyes Absent (EYA) and Zinc-finger homeodomain protein 1 (ZFH-1) (Boyle et al., 1997; Broihier et al., 1998). The posterior of male gonads contains an additional group of male-specific SGPs (msSGPs) specified in a separate location (PS13) (DeFalco et al., 2003). Once germ cells contact SGPs, these two distinct cell types sort away from neighboring mesodermal cells, forming a contiguous tissue along PS10-12. Germ cells and SGPs then undergo two concurrent, but separable, morphogenetic processes (germ cell ensheathment and gonad compaction) to form a properly patterned organ (Jenkins et al., 2003). As SGPs contact germ cells, they undergo dramatic cell shape changes to extend thin cellular protrusions, which ensheath individual germ cells (Jenkins et al., 2003). At the same time, the associated germ cells and SGPs compact together in PS10 to form a round organ with a distinct boundary (Boyle and DiNardo, 1995; Brookman et al., 1992; Jenkins et al., 2003).

Although many genes involved in germ cell migration and SGP specification have been characterized, only three downstream genes have been identified that specifically coordinate the morphogenetic movements of gonad formation. *traffic jam* encodes a Maf transcription factor required for germ cells and SGPs to intermingle properly in both embryonic and adult gonads, and may regulate the adhesive properties of SGPs (Li et al., 2003). The homophilic cell-adhesion molecule *Drosophila* E-cadherin [DE-cadherin, encoded by the *shotgun* (*shg*) locus (Tepass et al., 1996; Uemura et al., 1996)] is also important for regulating cell adhesion in the gonad. DE-cadherin is upregulated in the SGPs during gonad formation and is required for both gonad compaction and germ cell ensheathment (Jenkins et al., 2003). *fear of intimacy* (*foi*) is also required for gonad formation (Moore et al., 1998; Van Doren et al., 2003) and *foi* mutants exhibit a similar phenotype to *shg* mutants. *foi* and *shg* are both required for a specific aspect of tracheal development (tracheal branch fusion), suggesting that these genes may participate in a common pathway to coordinate tissue morphogenesis (Van Doren et al., 2003). Consistent with this, DE-cadherin protein levels are greatly reduced in *foi* mutant gonads (Jenkins et al., 2003), suggesting that *foi* may act upstream of DE-cadherin to regulate gonad formation. How DE-cadherin is regulated during gonad morphogenesis, and how this is influenced by FOI, has not been previously explored.

FOI (dZIP6/dSLC39A6) is a member of the Zrt, IRT-like protein (ZIP) family of ion transporters (Mathews et al., 2005) that control zinc influx into the cytoplasm from outside the cell or from intracellular stores (Gaither and Eide, 2001). Conserved from fungi to humans, the ZIP family is defined by its conserved transmembrane (TM) structure and several regions of homology (Eng et al., 1998) (Fig. 1B). These proteins often have a histidine-rich cytoplasmic loop, and some have a histidine-rich N-terminal extracellular domain (Fig. 1A). The most conserved region within the LIV-1 subfamily, to which FOI belongs, is known as the 'signature sequence' or HELP domain and is predicted to contain

Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA.

*Author for correspondence (e-mail: vandoren@jhu.edu)

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two amphipathic α-helices with several highly conserved histidine residues (Fig. 1B). Previously, we have shown that FOI is a zinc-specific transporter, and have identified key conserved regions that are crucial for zinc transport activity (Mathews et al., 2005).

Although it is clear how zinc transporters can act at the cellular level to regulate zinc homeostasis, it is more challenging to understand the complex phenotypes caused by changes in zinc transporter function. Little is known about the in vivo roles of the ZIP family in animals; however, recent work indicates that these proteins are essential for embryonic development and adult homeostasis. Two ZIP family members, FOI and zebrafish LIV1 (zSLC39A6), are required for specific morphogenetic events during embryonic development (Pielage et al., 2004; Van Doren et al., 2003; Yamashita et al., 2004). In addition, mutations in human ZIP4 (SLC39A4) are responsible for the genetic disorder acrodermatitis enteropathica (AE), the symptoms of which include epidermal lesions, gastrointestinal defects and infant mortality (Kury et al., 2003; Wang et al., 2002). AE can be treated with increased dietary zinc, and some human ZIP4 mutations associated with AE affect zinc transport activity (Wang et al., 2004), indicating that defects in zinc regulation are crucial to this disease. However, it is still unclear whether zinc can be a regulator of developmental events and if ZIP proteins act primarily through their zinc transport activity or have other essential functions relevant for their in vivo roles.

Here, we use an in vivo rescue assay to analyze FOI function during gonad formation. In particular, we investigate whether the function of FOI as a zinc transporter is crucial for its role in development by studying mutant forms of FOI that are defective for zinc transport. In addition, we investigate how FOI acts to regulate DE-cadherin protein expression in the gonad, and uncover a surprising role for post-transcriptional regulation in generating the proper expression pattern of DE-cadherin.

MATERIALS AND METHODS

Fly stocks

The following fly stocks were used: wild type, fafl (*ru st faf lacZ e ca*) (Moore et al., 1998) and w^{1118}; *foi* alleles; $foi^{20.71}$ and $foi^{16.33}$ (Moore et al., 1998); UAS-*mCD8::GFP* and *tubulin*-GAL4 (Lee and Luo, 1999); shg^{R69}, (Godt and Tepass, 1998), *tub-DE-cad* and shg^{R69}, *tub-DE-cad* flies [a gift from P. Rorth (Pacquelet et al., 2003)]; *twist*-GAL4 (mesoderm) (Baylies and Bate, 1996); *breathless*-GAL4 (trachea) (Shiga et al., 1996); and shg^{k03401} flies (obtained from the Bloomington Stock Center).

Plasmids

GAL4-dependent, C-terminal hemagglutinin-epitope (HA) tagged FOI (UAS-foi) is as previously described (Van Doren et al., 2003). Site-directed mutations were introduced into the UAS-foi construct using QuikChange (Stratagene): D308A, H554A, D551A/D558A, T557P, H583A/H587A, E584A/E588A/D591A and Y646A (this work) (Mathews et al., 2005). ΔN was generated using a PCR-based strategy to delete amino acids D23-D254 (Q22GRAK255). CATSUP TM6-8 was generated using a PCR-based strategy to replace TM6-8 in FOI with the CATSUP TM6-8 domain. Cloning details available upon request.

Immunolabeling

Antibody staining conducted as previously described (Jenkins et al., 2003; Moore et al., 1998), except for α-DCAD2 immunolabeling where embryos were devitellinized by sonication (Patel, 1994) in PBS containing 0.1% Triton X-100 with a 3 second constant pulse using a Branson Sonifier 250 (setting 1). Antibodies used (dilutions, source): rabbit α-VAS (1:5,000, R. Lehmann), chick α-VAS (1:5000, K. Howard), rabbit α-β-GAL (1:10,000, Capel), mouse α-β-GAL (1:10,000, Promega), rabbit α-GFP (1:2,000, Torrey Pines Biolabs), rabbit α-ZFH-1 (1:5,000, R. Lehmann), mouse α-EYA 10H6 (1:25, N. Bonini, Developmental Studies Hybridoma Bank, DSHB), mouse α-NRT BP106 (1:10, C. Goodman, DSHB), rat α-DE-cadherin (DCAD2, 1:20, T. Uemura, DSHB), sheep α-Digoxigenin (1:2000, Roche Diagnostics) and mouse α-HA (0.4 μg/ml, Roche Diagnostics). Secondary antibodies (Molecular Probes or Jackson Laboratory) were used at 1:500. Homozygous mutant embryos were identified using *TM3, Ubx-lacZ* (β-GAL). Embryos staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). *shg* mutant embryos were aged for 20 hours at 18°C after egg laying to yield collections of embryos stage 15 and older. Embryos were mounted in 70% glycerol containing 2.5% DABCO (Sigma) and visualized using a Zeiss LSM 510 meta confocal microscope.

In vivo rescue assay

Mutant UAS-*foi* transgenic lines were generated (Rubin and Spradling, 1982) and lines with expression levels similar to wild-type UAS-*foi* were selected by crossing to *breathless*-GAL4 and immunolabeling histochemically with α-HA. To express UAS-*foi* in a *foi* mutant, $foi^{20.71}$, UAS-*foi/TM3, Ubx-lacZ* flies were crossed to $foi^{20.71}$, *twist*-GAL4*/TM3, Ubx-lacZ*. Controls include *foi* mutants with the UAS-*foi* transgene or GAL4 driver alone. Rescue of gonad compaction was examined in at least two independent transgenic lines, analyzing at least 80 gonads for each line. Gonads were considered rescued when similar to wild type. A single representative line was chosen for further analysis of gonad compaction (α-ZFH-1, α-VAS), germ cell ensheathment (α-NRT, α-VAS) and DE-cadherin expression (α-DCAD2, α-VAS), and at least 30 gonads were analyzed. Rescue was scored as the presence of a greater amount of germ cell ensheathment or gonad-specific DE-cadherin expression than normally observed in *foi* mutants (i.e. not necessarily rescued to wild-type levels).

FOI subcellular localization and membrane topology

UAS-*foi* constructs were expressed in Schneider S2 cells by co-transfection with *actin*-GAL4 as described (Van Doren et al., 2003). Protein localization and C-terminal membrane topology were assessed using immunolabeling of permeabilized and non-permeabilized cells, followed by staining with DAPI to label DNA, as previously described (Mathews et al., 2005; Van Doren et al., 2003). Cells were visualized using a Zeiss Axiovert 5100 2TV microscope equipped for deconvolution microscopy (Deltavision, Applied Precision).

Whole-mount fluorescence in situ hybridization

The *shg* antisense probe was synthesized by digesting pBS-DEcad (gift from V. Hartenstein) with *Hin*dIII and transcribing with T3 RNA polymerase (Promega) using digoxigenin-labeled UTP (Boehringer-Mannheim). Embryos were fixed and devitellinized, as for immunostaining. Whole-mount in situ hybridization was performed as previously described (Lehmann and Tautz, 1994), except that instead of Proteinase K treatment, embryos were permeabilized in PBS containing 0.1% Tween-20 (PBTw) with two 3-second continuous pulses using a Branson Sonifier 250 and washed in PBTw. The in situ was developed with the HNPP Fluorescent Detection Set (Roche Diagnostics) according to the manufacturer's instructions. Following this a standard antibody staining was performed (as described above), except the NGS-blocking steps were removed. Embryos were mounted in 70% glycerol containing 2.5% DABCO (Sigma) and visualized using a Zeiss LSM 510 meta confocal microscope. At least 40 gonads were analyzed for each stage and genotype.

RESULTS

Zinc transport activity is essential for gonad coalescence and DE-cadherin expression

To determine how FOI affects gonad morphogenesis and DE-cadherin expression, we conducted a structure-function analysis of FOI using an in vivo transgenic rescue assay. Previously, we have shown that expression of wild-type or epitope-tagged versions of FOI can rescue the gonad coalescence defects observed in *foi* mutants (Mathews et al., 2005; Van Doren et al., 2003). Here, we use this assay to analyze the ability of mutant forms of FOI to rescue gonad compaction, germ cell ensheathment and DE-cadherin

expression. Importantly, we have previously tested a number of these mutant forms of FOI for their ability to function in zinc transport (Mathews et al., 2005). This allows us to determine whether it is the zinc transport activity of FOI, or some other function, that is important for regulating gonad morphogenesis and DE-cadherin expression.

Mutations in FOI

We generated a series of mutations in FOI (Fig. 1A,B) within the context of a C-terminal hemagglutinin (HA)-tagged version of the protein that retains wild-type activity in vivo (Van Doren et al., 2003). To determine the importance of the histidine-rich N-terminal domain, we deleted this region (ΔN). To assess whether the primary sequence of the TM domains, or just their transmembrane structure, is essential for FOI function, we mutated specific conserved amino acids in TM domains 2 (D309A) and 7 (Y646A), and also replaced TM domains 6-8 of FOI with TM domains 6-8 from the related *Drosophila* ZIP protein, CATSUP (Stathakis et al., 1999) (CAT TM6-8). Finally, we altered the highly conserved 'HELP' domain to determine the importance of conserved histidine residues (H554A and H583A/H587A), and to investigate whether disrupting the predicted amphipathic (D551A/D558A and E584A/E588A/D591A) or alpha-helical (T557P) nature of this domain would affect FOI function.

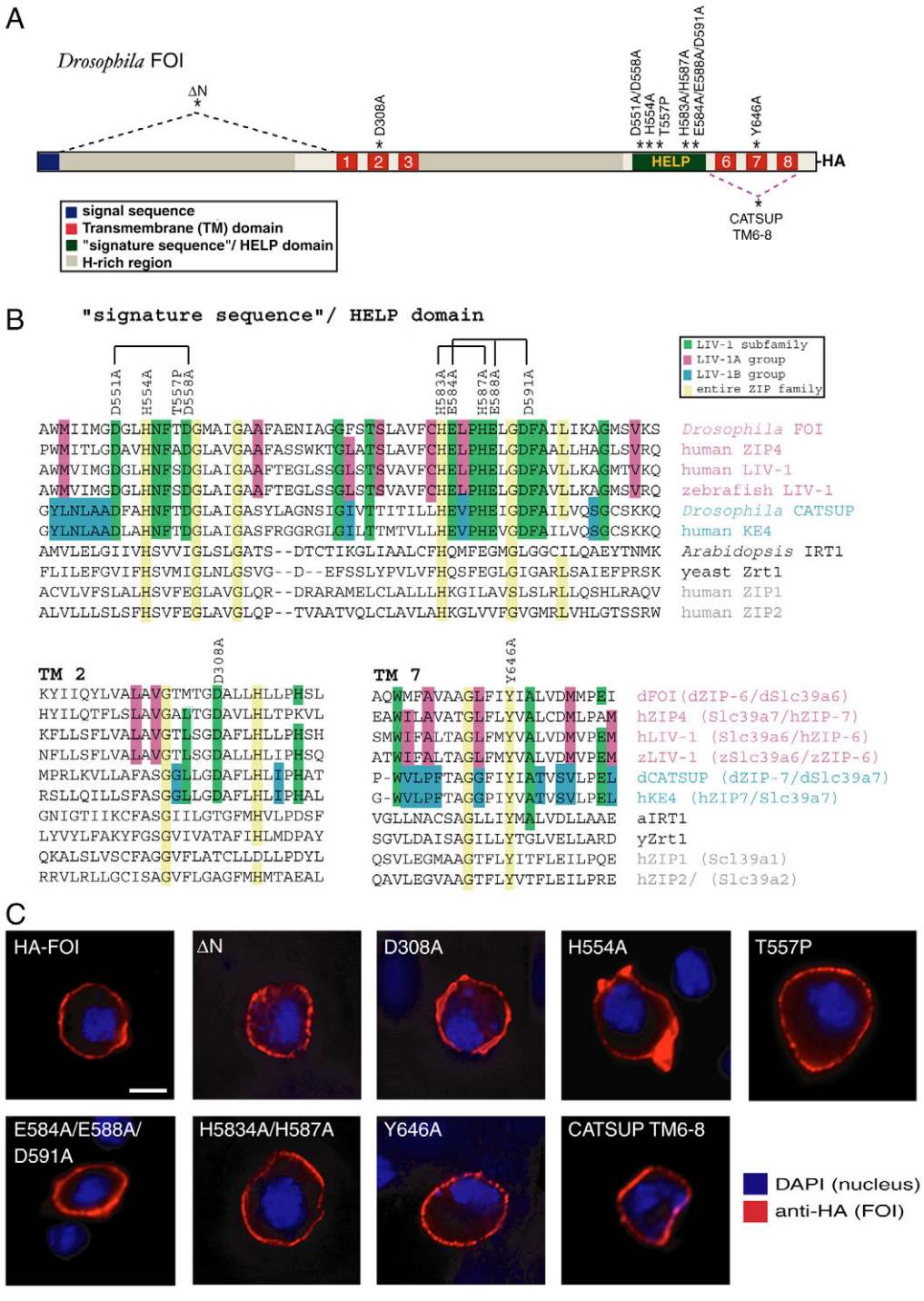

Fig. 1. FOI is a member of the ZIP family of zinc transporters. (**A**) Diagram of the FOI protein with conserved domains highlighted. Mutations introduced into HA-FOI are specified. (**B**) Sequence alignment of highly conserved domains (as indicated) of representative ZIP proteins from different subgroups. Adapted, with permission, from Mathews et al. (Mathews et al., 2005). Alternative nomenclature for these same proteins is included in the lower alignment. Residues tested for function in gonad morphogenesis are indicated (brackets link mutations introduced together). (**C**) Deconvolution images of *Drosophila* S2 cells transfected with the indicated UAS-*foi* constructs and immunolabeled with α-HA (red) before permeabilization. DAPI (blue) labels the nuclei. Scale bar: 10 μm.

We verified that the subcellular localization and membrane topology of FOI are not disrupted by our mutations. In each case, the C-terminal HA epitope tag was properly localized to the cell surface and was extracellular (could be detected without permeabilization) when the proteins were expressed in *Drosophila* S2 cells, similar to wild-type HA-FOI (Fig. 1C). Immunolabeling of permeabilized cells confirmed that a high percentage of each mutant protein was correctly localized to the cell surface (W.R.M. and M.V.D., unpublished). We then produced transgenic *Drosophila* lines where the mutant forms of FOI were expressed using the GAL4/UAS system (Brand and Perrimon, 1993), and selected those that exhibited expression levels in the embryo equal to or greater than wild-type HA-FOI control lines. Thus, any effects of these mutations on FOI function are unlikely to be due to changes in expression level, subcellular localization or membrane topology.

Rescue of gonad phenotypes

We next assessed the ability of mutant forms of FOI to rescue gonad defects observed in homozygous *foi*-mutant embryos. First, we examined gonad compaction, the process by which the germ cells and SGPs condense to form a round organ (Jenkins et al., 2003). Compaction was quantified using an immunohistochemical stain of Vasa (VAS), a germ cell-specific antigen (Fig. 2A), as performed previously (Van Doren et al., 2003). In addition, the association between germ cells and SGPs was characterized in embryos immunolabeled with antibodies recognizing VAS and ZFH1 (Fig. 2B). Proteins with mutations in the N-terminal, TM (except the Y646A mutation in TM7) and HELP domains of FOI were all defective in their ability to rescue the gonad compaction defects observed in *foi* mutants (Fig. 2A). In most cases, SGPs and germ cells remained loosely associated along several embryonic parasegments (e.g. Fig. 2E,F), similar to *foi* mutant controls (Fig. 2C), although some mutant proteins (e.g. E584A/E588A/D591A) exhibited rescued gonad compaction in a small percentage of embryos (Fig. 2A). These data indicate that the N-terminal, TM and HELP domains are essential for FOI function during gonad compaction. Only the Y646A mutant rescued the *foi* mutant gonad compaction defect with high penetrance (Fig. 2A). In embryos expressing Y646A, germ cells and SGPs properly associated with one another in a round gonad (Fig. 2G), similar to embryos expressing wild-type HA-FOI (Fig. 2D).

We next assessed germ cell ensheathment, the process in which SGP extensions surround individual germ cells in the gonad (Jenkins et al., 2003). Embryos were immunolabeled with anti-Neurotactin (anti-NRT) to reveal the SGP cell surface, including the extensions around germ cells (Jenkins et al., 2003). Unlike expression of HA-FOI, which fully rescues the germ cell ensheathment defects observed in *foi* mutant gonads, none of the mutant FOI proteins comparably rescued this phenotype (Fig. 3A). A fraction of embryos expressing either the Y646A or E582A/E588A/D591A proteins exhibited rescue of germ cell ensheathment (Fig. 3F,G compared with B,D), but most embryos expressing these or other mutant forms of FOI exhibited no rescue and were indistinguishable from *foi* mutant controls (Fig. 3E, compare with 3C). Together, these data indicate that the N-terminal, TM and HELP domains are all required for germ cell ensheathment.

Finally, we examined the ability of mutant versions of FOI to rescue DE-cadherin protein expression in the gonad. DE-cadherin is expressed at high levels in the gonad of wild-type embryos (Fig. 4B) but is greatly reduced in *foi* mutants (Fig. 4C) (Jenkins et al., 2003).

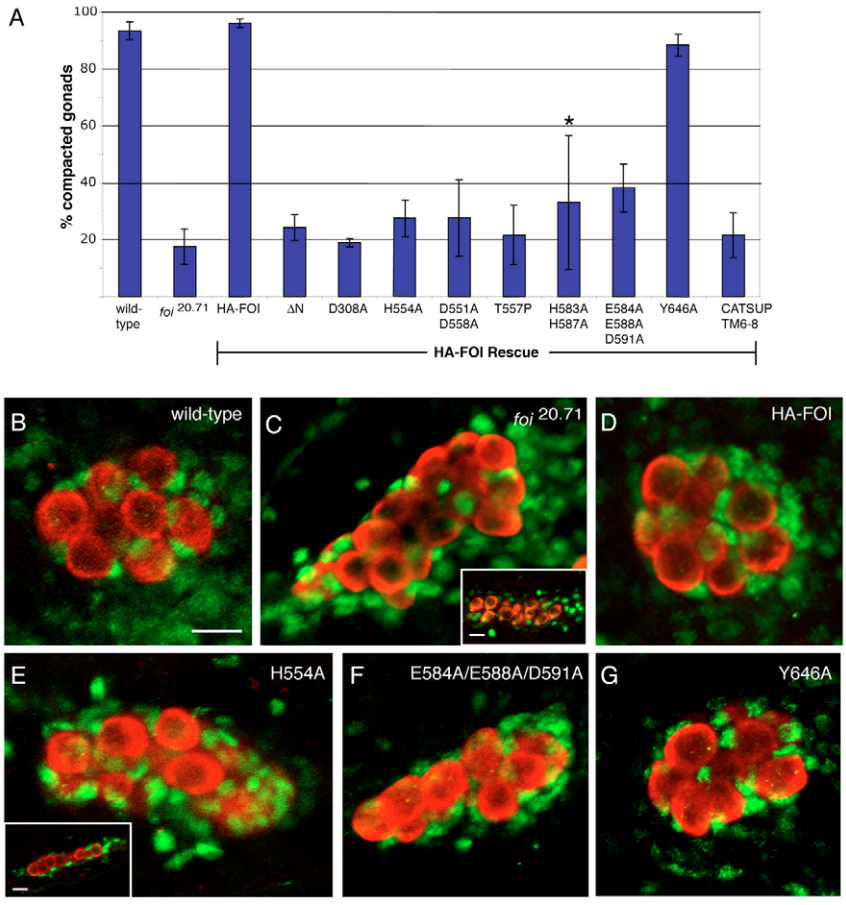

Fig. 2. Rescue of gonad compaction. Expression of indicated FOI proteins in *foi*-mutant embryos. (**A**) Graph indicates percentage of fully compacted gonads, as judged by α-VAS staining (average of two independent lines +s.d.; asterisk indicates construct for which three independent insertion lines were tested). (**B-G**) Confocal images of representative stage 15-16 gonads immunolabeled with α-VAS (germ cells, red) and α-ZFH-1 (SGPs, green). (B) Wild type, (C) *foi* mutant, (D-G) *foi* mutants with the indicated HA-FOI proteins expressed. Only expression of HA-FOI (D) or Y646A (G) rescues gonad compaction. Insets in C and E represent the most severe phenotype observed for these two genotypes; the full panels represent a less severe, but more representative, phenotype. Scale bars: 10 μm.

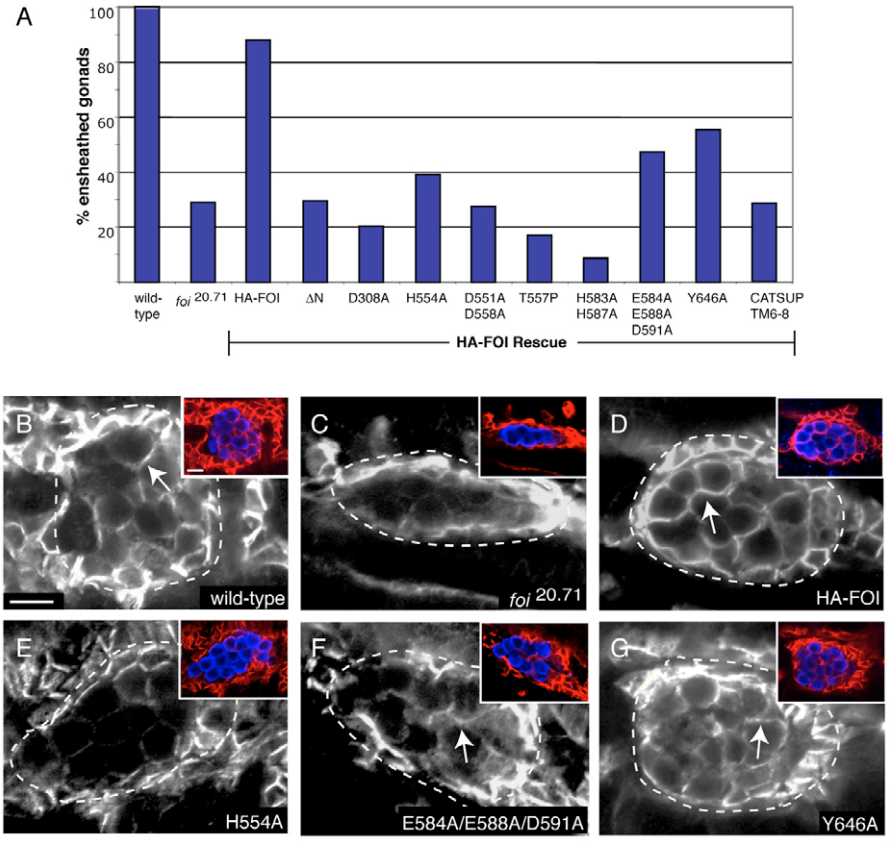

Fig. 3. Rescue of germ cell ensheathment. Expression of indicated FOI proteins in *foi*-mutant embryos. (**A**) Graph indicates percentage of gonads with ensheathed germ cells (as judged with α-NRT). Only expression of wild-type HA-FOI fully rescues germ cell ensheathment. (**B-G**) Confocal images of representative stage 15-16 gonads immunolabeled with α-NRT (SGP cell surface). Insets show location of germ cells (α-VAS, blue) relative to SGPs (α-NRT, red). (B) Wild type, (C) *foi* mutant, (D-G) *foi* mutants with the indicated HA-FOI proteins expressed. (F,G) Examples of gonads in which germ cell ensheathment is rescued in these genotypes (i.e. more ensheathment than observed in *foi* mutants). Arrows indicate examples of germ cell ensheathment. Perimeter of gonad is outlined. Scale bars: 10 μm.

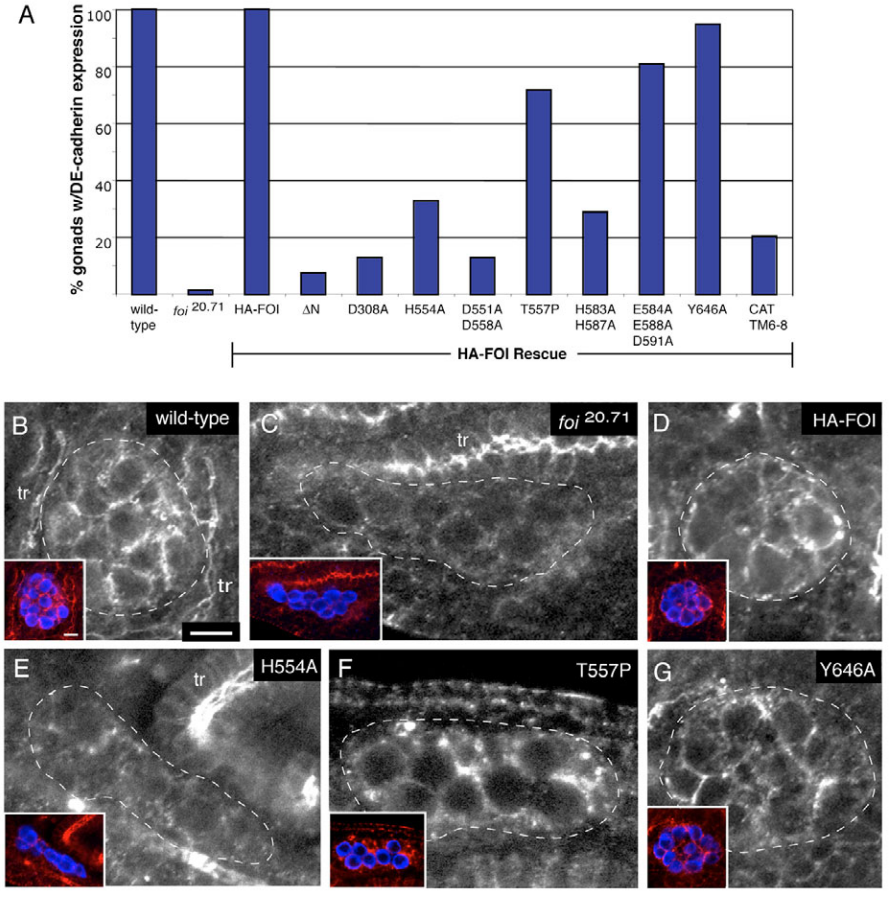

Fig. 4. Rescue of DE-cadherin expression. Expression of indicated FOI proteins in *foi*-mutant embryos. (**A**) Graph indicates the percentage of gonads with higher levels of DE-cadherin expression than *foi*-mutant controls. (**B-G**) Confocal images of representative stage 15-16 gonads, immunolabeled with α-DCAD2 (DE-cadherin). Insets show positions of germ cells (α-VAS, blue) relative to DE-cadherin (α-DCAD2, red). (B) Wild type, (C) *foi* mutant, (D-G) *foi* mutants with the indicated HA-FOI proteins expressed. Wild type (B), HA-FOI (D), T557P (F) and Y646A (G) gonads express DE-cadherin, while $foi^{20.71}$ (C) and H554A (E) gonads do not. DE-cadherin expression in gonads expressing T557P (F) is more diffuse than in wild type (B). DE-cadherin in neighboring tracheal branches is labeled (tr). Perimeter of gonad is outlined. Scale bars: 10 μm.

Three of the mutant FOI proteins (T557P, E584A/E588A/D591A and Y646A) rescued at least some DE-cadherin expression in the gonad (Fig. 4A,F,G). However, although DE-cadherin localization appeared normal in embryos expressing the Y646A protein, it appeared weaker and more diffuse in embryos expressing the T557P or E584A/E588A/D591A proteins. It is possible that the DE-cadherin protein observed in these embryos is mislocalized or inactive, consistent with the poor degree of rescue observed with the T557P and E584A/E588A/D591A proteins in our other assays. The remaining mutant forms of FOI were unable to rescue any DE-cadherin expression (Fig. 4A,E), indicating that the N-terminal, HELP and TM domains are all required for proper DE-cadherin expression during gonad coalescence.

FOI function and zinc transport

To determine whether the zinc transport activity of FOI is important for its role in development, we compared the in vivo rescue activity of the different mutant forms of FOI with their effects on zinc transport activity (Fig. 5) (Mathews et al., 2005). Several mutant FOI proteins (ΔN, H554A and D308A) that were strongly defective in zinc transport activity, including some with just a single amino acid change, also failed to rescue gonad morphogenesis and DE-cadherin expression. A protein with less severe defects in zinc transport (E584A/E588A/D591A) rescued some SGP-specific DE-cadherin expression, and partially rescued gonad compaction and germ cell ensheathment. And the Y646A protein, which fully rescued gonad compaction and DE-cadherin expression, and partially rescued germ cell ensheathment, had the least severe zinc transport defect. Thus, there is a direct correspondence between the zinc transporter activity of FOI and its function in regulating gonad morphogenesis and we have not uncovered mutations that separate these activities.

foi affects *shg (DE-cadherin)* RNA levels

As our data suggest that the zinc transport activity of FOI is required for proper DE-cadherin protein levels in the gonad, we wanted to determine how FOI and zinc might regulate DE-cadherin expression. Previously, it has been shown that zebrafish LIV-1, which is highly similar to FOI, can regulate the activity of the transcription factor SNAIL, a known regulator of DE-cadherin expression (Yamashita et al., 2004). To determine if FOI affects DE-cadherin transcript levels in the gonad, we analyzed *shg* mRNA expression using fluorescent in situ hybridization. In wild-type embryos, *shg* RNA was first detected in the gonad just prior to gonad coalescence (stage 13; Fig. 6A,A′), and was also detected at later stages after gonad coalescence (Fig. 6C,C′), similar to what is observed with DE-cadherin protein (Jenkins et al., 2003). *shg* RNA was primarily observed in the cells immediately surrounding the germ cells (SGPs), but was not observed in the germ cells themselves. *shg* RNA was also observed in other tissues that express high levels of DE-cadherin protein, including the salivary gland, dorsal epidermis and gut (Tepass et al., 1996). In *foi* mutants, gonad-specific *shg* RNA was not observed at either early (stage 13; Fig. 6B,B′) or later stages (through stage 16; Fig. 6D,D′), but was still observed in other tissues where DE-cadherin is normally expressed (W.R.M. and M.V.D., unpublished). Thus, within the gonad, FOI appears to regulate DE-cadherin at least in part by regulating *shg* RNA levels.

The decreased levels of *shg* RNA observed in *foi* mutants might reflect decreased levels of *shg* transcription, instability of the *shg* mRNA, or both. To investigate *shg* transcription, we analyzed the expression of a *shg* enhancer-trap, for which accumulation of β-galactosidase (β-GAL) is an indicator of *shg* promoter activity.

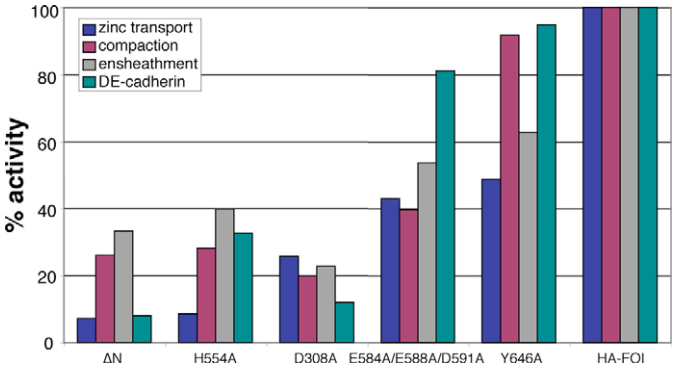

Fig. 5. The zinc transport activity of FOI is required for proper gonad morphogenesis in vivo. Graph represents a comparison of the amount of FOI function exhibited by each mutant version of FOI, relative to the activity of wild-type HA-FOI. Data include zinc transport activity, as assayed by zinc uptake in cultured cells [blue, data from Mathews et al. (Mathews et al., 2005)], and in vivo rescue activity, as judged by gonad compaction (red), germ cell ensheathment (gray) and DE-cadherin expression (green) (data from Figs 2-4 but normalized to HA-FOI controls). The zinc transport ability of each FOI protein directly correlates with its ability to rescue the *foi* gonad phenotype in vivo.

Wild-type gonads exhibited slightly increased expression of this enhancer-trap in SGPs, compared with surrounding tissues, just prior to gonad coalescence (stage 13) (A.B.M. and M.V.D., unpublished) and this was maintained throughout the rest of embryogenesis (Fig. 6E). No such increase was observed in *foi* mutant gonads (Fig. 6F), indicating that *foi* may have some effect on *shg* transcription. However, the upregulation of the *shg* enhancer trap in the gonad and the change in *foi* mutants appear much less dramatic than we observed for the endogenous *shg* RNA and DE-cadherin protein. This suggests that DE-cadherin might also be regulated at the post-transcriptional level.

DE-cadherin is regulated at the post-transcriptional level in the gonad

To investigate post-transcriptional regulation of DE-cadherin, we examined the pattern of DE-cadherin protein accumulation when DE-cadherin was expressed from a heterologous, ubiquitous promoter (*alpha-tubulin84B)*. Expression of a full-length *shg* cDNA using this promoter (*tub-DE-cad*) is sufficient to rescue the embryonic defects in *shg* null mutant embryos, and can even rescue a small percentage of flies to adult viability (Pacquelet et al., 2003). In wild-type embryos expressing *tub-DE-cad*, we observed the highest levels of DE-cadherin immunoreactivity in tissues that normally express endogenous DE-cadherin protein, such as the gonad, trachea, salivary gland and ectoderm (Fig. 7A,B; W.R.M. and M.V.D., unpublished). No gross morphological defects were observed in these embryos, indicating that additional DE-cadherin expressed from *tub-DE-cad* does not disrupt normal patterning. Virtually all of the gonads in these embryos exhibited high levels of DE-cadherin expression and had a wild-type morphology (Fig. 7B; 98% wild-type, *n*=58).

Next, we examined DE-cadherin protein in embryos where *tub-DE-cad* was the only source of zygotic DE-cadherin expression [*tub-DE-cad* expressed in embryos homozygous for a protein null mutant of *shg* (shg^{R69})] (Godt and Tepass, 1998; Pacquelet et al., 2003). Surprisingly, in shg^{R69}*; tub-DE-cad* embryos, we observed that DE-cadherin protein was present at much higher levels in the gonad than

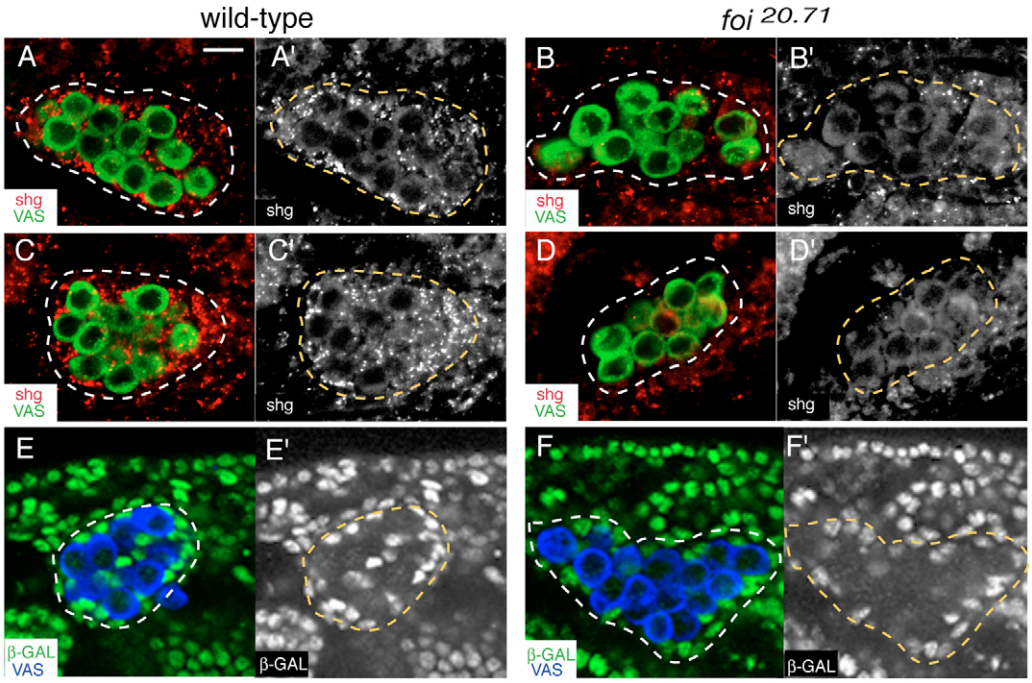

Fig. 6. *shg* mRNA is reduced in *foi* mutants. (**A-D**) Confocal images of embryos labeled by fluorescent in situ hybridization to reveal *shg* RNA (red) and immunolabeled with α-VAS (germ cells, green). (A′-D′) *shg* RNA channel alone. *shg* RNA is observed in SGPs of wild-type embryos both before (A, stage 13) and after (C, stage 15) gonad coalescence, but is not detected in *foi*-mutant gonads at either stage (B, stage 13; D, stage 15). (**E**,**F**) Confocal images of stage 15 wild-type (E) and *foi*-mutant (F) gonads heterozygous for the shg^{k03401} enhancer-trap immunolabeled with α-β-GAL (enhancer trap, green) and α-VAS (germ cells, blue). SGPs were identified using α-EYA (not shown). (E′,F′) Identical images showing only α-β-GAL. SGPs in wild-type gonads (E′) exhibit slightly increased shg^{k03401} expression compared with surrounding tissues, while SGPs in *foi*-mutants (F′) exhibit weaker expression than surrounding tissues. The perimeter of gonad is outlined. Scale bar: 10 μm.

in the surrounding tissue, even though it was being expressed from the ubiquitous *tubulin* promoter. DE-cadherin was expressed in a majority of gonads (92%, *n*=41, Fig. 7D), and the coalescence defects observed in *shg* mutants were largely rescued in these embryos. Gonad-specific DE-cadherin expression is due to the *tub-DE-cad* transgene and not residual maternally contributed DE-cadherin, as zygotic shg^{R69} mutant embryos alone exhibited little or none of the typical DE-cadherin staining in the gonad (Fig. 7C). The residual DE-cadherin 'ring' staining observed in the shg^{R69} mutant gonad is also present in embryos in which the *shg* gene is deleted and is not associated with the normal partners of DE-cadherin such as Armadillo/β-catenin (Jenkins et al., 2003). Furthermore, gonad-specific DE-cadherin expression is unlikely to be due to gonad-specific transcription from the *tubulin* promoter, as examination of a related *alpha-tubulin84B* promoter construct (*tubulin*-GAL4 crossed to UAS-*mCD8::GFP*) revealed that the activity of the *tubulin* promoter is actually lower in the gonad than in surrounding tissues (Fig. 7E). Thus, we conclude that the gonad-specific pattern of DE-cadherin expression is generated by post-transcriptional regulation.

foi affects DE-cadherin expression at the post-transcriptional level

To determine whether *foi* affects post-transcriptional regulation of DE-cadherin, we examined DE-cadherin protein when *tub-DE-cad* was expressed in a *foi*-mutant background. Strikingly, *tub-DE-cad* showed a greatly reduced ability to restore DE-cadherin immunoreactivity to *foi*-mutant gonads compared with *shg*-mutant gonads. Most $foi^{20.71}$, *tub-DE-cad* embryos (*n*=86) exhibited either weak punctate DE-cadherin expression (52%, Fig. 7F) or no DE-cadherin expression (14%). Only 34% (Fig. 7H) exhibited DE-cadherin immunoreactivity at levels more similar to shg^{R69}; *tub-DE-cad* controls (Fig. 7D). Therefore, *foi* is required for proper DE-cadherin protein expression even when DE-cadherin RNA is expressed from the ubiquitous *tubulin* promoter.

Interestingly, the *tub-DE-cad* transgene was still able to rescue the gonad phenotype in some *foi*-mutant embryos. Although *foi* mutant gonads rarely appear wild type (4%, *n*=42), when assessed for both SGP and germ cell morphology, 22% (*n*=86) of $foi^{20.71}$, *tub-DE-cad* embryos exhibited wild-type gonads (Fig. 7H; data not shown). The remaining embryos had gonads similar to *foi* mutants (Fig. 7G), and some also contained germ cells that retained a protrusive morphology. In general, the $foi^{20.71}$, *tub-DE-cad* embryos that exhibited increased DE-cadherin expression in the gonad appeared to be those with rescued gonad coalescence (compare Fig. 7F-H). Thus, the relatively low-level rescue of the *foi* mutant phenotype by *tub-DE-cad* may reflect the reduced expression of DE-cadherin protein in *foi* mutants or, alternatively, could reflect DE-cadherin-independent effects of *foi*. Regardless, expression of DE-cadherin was sufficient to rescue the *foi* mutant phenotype in some cases, indicating that one of the principal ways that *foi* affects gonad formation is through regulating DE-cadherin.

Finally, we wanted to investigate whether the post-transcriptional effects of *foi* on DE-cadherin expression occur at the level of the DE-cadherin protein or RNA. To do this, we investigated the pattern of *shg* RNA accumulation from the *tub-DE-cad* transgene. When *tub-DE-cad* was expressed in wild-type embryos, we observed a high level of *shg* RNA accumulation in the gonad by in situ hybridization (W.R.M. and M.V.D., unpublished). *tub-DE-cad* also restored *shg* RNA accumulation to a shg^{R69} mutant (Fig. 7J, compared with I,

81% of *shg*^{R69}*; tub-DE-cad* embryos exhibited high levels of *shg* RNA in the gonad, *n*=38). By contrast, when *tub-DE-cad* was expressed in *foi*-mutant embryos, *shg* RNA accumulation in the gonad was greatly reduced (Fig. 7K), and was detectable in only 24% of the gonads (Fig. 7L, *n*=42). This is comparable with the fraction of gonads that exhibited high levels of DE-cadherin protein expression in the *foi*$^{20.71}$, *tub-DE-cad* background. Thus, expression of DE-cadherin using the *tub-DE-cad* transgene is unable to fully restore *shg* RNA accumulation in *foi*-mutant gonads. As the activity of a related *alpha-tubulin84B* promoter construct (*tubulin*-GAL4) is unaffected in *foi* mutants (W.R.M. and M.V.D., unpublished), *foi* is likely to affect *shg* RNA stability.

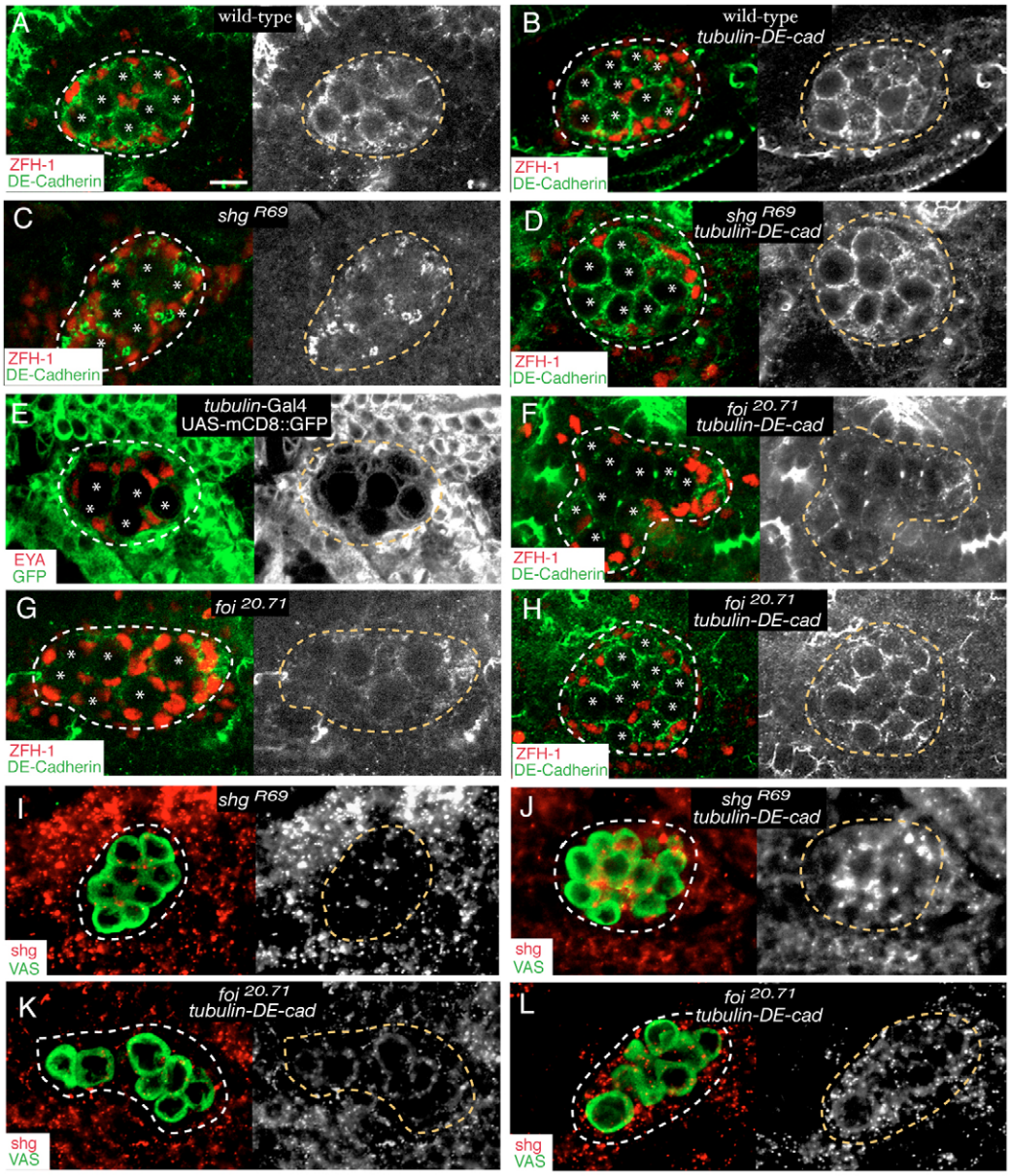

Fig. 7. *foi* regulates *shg* at the post-transcriptional level. (A-D,F-H) Confocal sections of stage 15-16 embryos immunostained with α-ZFH-1 (SGPs, red) and α-DCAD2 (DE-cadherin, green). The right-hand panels show DE-cadherin channel alone. Gonads and germ cells are indicated with broken lines and asterisks, respectively. Both wild-type (**A**) and wild-type, *tub-DE-cad* (**B**) embryos exhibit high levels of DE-cadherin in the gonad. (**C**) *shg*^{R69} mutant gonad lacking most DE-cadherin expression, but with some α-DCAD2 immunoreactivity in large ring-like structures that fail to co-localize with *armadillo* (Jenkins et al., 2003). (**D**) *shg*$^{R69;}$ *tub-DE-cad* gonad with high levels of DE-cadherin concentrated around germ cells where they are ensheathed by SGPs. (**E**) Confocal section of a stage 15 embryo, in which *tubulin*-GAL4 drives expression of UAS-*mCD8::GFP*, immunolabeled with α-GFP (green) and α-EYA (SGPs, red). The SGPs express less GFP than the surrounding mesodermal cells. (**F**,**H**) *foi*$^{20.71}$, *tub-DE-cad.* DE-cadherin is most often seen as weak punctae between germ cells (F) or is completely absent in gonads. In some embryos (H), more DE-cadherin protein is detected. (**G**) Little or no DE-cadherin expression is observed in the SGPs of *foi*$^{20.71}$ mutant gonads. msSGPs (posterior/right in this gonad) still express DE-cadherin in *foi* mutants, as has previously been reported (Jenkins et al., 2003). (**I-L**) Confocal sections of stage 15-16 embryos, labeled by fluorescent in situ hybridization to reveal *shg* RNA (red) and immunolabeled with α-VAS (green, germ cells). Right-hand images represent *shg* RNA alone. Very little *shg* RNA immunoreactivity is observed in *shg*^{R69} mutant gonads (I), but *shg* RNA is restored in *shg*^{R69}; *tub-DE-cad* (J). *tub-DE-cad* fails to restore *shg* RNA expression to most *foi*$^{20.71}$ mutant gonads (K), but a minority have *shg* RNA restored (L). Scale bar: 10 μm.

DISCUSSION

We have investigated how FOI regulates DE-cadherin expression and gonad development. We find that the zinc transport activity of FOI is essential for proper gonad morphogenesis and DE-cadherin expression. Thus, zinc is likely to act as a crucial regulator of these developmental processes. We further find that post-transcriptional regulation is an important aspect of how the tissue-specific pattern of DE-cadherin expression is generated. FOI regulates DE-cadherin at least in part by affecting this post-transcriptional control, highlighting the diverse mechanisms that are employed to control this essential cell-adhesion molecule.

Zinc transport activity of FOI is essential for gonad morphogenesis

Previously, it was unclear whether ZIP family members regulate developmental processes by acting as zinc transporters or through some other unidentified function. Our data now indicate that FOI regulates gonad formation through its zinc transporter activity. The ability of the mutant forms of FOI to rescue gonad morphogenesis and DE-cadherin expression corresponds directly with their ability to function as zinc transporters (Fig. 5). Mutations that strongly affect the zinc transport activity of FOI (e.g. H554A) also strongly reduce the ability of FOI to rescue gonad morphogenesis and DE-cadherin expression. Mutations that only partially affect the zinc transport activity of FOI (e.g. Y646A) retain some ability to rescue gonad morphogenesis and DE-cadherin expression. If FOI affects gonad formation through a function separate from zinc transport, we would expect to have identified conserved residues that affect these two activities independently. This was not the case. Indeed, even single amino acid changes in very different regions of FOI (e.g. D308A and H554A) affect both zinc transport and gonad morphogenesis. We conclude that the zinc transporter function of FOI is essential for gonad morphogenesis and regulation of DE-cadherin. This reveals a crucial role for zinc regulation in development and suggests that other ZIP family members with developmental roles (e.g. zebrafish LIV1) may also act via zinc transport.

Our in vivo analysis is also informative for revealing domains that are essential for FOI function. We find that, even though the N-terminal extracellular domain of FOI shows little sequence conservation with other family members (Mathews et al., 2005), and some ZIP family members lack an extended N-terminal domain, this domain is nevertheless important for FOI function. We also find that, in addition to their TM character, the specific sequence of the TM domains is crucial for FOI function. Mutations that are not predicted to affect the TM structure of FOI, such as mutating a single acidic residue in TM2 (D308A) or replacing TM6-8 of FOI with similar TM domains from the related protein CATSUP (CAT TM6-8), still disrupt the in vivo rescue activity of FOI. Finally, we analyzed several characteristics of the highly conserved HELP domain in FOI (which may or may not have TM structure) (Mathews et al., 2005). The predicted amphipathic α-helical nature of this domain appears to be crucial, as altering the pattern of acidic residues (D551A/D558A and E584A/E588A/D591A) or inserting a helix contorting proline residue (T557P) disrupts FOI function. In addition, conserved histidines in this domain are essential (H554A and H583A/H587A), and mutating even a single histidine has a dramatic effect in vivo. As FOI is a zinc transporter, it is likely that the specific sequences of the TM domains form the proper membrane pore for zinc, while histidines in the N-terminal and HELP domains act to coordinate zinc before and during transport.

Regulation of DE-cadherin by FOI

shg and *foi* are both required for proper gonad and tracheal morphogenesis (Uemura et al., 1996; Van Doren et al., 2003), and *foi* regulates DE-cadherin expression in the gonad (Jenkins et al., 2003). DE-cadherin protein levels are not reduced in *foi* mutants simply because the gonad has failed to coalesce; other mutations blocking gonad coalescence do not affect DE-cadherin (A.B.M., J. Weyers and M.V.D., unpublished). Thus, it is likely that *foi* affects DE-cadherin more directly and this is an important aspect of how *foi* functions in gonad and tracheal development. In support of this, we found that expression of DE-cadherin was sufficient to partially rescue *foi* mutant gonads.

As both DE-cadherin protein and *shg* RNA levels are reduced in *foi* mutant gonads, we investigated whether *foi* affects DE-cadherin transcription. Our analysis of a *shg* enhancer-trap suggests that some aspects of DE-cadherin regulation by *foi* may be at the transcriptional level. Recently, it has been shown that a related ZIP protein, zebrafish LIV1, can regulate the activity of the Zn-finger transcription factor SNAIL, which may also influence E-cadherin expression (Yamashita et al., 2004). Examples of how zinc transporters and zinc can modulate the function of transcription factors include regulating zinc-dependent DNA-binding domains (e.g. zinc fingers) (Dalton et al., 1997), regulating non-DNA binding zinc-finger domains (Bird et al., 2003) and affecting transcription factor subcellular localization (Smirnova et al., 2000; Yamashita et al., 2004).

However, although a majority of studies focus on transcriptional regulation of E-cadherin, it is likely that this essential cell-adhesion molecule is often regulated at many levels, including through post-transcriptional and post-translational mechanisms (Gumbiner, 2000). Here, we present clear evidence that DE-cadherin is regulated at the post-transcriptional level in the embryonic gonad. Expression of DE-cadherin from the general *tubulin* promoter (*tub-DE-cad*) (Pacquelet et al., 2003) was sufficient to restore gonad-specific DE-cadherin protein accumulation in *shg* mutants (Fig. 7). Recent work suggests that DE-cadherin localization within the ovary is also regulated partly through a post-transcriptional mechanism (Becam et al., 2005). Thus, post-transcriptional regulation may be sufficient to generate tissue-specific patterns of DE-cadherin expression in many contexts. *tub-DE-cad* was much less able to restore DE-cadherin protein to the gonad in *foi* mutants. This indicates that FOI is required for positive, post-transcriptional regulation of DE-cadherin. One component of this regulation is likely to act on *shg* RNA stability, as *foi* affects the gonad-specific accumulation of *shg* RNA from *tub-DE-cad* (Fig. 7), but does not affect the activity of the *tubulin* promoter. Thus, the steady-state pattern of *shg* RNA accumulation does not merely reflect *shg* promoter activity but may have a significant post-transcriptional component. In principle, zinc could regulate the activity of RNA-binding proteins that affect RNA stability in the same way it regulates DNA-binding transcription factors. In addition, DE-cadherin may be further regulated at the protein level in the gonad, such as through regulation of translation or protein stability.

Role of ZIP proteins in development and disease

Recent in vivo work on several ZIP proteins suggests that these zinc transporters play essential roles in development and disease that may broadly involve regulation of cell-cell adhesion (Fig. 8). In zebrafish, regulation of SNAIL by LIV1 is essential for the anterior migration of zebrafish organizer cells and may regulate E-cadherin expression in this tissue (Yamashita et al., 2004). According to this model, LIV1 activates SNAIL activity, which leads to downregulation of E-

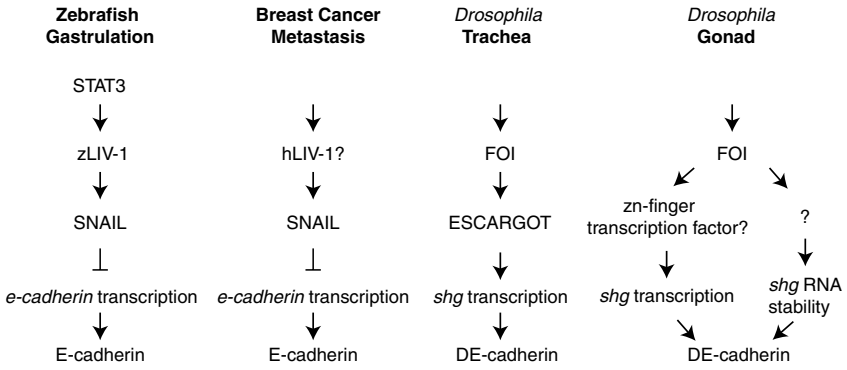

Fig. 8. Models of zinc regulation of DE-cadherin. Human and zebrafish LIV1 proteins may function as zinc transporters to activate SNAIL, a repressor of E-cadherin transcription. *Drosophila* FOI may activate Escargot (an activator of DE-cadherin) in the trachea, and is required for both DE-cadherin transcription and RNA stability in the gonad.

cadherin and the decreased cell adhesion necessary for cell migration (Yamashita et al., 2004). Interestingly, SNAIL is also thought to be an important regulator of E-cadherin during the progression and metastasis of certain cancers, such as breast cancer. As a tumor gains metastatic potential, SNAIL expression is upregulated and E-cadherin is downregulated (Nieto, 2002). As human LIV-1 is strongly expressed in breast cancer cell lines (Manning et al., 1988), and has been implicated in breast cancer metastasis (Manning et al., 1994), it may function to activate the activity of SNAIL as a transcriptional repressor of E-cadherin, again allowing for cell migration and metastasis. A similar, but opposite, relationship may exist in the *Drosophila* tracheal system, where the SNAIL family member Escargot (ESG) is a positive regulator of E-cadherin during the fusion of neighboring tracheal branches (Tanaka-Matakatsu et al., 1996). As FOI is also required for this process (Van Doren et al., 2003), it may act by promoting ESG activity. In this case, FOI and ESG would activate DE-cadherin expression, which is necessary for cell-cell attachment during tracheal branch fusion. In the gonad, FOI is also positively required for DE-cadherin expression. Although ESG is present in the gonad (Boyle and DiNardo, 1995), we have not observed changes in DE-cadherin expression during gonad coalescence in *esg* mutants (S. Le Bras and M.V.D., unpublished), indicating that some other target for regulation by FOI and zinc must exist in this tissue. An important theme in the action of ZIP proteins may be to influence the activity of zinc-regulated transcription factors, with cell-cell adhesion molecules being important targets of such regulation. However, as discussed above, we have found that additional, post-transcriptional mechanisms are crucial in the gonad for regulation of DE-cadherin protein expression by FOI. Thus, it will be very important to analyze the contribution of post-transcriptional regulation of E-cadherin to other developmental and disease processes. Indeed, there is even evidence that the same crucial factor, SNAIL, can influence post-transcriptional regulation (Ohkubo and Ozawa, 2004).

An important issue relevant to the role of zinc and zinc transporters in development and disease is whether they play an instructive or permissive role. Is zinc merely required at a minimum threshold level in various tissues or does regulation of intracellular zinc concentration play a signaling role at specific times and places? Existing evidence suggests that zinc may play an instructive role. Both *Drosophila foi* and zebrafish LIV1 have highly tissue-specific patterns of expression and affect the development of selected tissues, while others remain unaffected (Pielage et al., 2004; Van Doren et al., 2003; Yamashita et al., 2004). Mammalian ZIP and Cation Diffusion Facilitator family members also have tissue-specific expression patterns (e.g. Dufner-Beattie et al., 2003; Palmiter et al., 1996). Thus, zinc transporters have the necessary spatial and temporal resolution to play an instructive role. In addition, zinc transporters have clear roles as modulators of intracellular signals. They have the capacity to modulate signaling pathways, for example the *ras* pathway (Bruinsma et al., 2002), and can influence transcription factor activity and gene expression (Smirnova et al., 2000; Bird et al., 2003; Yamashita et al., 2004). Because we show here that the zinc transport activity of FOI is crucial for its developmental role, it is likely to act by modulating zinc concentration. Thus, zinc has the potential to be an important and dynamically regulated signaling molecule during development and adult homeostasis.

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