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zfh-1 is required for germ cell migration and gonadal mesoderm development in *Drosophila*

Heather Tarczy Broihier[1,2], Lisa A. Moore[1,2], Mark Van Doren[1], Stephanie Newman[1] and Ruth Lehmann[1,3,*]

[1]Skirball Institute, New York University Medical Center, NY, NY, 10016, USA
[2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA
[3]Howard Hughes Medical Institute
*Author for correspondence (e-mail: lehmann@saturn.med.nyu.edu)

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SUMMARY

In *Drosophila* as well as many vertebrate systems, germ cells form extraembryonically and migrate into the embryo before navigating toward gonadal mesodermal cells. How the gonadal mesoderm attracts migratory germ cells is not understood in any system. We have taken a genetic approach to identify genes required for germ cell migration in *Drosophila*. Here we describe the role of *zfh-1* in germ cell migration to the gonadal mesoderm. In *zfh-1* mutant embryos, the initial association of germ cells and gonadal mesoderm is blocked. Loss of *zfh-1* activity disrupts the development of two distinct mesodermal populations: the caudal visceral mesoderm and the gonadal mesoderm. We demonstrate that the caudal visceral mesoderm facilitates the migration of germ cells from the endoderm to the mesoderm. Zfh-1 is also expressed in the gonadal mesoderm throughout the development of this tissue. Ectopic expression of Zfh-1 is sufficient to induce additional gonadal mesodermal cells and to alter the temporal course of gene expression within these cells. Finally, through analysis of a *tinman zfh-1* double mutant, we show that *zfh-1* acts in conjunction with *tinman*, another homeodomain protein, in the specification of lateral mesodermal derivatives, including the gonadal mesoderm.

Key words: *zfh-1*, *tin*, *dpp*, *cli*, Germ cell, Gonadal mesoderm, Migration, Mesoderm, *Drosophila*

INTRODUCTION

Germ cells segregate from somatic cells early in the development of many species. In *Drosophila*, primordial germ cells form at the posterior pole of the embryo and cellularize while the somatic nuclei remain syncytial. Although this sets germ cells apart from somatic cells, they will interact with somatic cells throughout the life of the fly. These interactions are initiated in the embryo when germ cells migrate toward gonadal mesodermal cells and continue as germ cells differentiate into sperm or eggs. We are interested in uncovering the molecules responsible for mediating the initial interactions between germ cells and somatic cells as they may provide insights into general mechanisms of cell migration and organogenesis.

Germ cells relinquish their extraembryonic location during gastrulation when they migrate toward the somatic gonadal precursors (SGPs), which will give rise to the gonadal mesoderm. As the germband extends, the germ cells initially remain attached to the posterior pole as it sinks into the embryo and forms the posterior midgut (PMG). Germ cells extend cytoplasmic projections and migrate through the blind end of the PMG during early stage 10 (Callaini et al., 1995; Jaglarz and Howard, 1995). After germ cells move through the endoderm, they migrate on its basal surface until they contact overlying mesodermal cells. Beginning late in stage 10, germ cells transfer from the endoderm into mesoderm where they will associate with SGPs.

Somatic gonadal precursors (SGPs) form in three bilateral clusters in PS10-12, which are located immediately ventral to the precursors of the visceral mesoderm (Boyle et al., 1997; for a review of mesoderm development, see Riechmann et al., 1997). Here we refer to all mesoderm located at this dorsovental position as lateral mesoderm. SGP clusters are identifiable beginning at stage 11 via their expression of Clift/Eyes absent (Cli) protein, which is a nuclear protein of novel sequence (Bonini et al., 1993). Furthermore, *cli* function is required for SGP development, as in *cli* mutant embryos, SGPs are specified but fail to differentiate (Boyle et al., 1997). During stage 12, the SGP clusters and their associated germ cells migrate toward each other so that, by stage 13, the three clusters are contiguous (Boyle and DiNardo, 1995; Boyle et al., 1997). Coalescence of germ cells and SGPs occurs in PS10 during stage 14. Germ cells are not required for expression of SGP-specific markers or for coalescence of these cells (Brookman et al., 1992; Boyle et al., 1997).

Some genes involved in SGP specification have recently been identified. The homeobox-containing gene *tinman (tin)* is expressed throughout the mesoderm as it invaginates into the embryo. *tin* expression is subsequently maintained only in dorsal mesoderm, where it is required for the specification of the heart and the visceral mesoderm (Azpiazu and Frasch,

1993; Bodmer, 1993). Expression of *tin* in dorsal mesoderm depends on *decapentaplegic* (*dpp*), which is expressed in the overlying dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Although SGPs arise ventral to visceral mesoderm, and therefore outside of the dorsally restricted *tin* expression domain, SGP-specific gene expression is not initiated properly in *tin* mutant embryos (Boyle et al., 1997). The homeotic gene *abdominal A (abdA)* has also been shown to be required for the specification of SGPs (Cumberledge et al., 1992; Boyle and DiNardo, 1995; Greig and Akam, 1995; Boyle et al., 1997). In the absence of proper SGP specification in *tin* or *abdA* mutant embryos, germ cells still migrate toward and associate with laterally positioned mesodermal cells (Boyle et al., 1997; Moore et al., 1998a).

The fact that genes known to be involved in SGP development are not required for germ cell migration to lateral mesoderm raises the question of what genes are required to guide germ cells to the SGPs. Before germ cells are positioned next to the lateral mesoderm, they migrate over the surface of the endoderm. The oriented migration of germ cells on the endoderm requires the product of the *wunen (wun)* locus (Zhang et al., 1996). *wun* is a transmembrane protein expressed on the PMG, which repels migratory germ cells and thereby limits the region of the gut over which they can migrate (Zhang et al., 1997). It is possible that, in addition to the repellent *wun* signal, germ cell migration on the gut might be oriented by the overlying mesoderm. However, in *twist* (*twi*) *snail* (*sna*) double mutants, which lack mesoderm, germ cells migrate properly on the PMG surface (Jaglarz and Howard, 1994). In the absence of mesoderm, however, subsequent steps of germ cell migration are blocked and germ cells remain attached to the gut. Since *twi* and *sna* were the only mesodermally acting genes known to block germ cell migration to the mesoderm, it was proposed that germ cells would navigate toward and adhere to any mesodermal cells (Williamson and Lehmann, 1996). The isolation of additional mutations interfering with germ cell migration from the endoderm to the mesoderm indicates that this is not the case, as at least some components of mesoderm still develop in these mutants (Moore et al., 1998a). Here we analyze the role of one such gene, *zfh-1*, in mediating the initial interactions between germ cells and mesoderm.

zfh-1, or *zinc finger homeodomain protein-1*, codes for a transcription factor containing nine zinc fingers and a single homeodomain, which is expressed in the central nervous system and in numerous mesodermal lineages including the dorsal vessel, muscle precursors, and the mesoderm of coalesced gonads (Fortini et al., 1991; Lai et al., 1991). Previous phenotypic analysis of *zfh-1* mutant embryos revealed defects in heart and muscle formation (Lai et al., 1993).

We show here that *zfh-1* is required for the initial interactions between germ cells and mesoderm, as germ cells do not correctly migrate to the lateral mesoderm in *zfh-1* mutant embryos. Mutant analysis demonstrates that *zfh-1* is necessary for the development of two distinct mesodermal lineages which both function in germ cell migration: the caudal visceral mesoderm and the somatic gonadal precursors. Analysis of embryos in which Zfh-1 is ectopically expressed demonstrates that *zfh-1* is sufficient for the development of additional SGPs. Furthermore, we demonstrate that *tin* and *zfh-1* have overlapping functions in the development of lateral mesoderm derivatives, including gonadal mesoderm and the fat body. Finally, by studying the spatial and temporal association of germ cells with mesodermal cells, we find that germ cells follow multiple migratory paths to reach the SGPs.

MATERIALS AND METHODS

Fly stocks

We used a *trans*heterozygous combination of two EMS-induced *zfh-1* alleles, $zfh\text{-}1^{65.34}/zfh\text{-}1^{75.26}$, for all analyses (Moore et al., 1998a). The alleles show the same phenotype in *trans* to each other, in *trans* to a deficiency uncovering *zfh-1* (Df(3R)20 e der^{177}), or when *trans*heterozygous to another strong *zfh-1* allele, $zfh\text{-}1^{2}$. (Both of these stocks were provided by Z. C. Lai.) An anti-Zfh-1 antibody does not recognize protein in $zfh\text{-}1^{65.34}$ or $zfh\text{-}1^{75.25}$ embryos, but both alleles produce *zfh-1* RNA. These same *zfh-1* alleles were used to make germline clones.

Two overlapping deficiencies, Df(3L)vin^4 and Df(3L)vin^6, were used to uncover the *byn* locus (Bloomington). In addition the following stocks were used: Df(3R)GC14 (tin^{-}) (M. Frasch), $abdA^{MX1}$ (W. Bender), dpp^{H46} (B. Gelbart), srp^{9L} (Bloomington), cli^{1} (Bloomington), $cli^{14.40}$ (generated in the Lehmann laboratory), cli^{D1} (M. Boyle) and *HSzfh-1/CyO* (Z. C. Lai), described in Lai et al. (1991).

The *tin zfh-1* double mutant was made by standard meiotic recombination. Recombinants were generated between Df(3R)GC14 and both $zfh\text{-}1^{65.34}$ and $zfh\text{-}1^{75.26}$. The *cli;zfh-1* double mutant was generated using the cli^{D1} allele and both $zfh\text{-}1^{65.34}$ and $zfh\text{-}1^{75.26}$. The phenotypes of both double mutants were identical with either $zfh\text{-}1^{65.34}$ or $zfh\text{-}1^{75.26}$. In all experiments, we used balancers carrying P-lacZ transgenes: P{UbxlacZ-TM3} or P{ftzlacZ-CyO} to distinguish homozygous mutant embryos from balancer-bearing siblings.

Whole-mount antibody staining

Antibody staining was performed using either horseradish peroxidase with biotinylated secondary antibodies and the Elite kit (Vector Laboratories), or with alkaline phosphatase (AP) with directly conjugated secondary antibodies. Embryos were fixed by gentle shaking for 25 minutes in 8 ml heptane, 0.25 ml 37% formaldehyde and 1.75 ml PBS, 50 mM EDTA and devitellinized as for in situ hybridization (Ephrussi et al., 1991). Following rehydration, antibody staining using biotinylated secondaries was conducted as described in Eldon and Pirrotta (1991). Antibody staining with multiple primary antibodies was carried out as described in Patel (1994). Whole-mount embryos were embedded in PolyBed812 (Polysciences) following the protocol of Ephrussi et al. (1991), or in 85% glycerol, then analyzed with a Zeiss Axiophot using Nomarski optics. Slides were taken using either 64T or 160T Kodak film.

After staining, embryos to be sectioned were dehydrated stepwise (10 minutes each) in 30%, 50%, 70%, 90% and 95%, and twice in 100% ethanol. Embryos were then transferred to a crystallizing dish and incubated for 2 hours in 1:1 100% ethanol:PolyBed812, at which point the 1:1 mixture was removed and fresh PolyBed812 was added. Embryos were left in the resin overnight and embedded in rubber molds from Ted Pella, Inc. 2 μM sections were cut on a Leica UltraCut UCT and embedded in Permount (Fisher). They were visualized using phase-contrast optics.

The following primary antibodies were used in this work: affinity-purified anti-Zfh-1d (Z.C. Lai) at 1:500; anti-Vasa (A. Williamson) at 1:5,000; anti-β-gal (Cappel) at 1:10,000; anti-Srp (M. Leptin) at 1:1,000; and anti-Cli (M. Boyle and N. Bonini) at 1:1000. The following secondary antibodies were used: biotinylated-goat anti-rabbit, biotinylated-goat anti-mouse, and AP-goat anti-rabbit; all from Jackson ImmunoResearch. Anti-Vasa, anti-β-gal, anti-Srp and secondary antibodies were diluted 1:10 and preabsorbed against an overnight collection of wild-type embryos prior to use.

Whole-mount in situ hybridizations

Whole-mount in situ hybridizations were performed as in Lehmann and Tautz (1994). Embryos were typically incubated with probe for 36 hours at 55°C. Following the in situ hybridization protocol, embryos that were to be double stained with antibodies were first stored in 70% ethanol, from overnight to one week. Antibody staining was then conducted as Lehmann and Tautz (1994).

Antisense digoxigenin-coupled RNA probes were synthesized using the Boehringer-Mannheim 'Genius' 4 kit as described in Gavis and Lehmann (1992). The *lacZ* riboprobe was made with the pC4β-gal plasmid and T7 polymerase (Thummel et al., 1988). The *crocodile* riboprobe was made with a pNB40-derived plasmid from the Jäckle laboratory and T7 polymerase. The *bagpipe* riboprobe was generated with a pGem1-derived plasmid from the Frasch laboratory and T7 polymerase. The riboprobe for the 412 retrotransposon was generated using the pSK2.4#3 plasmid (Brookman et al., 1992) and T7 polymerase. The biotinylated 412 RNA probe was made using biotin-21-UTP from Clontech according to the method in Lehmann and Tautz (1992). Embryos were mounted as described above.

SGP counts

The number of SGPs present in *tin* homozygotes and heterozygotes was compared. At stage 11, we found 19.2 SGPs (s.d.=4.3, n=12) present in *tin* homozygotes and 32.6 SGPs (s.d.=2.1, n=8) in *tin* heterozgygotes. At stage 12, there were 7.8 SGPs (s.d.=3.5, n=8) present in *tin* homozygotes and 34 SGPs(s.d.=0.9, n=6) in *tin* heterozygotes. A two-sided t-test assuming equal variance gives $P=2.4\times10^{-8}$ and $P=1.5\times10^{-10}$ for the two stages, respectively, demonstrating that the two populations have different means.

The number of SGPs in *HSzfh-1* embryos at late stage 12 or stage 13 was compared to OR embryos subjected to an identical heat shock. There are an average of 48 SGPs (s.d.=13.6, n=18) in *HSzfh-1* embryos at this stage. In wild-type (Oregon R) controls, there are 28.3 SGPs (s.d.=3.8, n=16). A two-sided t-test assuming equal variance gives $P=9.0\times10^{-7}$. Thus, more SGPs are present in *HSzfh-1* embryos than in wild type.

Heat-shock protocol

A P{*hsp70-zfh-1*}/CyO stock was kindly provided by Z. C. Lai. 1 or 2 hour embryo collections were aged until the midpoint of the collection was at 5 hours AEL. Embryos were then subjected to a 1 hour 37°C heat shock by floating the apple juice plates in a water bath. The embryos were subsequently aged according to the stage of development to be analyzed. Embryos were aged 3 hours for analysis of *bap* RNA expression at stage 10. When expression of *croc* RNA (stage 11) was investigated, embryos were aged 4 hours, fixed as above and stained. Embryos were aged 7 hours (stage 13, 14) prior to fixation and antibody staining in the analysis of Cli expression. In all experiments, Oregon R embryos were collected, heat-shocked, aged, fixed and stained in parallel to *HSzfh-1*-bearing embryos as controls.

RESULTS

Zfh-1 is necessary for germ cell migration

In a large-scale mutagenesis screen aimed at identifying genes required for germ cell migration and gonad formation (Moore et al., 1998a), we isolated six alleles of *zfh-1* (Fortini et al., 1991; Lai et al., 1993). Germ cell formation and the initial steps of germ cell migration are not affected in *zfh-1* mutant embryos. The first deviation from wild-type development occurs in late stage 10 when germ cells are positioned on the endoderm in close juxtaposition with mesoderm. In wild-type embryos, germ cells leave the endoderm and navigate into lateral mesoderm where they contact somatic gonadal

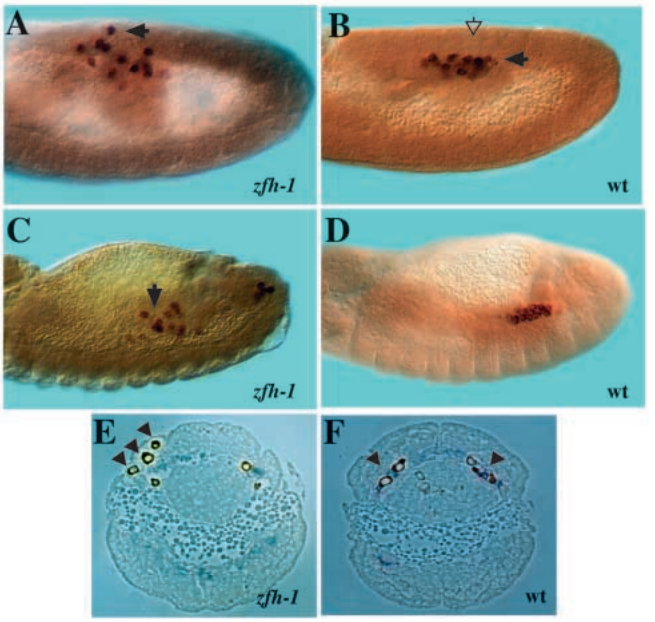

Fig. 1. Germ cells in *zfh-1* mutant embryos do not successfully navigate to the SGPs. Whole-mount embryos in A-D are anterior left and dorsal up; transverse sections (E-F) are dorsal up. *zfh-1*$^{65.34}$/*zfh-1*$^{75.26}$ embryos (A,C,E) or wild-type embryos (B,D,F). Germ cells labeled with anti-Vasa in brown (all panels) and gonadal mesoderm with the 412 retrotransposon RNA in blue (E,F). (B) Wild-type embryo at stage 11, germ cells attach to lateral mesoderm (filled arrow). (A) *zfh-1* mutant embryo at stage 11, germ cells migrate past their target mesodermal cells, into posterior or ectodermal regions (arrow). Open arrow in (B) denotes A/P level of sections in E,F. (E) Section of *zfh-1* mutant embryo at stage 11 with ectodermally located germ cells (arrowheads). (F) Wild-type embryo at the same stage with germ cells (arrowheads) adhering to gonadal mesoderm cells. (D) Wild-type embryo at stage 13 with germ cells within lateral mesoderm. In stage 13 *zfh-1* mutant embryo (C), lost germ cells are found near the PMG (arrow) and at the posterior end of the embryo, reflecting those germ cells that migrated too far posteriorly at stage 11.

precursors (SGPs) (Fig. 1B) (Boyle et al., 1997). Germ cells and SGPs remain in contact through the remainder of embryogenesis until the two cell types coalesce at stage 14 to form the embryonic gonad (Fig. 1D). In *zfh-1* mutant embryos, the majority of germ cells do not attach to the SGPs at stage 11 (Fig. 1A). Instead, some germ cells remain attached to the gut, leading to a cluster of germ cells in the middle of the embryo during later stages of development (Fig. 1C). Germ cells that enter the mesoderm in *zfh-1* mutant embryos disperse throughout the posterior mesoderm with some cells migrating across the mesodermal layer and into the overlying ectoderm (Fig. 1E, arrowheads). Occasionally, small gonads form in mutant embryos (data not shown).

The germ cell migration defect that we observe in *zfh-1* mutant embryos demonstrates that *zfh-1* function is required for the transition of germ cells from the endoderm to the mesoderm and suggests that *zfh-1* is required for interactions between germ cells and SGPs. Zfh-1 protein is found in the germ cells as well as in many mesodermal derivatives (Lai et al., 1991; see below). Thus, *zfh-1* could be required either in the germ cells or in the mesoderm to promote the interaction between these two cell types. In order to distinguish between

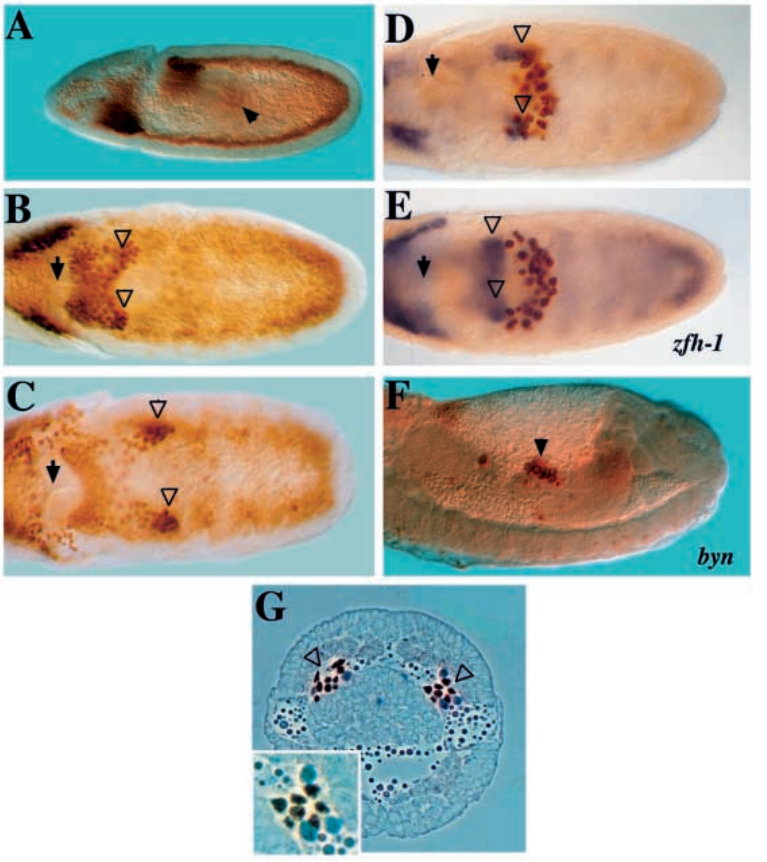

Fig. 2. Caudal visceral mesodermal cells express Zfh-1 and interact with migratory germ cells. Embryos in (A-D) and (G) wild type, (E) *zfh-1*$^{65.34}$/*zfh-1*$^{75.26}$, (F) *byn*^{vin4}/*byn*^{vin6}. (A-C) Labeled with anti-Zfh-1; (D-E) labeled with anti-Vasa (brown) marking germ cells and a *zfh-1* riboprobe (blue) marking the caudal visceral mesoderm. Open arrowheads point to the caudal visceral mesoderm which is in a slightly deeper plane of focus than the germ cells. We were able to use *zfh-1* RNA as a caudal visceral mesoderm marker, as the *zfh-1* alleles used in this analysis transcribe *zfh-1* RNA. (F) Stained with anti-Vasa, and the section in G is labeled with anti-Zfh-1(brown) and anti-Vasa (blue). (A) Wild-type Zfh-1 expression at stage 9. The anteriormost cells are hemocyte precursors, whereas the posteriormost cells are caudal visceral mesoderm. Maternal Zfh-1 is still faintly visible in germ cells (arrowhead) at this stage. (B-E) Open arrowheads indicate caudal visceral mesoderm and black arrows mark the posterior end of the embryo for reference. (B) Early stage 10 and (C) late stage 10 demonstrate normal caudal visceral mesoderm migration. (D,E) Embryos compare the positions of caudal visceral mesoderm and germ cells at late stage 10 in wild-type (D) and *zfh-1* mutants (E). In wild type, caudal visceral mesodermal cells have migrated anteriorly to the position of the germ cells on the PMG. In *zfh-1* mutants, caudal visceral mesoderm remains posterior to the germ cells. Transverse section in G illustrates the association of germ cells (blue) and caudal visceral mesoderm (brown, open arrowheads), in wild type. (F) *byn* mutant embryo at stage 13. A cluster of germ cells is situated on the PMG surface. There are small gonads in this embryo, but they are out of the plane of this photograph.

these two possible modes of *zfh-1* function, we analyzed the requirement for *zfh-1* activity in germ cells and in mesodermal lineages.

Maternal Zfh-1 is not required for germ cell migration

zfh-1 RNA and protein are localized to the posterior pole of the oocyte and incorporated into germ cells. Zfh-1 protein is detectable in germ cells until they migrate through the PMG during stage 10 (Lai et al., 1991; Fig. 2A). The protein in germ cells is maternally provided as it is present in embryos homozygous mutant for a *zfh-1* protein null allele (data not shown). We do not detect any subsequent zygotic transcription of *zfh-1* in germ cells.

In order to assess whether or not the maternal *zfh-1* product is required for proper germ cell migration, we generated *zfh-1* mutant germline clones using the FLP-FRTovo^{D} recombination system (Chou and Perrimon, 1992; Xu and Rubin, 1993). We find that embryos derived from *zfh-1* homozygous mutant germline clones develop normally as long as a wild-type copy of *zfh-1* is zygotically provided. Furthermore, the germ cell migration phenotype of *zfh-1* homozygous embryos is not exacerbated when the embryos are derived from a *zfh-1* homozygous mutant germline (data not shown). Thus, maternal Zfh-1 is not required for proper germ cell migration.

Proper germ cell migration requires caudal visceral mesoderm migration

Zfh-1 protein is expressed in a dynamic pattern in the mesoderm (Lai et al., 1991). At stage 9, it is expressed in all mesodermal cells. By stage 10, Zfh-1 levels have declined in most mesodermal cells, although high levels are maintained in extreme anterior and posterior mesodermal cells (Fig. 2A). The cells within the anterior cluster are likely to be hemocytes, as they show migratory behavior characteristic of these cells. Moreover, their number is reduced in *serpent (srp)* mutants (data not shown), in which the proliferation of the hemocyte precursors is affected (Rehorn et al., 1996). Zfh-1-expressing mesodermal cells located at the posterior end of the embryo migrate anteriorly in two bilaterally symmetric groups between the endoderm and the interior of the dorsal mesoderm during stage 10 (compare position of open arrowheads in Fig. 2B,C). These cells have been termed the ‘caudal visceral mesoderm’ as they contribute to the midgut musculature at later stages (R. Reuter, personal communication).

Zfh-1 expression within the caudal visceral mesoderm raises the possibility that *zfh-1* plays a role in the development of this tissue. We used *crocodile (croc)*, a gene encoding a forkhead domain protein (Häcker et al., 1995), as a marker for the caudal visceral mesoderm and find that it is not expressed in the caudal visceral mesoderm in *zfh-1* mutant embryos (data not shown). We also analyzed caudal visceral mesoderm migration in *zfh-1* mutants. Whereas in late stage 10 wild-type embryos, the caudal visceral mesoderm has migrated anteriorly to the end of the PMG (Fig. 2D, open arrowheads), in *zfh-1* mutants, it remains posterior to germ cells (Fig. 2E, open arrowheads). Zfh-1 function is therefore required for aspects of caudal visceral mesoderm-specific gene expression and for the migration of these cells.

To determine whether Zfh-1-expressing caudal visceral

mesoderm may come into contact with migrating germ cells, we analyzed embryos double-labeled with anti-Vasa, a germ cell marker, and anti-Zfh-1. We find that the caudal visceral mesoderm is in close proximity to migratory germ cells during late stage 10. Analysis of sections of these embryos indicates that the caudal visceral mesoderm reaches the end of the posterior midgut at the time when the germ cells migrate from the midgut into the mesoderm (Fig. 2D,G). Only once the caudal visceral mesoderm has reached this position are germ cells found in lateral mesoderm. Furthermore, we always observed germ cells in contact with caudal visceral mesodermal cells in late stage 10 wild-type embryos (14/14 embryos sectioned, Fig. 2G). These observations suggest that, in wild type, many germ cells migrate to lateral mesoderm via the caudal visceral mesoderm.

We cannot directly assess the role of the caudal visceral mesoderm in guiding germ cells in *zfh-1* mutant embryos because *zfh-1* is required for both the development of the caudal visceral mesoderm and the somatic gonadal precursors (see below). In order to further investigate the role of the caudal visceral mesoderm in germ cell migration, we analyzed germ cell migration in *brachyenteron (byn)* mutant embryos (Singer et al., 1996). Like *zfh-1*, *byn* is required for the migration of the caudal visceral mesoderm, but unlike *zfh-1*, it is not required for gonadal mesoderm development (R. Reuter, personal communication, our observations). In *byn* embryos, many germ cells remain attached to the PMG surface (arrowhead in Fig. 2F), while some navigate correctly to the SGPs. The germ cell migration defect in *byn* embryos suggests that the germ cell migration phenotype of *zfh-1* embryos may be at least in part attributable to a defect in caudal visceral mesoderm migration. Since *byn* and *zfh-1* both disrupt caudal visceral mesoderm migration and show similar defects in germ cell migration, we propose that in wild-type embryos, the caudal visceral mesoderm facilitates the transition of many germ cells from the endoderm to the lateral mesoderm (Fig. 8A).

SGPs express high levels of Zfh-1

Boyle et al. (1997) have demonstrated that gonadal mesoderm originates from three bilateral clusters of SGPs located within the lateral mesoderm of PS10-12. Since *zfh-1* is necessary for germ cell migration and is expressed in the coalesced gonad (Lai et al., 1991), we traced Zfh-1 protein expression in the developing gonadal mesoderm. We find that Zfh-1 is present in clusters of lateral mesoderm in PS2-14 beginning at stage 10 (circled cells in Fig. 3A). Cells within the PS10-12 clusters give rise to the SGPs, whereas the clusters present in other segments give rise to the fat body, as they will adopt the characteristic morphology of this tissue (data not shown). During stage 11, Zfh-1 levels increase, particularly within PS10-12 (Fig. 3B,C). The upregulation of Zfh-1 in PS10-12 correlates with the specification of these cells as SGPs, as they express gonadal mesoderm-specific markers and interact with germ cells at this stage (Boyle et al., 1997; see below). Zfh-1 continues to be expressed at high levels in all SGPs through the remainder of embryogenesis (Fig. 3E, arrowhead).

abdA is required for gonadal mesoderm specification (Lewis, 1978; Cumberledge et al., 1992; Brookman et al., 1992; Boyle and DiNardo, 1995; Greig and Akam, 1995). We analyzed Zfh-1 expression in *abdA* mutants and find that Zfh-1 is expressed normally in mesodermal clusters at stage 10; however, its levels are not enhanced in PS10-12 during stage 11 (Fig. 3D). The loss of high Zfh-1 expression correlates with the failure of SGP specification in *abdA* mutants.

Although *abdA* is required for SGP specification, the initial stages of germ cell migration are unaffected in *abdA* mutant embryos. In *abdA* mutants, germ cells migrate properly from the endoderm into the lateral mesoderm during stage 11; lost

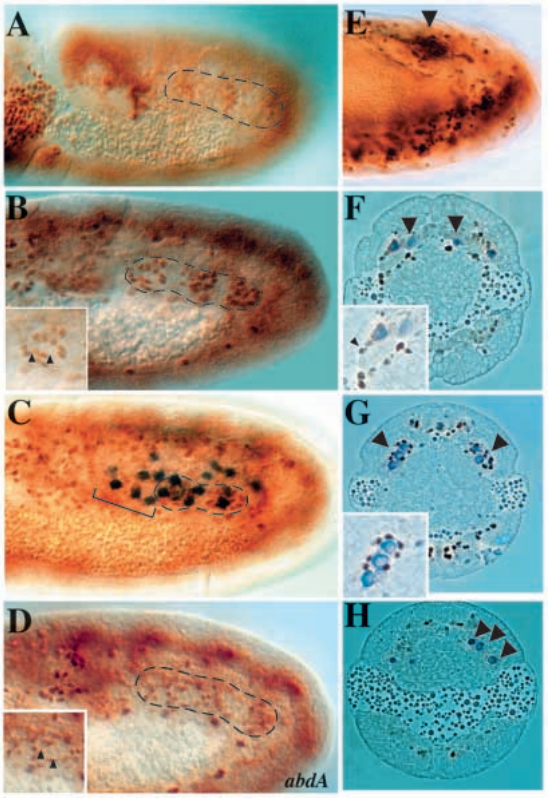

Fig. 3. Zfh-1 is expressed in somatic gonadal precursors from stage 10 through stage 14. (A-E) Whole-mount embryos are anterior left and dorsal up; (F-H) transverse sections are dorsal up. All panels except D. Wild type; (D) $abdA^{MX1}$. All embryos are stained with anti-Zfh-1 in brown. (C, F-H) Also labeled with the germ cell marker anti-Vasa in blue. (A) Stage 10 embryo with Zfh-1 expression in lateral mesoderm clusters (circled). (B) The expression of Zfh-1 in lateral mesoderm clusters increases at stage 11 when these cells are specified as SGPs (circled) and remains at high levels in these cells until they coalesce with germ cells at stage 14 (E). Unlabeled germ cells associate with the PS12 cluster at stage 11 (arrowheads in inset in B). (C) Embryo of the same stage as in B with both germ cells and the SGPs labeled. Some germ cells associate with Zfh-1-expressing SGPs (circled) while others remain posterior (bracket). (F-H) These spatial relationships are highlighted in transverse sections. (F) Germ cells migrate from the PMG toward SGPs during early stage 11. Two germ cells (arrowheads) migrating around the visceral mesoderm toward Zfh-1-expressing SGPs (arrowhead in inset). (G) Germ cells are surrounded by Zfh-1-expressing SGPs at stage 11 (magnified in inset). (H) Section of a stage 11 embryo posterior to the SGP clusters. Germ cells (arrowheads) are in the mesoderm, but they are not in the vicinity of SGPs. (D) *abdA* mutant at stage 11, illustrating Zfh-1 expression in lateral mesodermal clusters (circled). Zfh-1 levels are comparable to those of a stage 10 wild-type embryo (compare to A). Unlabeled germ cells associate with Zfh-1-expressing cells in *abdA* mutants (arrowheads in inset in D).

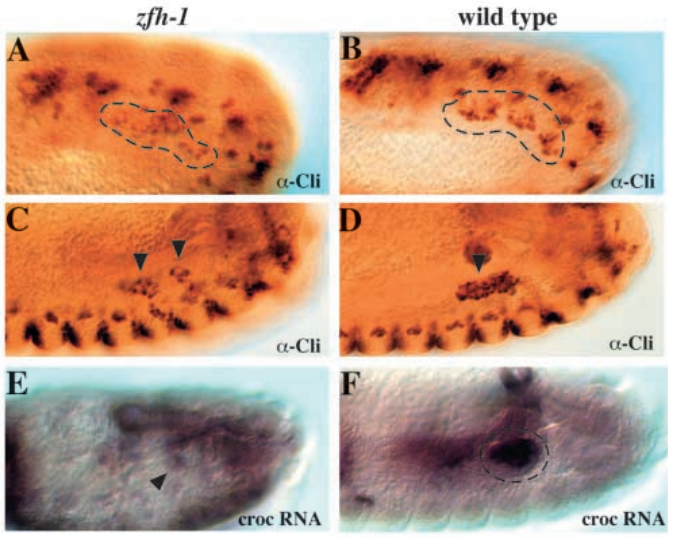

Fig. 4. *zfh-1* is necessary for SGP-specific gene expression. (A,C,E) *zfh-1*$^{65.34}$/*zfh-1*$^{75.26}$ mutant embryos; (B,D,F) wild-type embryos. (A-D) Stained with anti-Cli (brown) to label the SGPs. (The germ cells are faintly brown in the *zfh-1* mutant embryos in A,C as *zfh-1* mutant lines carry a lacZ-containing transgene expressed in their germ cells and anti-β-gal was used in order to distinguish the balancer-containing embryos. Germ cells are distinguishable from SGPs in A,C due to their large unstained nuclei.) The number of Cli-expressing SGPs is reduced at stage 11 in *zfh-1* mutants (A) compared to wild type (B). Residual SGPs remain attached to germ cells at stage 13 in *zfh-1* mutants (C, arrowheads). *croc* RNA is expressed strongly in SGPs at stage 14 in wild-type embryos (F), while *croc* RNA is absent in *zfh-1* mutants, even when small gonads are visible (E, arrowhead).

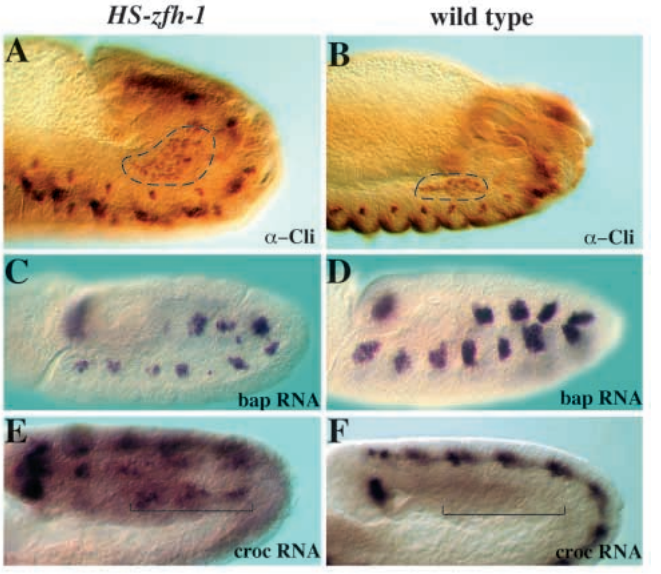

Fig. 5. Ectopic Zfh-1 alters the timing of SGP-specific gene expression and the size of the gonadal mesoderm primordium. (A-F) Anterior left and dorsal up. (A,C,E) *HSzfh-1* embryos; (B,D,F) wild-type embryos after an identical heat-shock regimen. (A,B) Stage 13 embryos stained with anti-Cli antibody. Ectopic Zfh-1 interferes with germband retraction, explaining the difference in appearance of the two embryos. (C,D) Stage 10 embryos labeled with *bagpipe* riboprobe. (E,F) Stage 11 embryos labeled with *crocodile* riboprobe. In *HSzfh-1* embryos (A, circled), the number of Cli-expressing cells is almost double that in wild type (B, circled). The A/P extent of the gonadal mesoderm is not expanded in the *HSzfh-1* embryo relative to wild type; rather, the gonadal mesoderm appears broader along the D/V axis. The number of *bagpipe*-expressing cells is reduced in *HSzfh-1* embryos (C) relative to wild type (D). *bagpipe* is expressed in visceral mesodermal cells, which originate immediately dorsal to SGPs. In *HSzfh-1* embryos (E), *croc* is expressed in the SGPs at stage 11 (bracket). In wild type, *croc* expression in the mesoderm is undetectable at stage 11 (F).

germ cells are not apparent until mid stage 12 (Moore et al., 1998a). Given that Zfh-1 is expressed at low levels in lateral mesodermal clusters in PS10-12 in an *abdA*-independent manner, we asked whether germ cells adhere to these cells in *abdA* mutant embryos. Indeed, germ cells specifically associate with mesodermal cells expressing low levels of Zfh-1 in *abdA* mutants (Fig. 3D), suggesting that germ cells can migrate toward lateral mesodermal cells that are not specified as gonadal mesoderm, but do express Zfh-1. Germ cells maintain this association until mid stage 12, when they disperse, presumably because SGP-specific gene products are required for the continued association of germ cells and gonadal mesoderm.

Germ cells follow different migratory paths to SGPs

To study the temporal and spatial relationship between migratory germ cells and SGPs in wild type, we prepared sections of embryos labeled with anti-Vasa to detect germ cells and anti-Zfh-1 to mark SGPs. In early stage 11 embryos, a few germ cells are still attached to the endoderm. These germ cells migrate into the mesodermal layer during stage 11 in a caudal visceral mesoderm-independent manner, as the caudal visceral mesoderm has migrated anteriorly past the end of the PMG by this stage (Fig. 8B; see Discussion). By mid stage 11, almost all germ cells are in lateral mesoderm. However, most of them have not yet reached the SGPs and are frequently situated next to Bagpipe-expressing visceral mesodermal cells that have migrated between the SGPs and the PMG (Azpiazu and Frasch, 1993; Fig. 3F, arrowheads; Fig. 8B). Shortly thereafter, germ cells associate with Zfh-1-expressing SGP clusters in PS11 and 12 (Fig. 3G). Germ cells are not observed with the PS10 cluster at stage 11, presumably because the PMG does not underlie the mesoderm in this parasegment. About one-third of germ cells migrate from the PMG into PS13 and are therefore posterior to all SGP clusters at stage 11 (Fig. 3C, brackets; Fig. 3H; Fig. 8C,D). Because all germ cells are associated with SGPs by the beginning of stage 13, the more posteriorly situated germ cells are likely to migrate anteriorly toward SGPs during stages 11 and 12. Thus, while some germ cells reach the SGPs by stage 11, other germ cells take a more indirect route and do not contact SGPs until stage 13. We conclude that germ cells do not follow a single invariant path to reach SGPs (see Discussion).

Zfh-1 is necessary for gonadal mesoderm development

Both the expression pattern of Zfh-1 in wild-type embryos and the germ cell migration phenotype in *zfh-1* mutants suggest that *zfh-1* is required for gonadal mesoderm development. SGP-specific gene expression in *zfh-1* mutants was analyzed in order

to test this hypothesis. Cli is expressed in SGPs soon after they are specified and *cli* activity is required for SGP development (Boyle et al., 1997). Using an anti-Cli antibody, we find that the number of Cli-expressing cells is greatly reduced in *zfh-1* mutants as compared to wild type. This reduction is apparent at stage 11, when SGPs first express Cli in wild type (Fig. 4A). Interestingly, the remaining SGPs encapsulate germ cells, suggesting that they retain aspects of SGP identity (Fig. 4C). Therefore, most, but not all, SGPs require *zfh-1* for expression of Cli. Consistent with this conclusion, we find that high Zfh-1 levels are expressed in SGPs at stage 11 in *cli* mutants. However, the number of cells expressing Zfh-1 decreases dramatically during stage 12 in *cli* mutants (data not shown), consistent with the proposed role of *cli* in the maintenance of SGP cell fate (Boyle et al., 1997).

An analysis of the expression of other SGP-specific markers confirms that *zfh-1* is required for gonadal mesoderm development. Both the 412 retrotransposon and *crocodile* RNA are expressed in the gonadal mesoderm at stage 14 (Brookman et al., 1992; U. Häcker and H. Jäckle, personal communication). At this stage, we find only a few 412-expressing SGPs in *zfh-1* mutants (Moore et al., 1998a). *croc* shows a more strict dependence on *zfh-1* function as it is undetectable in *zfh-1* mutants, even when small gonads are visible (Fig. 4E, arrowhead).

The observation that SGPs are not completely absent in *zfh-1* mutants demonstrates that a small number of SGPs are specified in a *zfh-1*-independent manner. The specification of these SGPs does not require *cli* function as they are still present in *cli; zfh-1* double mutants (data not shown). We present evidence below demonstrating that the *tin* gene is required for the specification of these SGPs.

Ectopic Zfh-1 is sufficient to induce additional SGPs

To obtain stronger evidence that *zfh-1* is an important regulator of gonadal mesoderm cell fate, we analyzed SGP development in embryos bearing a P{*hsp70-zfh-1*} transgene (Lai et al., 1991). Ectopic Zfh-1 rescues the germ cell migration phenotype of *zfh-1* mutants and *HSzfh-1* does not produce a germ cell migration phenotype in an otherwise wild-type background (data not shown). Additional gonadal mesodermal cells are present in *HSzfh-1* embryos when a 1-hour heat shock is administered at 5 hours AEL (stage 9; see Materials and Methods). Using the anti-Cli antibody, we detect an average of 48 SGPs in *HSzfh-1* mutant embryos at stage 13 compared to 28 SGPs in wild-type embryos (Fig. 5A,B). A similarly increased number of SGPs appears to be present in *HSzfh-1* embryos at stage 11, although the clusters are more disorganized relative to wild type, making quantitation difficult.

In order to determine if additional SGPs arise in place of other mesodermal derivatives, we examined *bagpipe (bap)* expression in *HSzfh-1* embryos. *bap* is expressed in the visceral mesoderm that forms immediately dorsal to SGPs (Azpiazu and Frasch, 1993; Boyle et al., 1997). We find a reduction in the number of *bap*-expressing cells in embryos with ectopic Zfh-1 (Fig. 5C,D). In 61/73 (84%) *HSzfh-1* embryos at stage 10, the majority of *bap*-expressing clusters were reduced in size relative to wild-type controls. The loss of *bap* expression in *HSzfh-1* embryos suggests that visceral mesodermal cells located in PS10-12 may be recruited to adopt a gonadal mesodermal cell fate by ectopic Zfh-1. However, the reduction in the size of the visceral mesoderm is not restricted to PS10-12, indicating that the presence of ectopic gonadal mesoderm is not a prerequisite for the loss of visceral mesoderm.

We verified that additional SGPs are present at stage 13 in *HSzfh-1* embryos using another marker, *croc* RNA (data not shown). Surprisingly, we also find that *croc* is expressed prematurely in SGPs when *HSzfh-1* embryos are subjected to an identical 1-hour heat shock at stage 9. In wild type, *croc* expression is not initiated in SGPs until stage 13. We find that in *HSzfh-1* embryos, *croc* transcription is detectable at stage 11 (compare Fig. 5E and F). It is activated in all three SGP clusters, although its expression is most pronounced in the PS12 cluster, presumably reflecting the fact that, in wild type, *croc* expression is restricted to posterior SGPs at stage 15. We were unable to detect *croc* transcript in SGPs prior to stage 11 even when ectopic Zfh-1 was induced at earlier stages (data not shown), suggesting that *croc* expression requires additional, *zfh-1*-independent signals that are not present until stage 11. Taken together, these results suggest that *zfh-1* is a primary regulator of gonadal mesoderm, as it is both able to promote ectopic gonadal mesoderm formation and to alter the temporal course of gene expression in these cells.

Only the pan-mesodermal expression of *tinman* is required for SGP formation

tinman (tin) encodes a homeobox protein that is expressed in all mesodermal cells until stage 9 (Azpiazu and Frasch, 1993; Bodmer, 1993), but subsequently maintained only in dorsal mesoderm. The dorsally restricted expression of *tin* requires

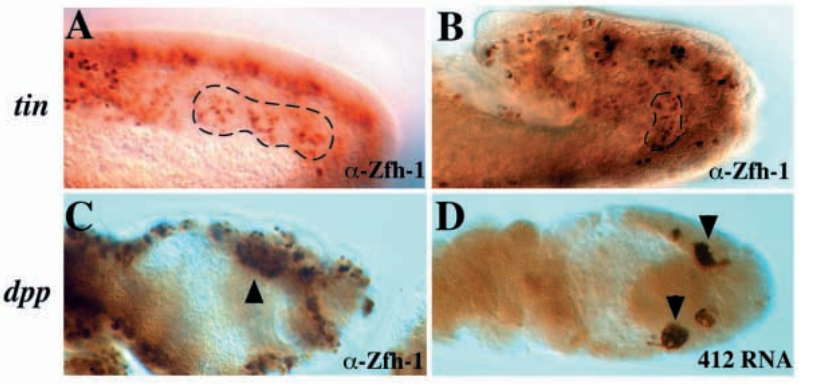

Fig. 6. Tin, but not Dpp, is required for SGP development. (A-D) Anterior left; (A,B) dorsal up, (C,D) 'frontal' views. (A,B) $tin^{Df(3R)GC14}$ mutant embryos labeled with anti-Zfh-1. (C,D) dpp^{H46} embryos stained with anti-Zfh-1 (C) or 412 riboprobe (D). The number of Zfh-1-expressing SGPs is reduced by 41% at stage 11 in *tin* mutants (A, circled) compared to wild type (see Fig. 3B). By stage 12, the number of Zfh-1-expressing cells is reduced by 76% in *tin* mutants (B, circled) compared to wild type. In this analysis, mesodermal cells were counted as SGPs if they express high levels of Zfh-1 and reside within PS10-12. SGP specification or coalescence does not require the presence of Dpp, and therefore dorsally restricted *tin* expression, as judged by either Zfh-1 protein expression (C) or 412 RNA expression (D).

Dpp, which is present in the overlying dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Although *tin* is not expressed in SGPs, it is required for the expression of SGP-specific markers, such as Cli protein and *Dwnt2* RNA (Boyle et al., 1997). However, the analysis of germ cell migration in *tin* mutants suggests that aspects of SGP specification are independent of *tin*. In *tin* mutants, germ cells align with mesodermal cells at stage 13, although they disperse shortly thereafter (Moore et al., 1998a). Because the onset of this phenotype is later than the onset of the germ cell migration phenotype in *abdA*, in which SGPs are not specified, SGPs may initially be present in *tin* embryos.

We analyzed *zfh-1* expression in *tin* mutants and find that *tin* is not required for early *zfh-1* expression throughout the mesoderm or for the refinement of this expression to lateral mesodermal clusters during stage 10. *tin* activity is, however, required for aspects of *zfh-1* expression beginning at stage 11. At this stage in wild-type embryos, *zfh-1* expression is enhanced in SGPs located within PS10-12. Using high Zfh-1 levels in PS10-12 as an assay for SGP specification, we find that fewer SGPs are specified in *tin* mutants, and those that are specified fail to maintain their differentiated state. In stage 11 wild-type embryos, on average 32.6 Zfh-1-expressing SGPs are present on each side of the embryo, compared to 19.2 in *tin* mutants (compare Fig. 6A to 3B). By stage 12, we find only 7.8 SGPs remaining in *tin* mutant embryos (Fig. 6B), while 34 are present in wild type. By stage 13, the first stage at which a germ cell migration phenotype is evident in *tin* mutant embryos, only 1 or 2 somatic gonadal precursors remain (data not shown). These data demonstrate that *tin* is required for the specification of some SGPs, as fewer Zfh-1-expressing cells are present at stage 11. *tin* also appears to be required for SGP differentiation, as the number of SGPs continues to decrease from stage 11 to stage 14.

To determine whether it is the early, pan-mesodermal expression or the late, dorsally restricted expression of *tin* that is important for SGP development, we analyzed gonadal mesoderm formation in a *dpp* mutant background. In *dpp* mutants, the initial, pan-mesodermal expression of *tin* is unaffected while the late expression is abolished (Staehling-Hampton et al., 1994; Frasch, 1995). We find that gonadal mesoderm development is largely unaffected in *dpp* mutants. Using multiple markers for SGP development, such as anti-Zfh-1 (Fig. 6C) and the 412 retrotransposon (Fig. 6D), we observe that the gonadal mesoderm coalesces in *dpp* mutant embryos. This demonstrates that SGP development depends on the early, unrestricted expression of *tin* throughout the mesoderm. While gonadal mesoderm develops normally, germ cells do not migrate from endoderm to the mesoderm, presumably due to the gastrulation defects in *dpp* mutant embryos (data not shown).

Tinman and Zfh-1 cooperate in gonadal mesoderm development

Both *tin* and *zfh-1* are important regulators of gonadal mesoderm cell fate. It is unlikely, however, that they fit neatly into a linear hierarchy controlling gonadal mesoderm determination. We have demonstrated that the early broad expression of *tin* is required for SGP development. However, *zfh-1* is not required for this expression (data not shown), suggesting that *zfh-1* is not upstream of *tin*. Furthermore, since

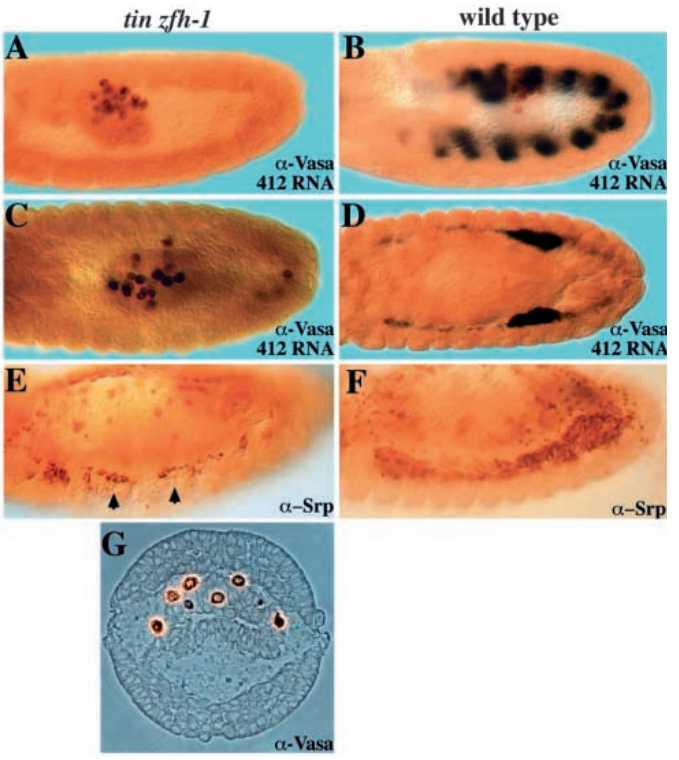

Fig. 7. *tin* and *zfh-1* cooperate in SGP specification. (A-F) anterior left, (A,B,E,F,G) dorsal up, (C,D) dorsal views. (A,C,E,G) *tin zfh-1* double mutant embryos. (B,D,F) Wild-type embryos. (A-D) Labeled with anti-Vasa to mark germ cells (brown) and with 412 RNA to mark gonadal mesoderm (blue). (E,F) The fat body precursors are labeled with anti-Srp. The transverse section in G is labeled with anti-Vasa. (A) While germ cells congregate on the dorsal side of the PMG in stage 11 *tin zfh-1* mutants, they do not migrate into the mesoderm (compare to wild-type embryo in Fig. 1B). Section in G demonstrates that mesodermal cells contact germ cells on the PMG in *tin zfh-1* double mutants. (C) Germ cells remain in the vicinity of the endoderm at stage 13. No gonadal mesoderm differentiates in *tin zfh-1* mutants as demonstrated by the absence of 412 RNA expression in A and C, compare to 412 expression in wild-type embryos in B and D. The number of fat body cells is drastically reduced in *tin zfh-1* mutants (E, arrowheads), compare to F.

germ cell association with SGPs is blocked in *zfh-1* mutants but not in *tin* mutants, it seems unlikely that *tin* acts upstream of *zfh-1* in SGP development. These observations suggest that *tin* and *zfh-1* function in parallel in gonadal mesoderm development.

To test this idea, a *tin zfh-1* double mutant was constructed. Whereas in both single mutants most germ cells migrate into the mesoderm, in *tin zfh-1* mutant embryos germ cells are unable to transfer between germ layers (Fig. 7A). Most germ cells adhere to the endoderm throughout embryogenesis, whereas some scatter near the gut at late stages (Fig. 7C). This phenotype is identical to that of *twi sna* mutants (Jaglarz and Howard, 1994; Warrior, 1994), in which no mesoderm forms, suggesting that *tin* and *zfh-1* regulate all mesodermal genes required for germ cell migration from the endoderm into the mesoderm. Stage 11 *tin zfh-1* mutant embryos were sectioned to exclude the possibility that germ cells cannot migrate into the mesoderm because the mesodermal layer is absent or

thinner, and therefore does not contact germ cells on the gut. The mesodermal layer in *tin zfh-1* mutants appears to contain approximately the correct number of cells and does contact germ cells positioned on the endoderm (Fig. 7G).

Gene expression in the mesoderm was analyzed in double mutant embryos in order to elucidate the cause of the germ cell migration phenotype. We assayed for the presence of gonadal mesoderm with multiple markers, such as the anti-Cli antibody (data not shown) and the 412 retrotransposon (Compare Fig. 7A,B). While 412-expressing cells are detectable in either *tin* or *zfh-1* single mutants, they are abolished in double mutant embryos. This demonstrates that *tin* and *zfh-1* have parallel functions in gonadal mesoderm formation. We next tested whether *tin* and *zfh-1* act together in fat body development, as the fat body is another lateral mesoderm derivative. While both *tin* and *zfh-1* are involved in the development of the fat body, neither is absolutely required (Moore et al., 1998b). Anti-Serpent (Srp) was utilized to label fat body precursors (Rehorn et al., 1996). Only a few residual Srp-expressing cells remain in *tin zfh-1* embryos (Fig. 7E), demonstrating that *tin* and *zfh-1* cooperate in the specification of two tissues derived from lateral mesoderm: the gonadal mesoderm and the fat body.

DISCUSSION

We show here that *zfh-1* is a primary regulator of gonadal mesoderm cell fate. This conclusion is supported by analysis of both loss-of-function and gain-of-function situations. In the absence of *zfh-1*, few SGPs are present, while ectopic Zfh-1 induces the formation of additional SGPs and alters the timing of gene expression within these cells. However, *zfh-1* does not act alone in the specification of SGPs. Analysis of a *tin zfh-1* double mutant indicates that these two genes cooperate in SGP specification. Lastly, we have found a novel relationship between the caudal visceral mesoderm and migratory germ cells.

The caudal visceral mesoderm contacts migratory germ cells

Caudal visceral mesodermal cells migrate in two bilaterally symmetric groups between the interior surface of the mesoderm and the posterior midgut. We show here that Zfh-1 is strongly expressed in these cells and is required for their differentiation and migration. Germ cells associate with caudal visceral mesodermal cells in wild type, suggesting that they may help guide germ cells toward the somatic gonadal precursors. In support of this hypothesis, many germ cells do not migrate successfully to the SGPs in *byn* embryos, in which caudal visceral mesoderm migration is blocked, but gonadal mesoderm development is unaffected.

Why do germ cells associate with caudal visceral mesodermal cells? We can imagine several means by which the caudal visceral mesoderm may act to increase the fidelity of germ cell migration. It is possible that these cells are important in repositioning the posterior midgut (PMG) close to the mesodermal layer during stage 10. We have previously observed that, in the wild type, germ cells transfer from the endoderm to the mesoderm only after the endoderm has flattened and comes to lie directly beneath the mesoderm (S. N. and R. L., unpublished data). Since caudal visceral mesoderm cells adhere to both the PMG and the mesoderm as they migrate, they may 'pull' the endoderm closer to the mesoderm as they move anteriorly. In this way, they may enable the germ cells to migrate to the mesodermal layer in a timely fashion, perhaps allowing them to respond to a temporally restricted signal from the mesoderm. It is also possible that the caudal visceral mesoderm initiates the bilateral symmetry of germ cells. In its presence, germ cells transfer directly from the PMG to lateral mesoderm close to the SGPs. This may be important if the signal attracting the germ cells to the SGPs is spatially restricted.

Specification of somatic gonadal precursors occurs stepwise

Analysis of germ cell migration and Zfh-1 expression in wild-type and mutant embryos indicates that cells acquire somatic gonadal precursor character progressively. The origin of gonadal mesodermal cells has been traced to stage 11 when SGPs are present as clusters of cells in PS10-12 (Boyle et al., 1997); however, earlier stages of SGP development can be followed with Zfh-1 antibody. At stage 10, shortly after the mesoderm has spread beneath the ectoderm, Zfh-1 is expressed in clusters of lateral mesodermal cells in PS2-14. We do not yet know what determines the position of these clusters along the dorsoventral axis, although their formation is independent of *dpp* activity, indicating that they form ventral to the precursors of the visceral mesoderm. As a result of *abdA* activity, levels of Zfh-1 protein are greatly enhanced in three clusters of SGPs in PS10-12 during stage 11.

Analysis of embryos with ectopically expressed Zfh-1 also suggests that SGPs are not specified until stage 11. In wild-type embryos, *croc* expression is not initiated until stage 13. However, in *HSzfh-1* embryos, *croc* expression was initiated at stage 11, but never earlier. Furthermore, ectopic Zfh-1 does not result in premature expression of Cli, which is normally expressed at stage 11. These results suggest that lateral mesoderm cells require additional, *zfh-1*-independent factors that are not present until stage 11 before they are competent to express SGP markers. While we do not know what these factors are, they may be additional targets of *abdA*, as *abdA* acts at stage11 to specify lateral mesodermal cells in PS10-12 as SGPs.

We propose that Zfh-1 expression in the lateral mesoderm, but not necessarily high Zfh-1 expression in the SGPs, is responsible for guiding germ cells from the endoderm into mesodermal tissue. Although we do not yet know how the lateral mesoderm attracts migratory germ cells, our data suggest that this 'attractant' does not depend on *abdA*, and therefore may not be produced exclusively within PS10-12. First, in wild-type embryos, germ cells begin migrating into the mesoderm during late stage 10, before *abdA* activity has resulted in high Zfh-1 levels. Additionally, in *abdA* mutant embryos, the initial association of germ cells and lateral mesodermal cells is not disrupted. Germ cells navigate toward and adhere to mesodermal cells expressing low Zfh-1 levels; however, this association is not maintained. The importance of lateral mesoderm in regulating germ cell migration is further demonstrated by the phenotype of *tin zfh-1* mutant embryos. In this background, lateral mesodermal derivatives are abolished and germ cells do not detach from the endoderm.

Zfh-1 is a primary regulator of gonadal mesoderm cell fate

zfh-1 is necessary for development of the gonadal mesoderm, as the number of cells expressing SGP markers is greatly reduced in *zfh-1* mutant embryos. It is possible that the correct number of SGPs are initially specified in *zfh-1* mutants, but they do not continue to differentiate as gonadal mesoderm. However, we favor the idea that *zfh-1* is required for the specification of mesodermal cells as SGPs. First, *zfh-1* is necessary for Cli expression in SGPs. Other than *zfh-1*, *cli* is the only gene known to be expressed in SGPs at stage 11, so it serves as the best marker for the specification of these cells at this early stage. Second, while a few SGPs are present in *tin* and *zfh-1* single mutants, none are specified in *tin zfh-1* double mutants embryos. While this demonstrates that *zfh-1* does not act alone in SGP specification, it shows that *zfh-1* functions in parallel with *tin* to define this mesodermal cell type. Third, ectopic expression of Zfh-1 induces additional gonadal mesodermal cells. Because the number of visceral mesodermal cells is also reduced in these embryos, it is possible that cells are diverted from a visceral mesodermal fate to a gonadal mesodermal fate. Taken together, these data argue that *zfh-1* is required for SGP specification.

There are almost twice as many SGPs in *HSzfh-1* embryos as in wild type, suggesting that the primordium of the gonadal mesoderm is enlarged. Additional SGPs have also been shown to be specified in the presence of *HSabdA* or *HSwg* (Boyle and DiNardo, 1995; Boyle et al., 1997; Greig and Akam, 1995). All three of these transgenes affect SGP specification differently, however. Ectopic AbdA increases the number of segments competent to become gonadal mesoderm, whereas, in *HSwg* embryos, the SGP clusters normally present at stage 11 become a continuous band of cells. These phenotypes suggest that, in the wild type, *abdA* activity restricts SGP formation to PS10-12 and *wg* activity is involved in the anterior-posterior positioning of SGPs within these parasegments. In *HSzfh-1* embryos, SGPs are still largely specified as clusters of cells within PS10-12; however, the number of cells within each cluster is increased. The SGP clusters appear broader along the dorsoventral axis, consistent with the finding that fewer visceral mesoderm cells are present in *HSzfh-1* embryos. A similar antagonistic relationship between SGPs and precursors to the visceral mesoderm was observed by Boyle et al. (1997). They found that, in *bap* null embryos, the number of SGPs is increased, apparently in the dorsal direction. Thus, cells appear to be able to be recruited from a visceral mesoderm fate to a gonadal mesoderm fate both by blocking visceral mesoderm development and by promoting gonadal mesoderm development. Additionally, these results imply that, although Dpp is required for the specification of dorsal mesoderm derivatives, cells are not irreversibly committed to a dorsal mesoderm fate by receiving the Dpp signal, as they still retain the ability to develop as more lateral derivatives.

Two homeodomain proteins, Zfh-1 and Tin, cooperate in gonadal mesoderm specification

Analysis of loss-of-function mutations reveals that *zfh-1* activity is necessary for SGP development. However, in *zfh-1* mutant embryos, a few SGPs form and associate with germ cells. It is unlikely that this is the result of residual *zfh-1* gene function. First, Zfh-1 protein is undetectable in the embryos that we used in our analyses. Second, the phenotypes of the alleles used are the same when homozygous as when heterozygous with a deficiency for the region. Lastly, maternal Zfh-1 is not required for germ cell migration indicating that it is not substituting for the absence of zygotic product in mutant embryos.

Instead, we favor the hypothesis that *zfh-1* cooperates with *tin* in SGP specification. In support of this hypothesis, we have shown that, whereas aspects of SGP identity are present in both single mutants, SGP specification is abolished in *tin zfh-1* double mutant embryos. As a result, germ cells do not migrate from the endoderm to the mesoderm in double mutant embryos. This germ cell migration phenotype is much more severe than when only one of the genes is absent. This suggests that wild-type function of either *tin* or *zfh-1* is able to regulate the expression of enough downstream genes to facilitate the migration of germ cells toward the mesoderm. However, when both gene products are absent, downstream targets are not expressed and thus germ cells are not guided toward the mesoderm.

It is possible that *tin* and *zfh-1* regulate non-overlapping groups of target genes which function in parallel in gonadal mesoderm development. Alternatively, it is possible that *tin* and *zfh-1* have the capacity to regulate identical targets. Characterization of *tin* and *zfh-1* targets should enable us to distinguish between these two models. Since Zfh-1 contains both zinc fingers and a homeodomain, an analysis of the DNA-binding domains in Zfh-1 may also help elucidate the manner by which *tin* and *zfh-1* cooperate in the formation of the gonadal mesoderm.

tin function in gonadal mesoderm development depends on its initial expression throughout the mesoderm and not its subsequent expression within the dorsal mesoderm. We do not yet know when *zfh-1* function is required for gonadal mesoderm development. It is possible that, like *tin*, *zfh-1* is required at an early developmental stage, although its persistent expression in clusters of lateral mesoderm suggests a continued function in gonadal mesoderm development. Analysis of the regulatory regions of the *zfh-1* locus may help us to dissect the temporal and spatial requirements for *zfh-1* function in SGP specification.

Germ cells navigate along different paths to reach the gonadal mesoderm

Cell migration within developing tissues such as the tracheal system or the nervous system occurs along highly stereotyped paths (Van Vactor et al., 1993; Samakovlis et al., 1996). Our analysis of germ cell migration indicates that there is a surprising degree of plasticity in the routes germ cells take toward the gonadal mesoderm. The first germ cells to migrate from the PMG to the mesoderm do so along the caudal visceral mesoderm (Fig. 8A). These germ cells take the most direct route from the PMG to the SGPs, as caudal visceral mesodermal cells touch SGPs at late stage 10. At mid stage 11, germ cells transfer to the mesoderm without directly contacting caudal visceral mesoderm, as these mesodermal cells have migrated past the germ cells. Germ cells leaving the PMG at this time migrate around other mesodermal cells toward SGPs (Fig. 8B). In particular, they navigate around *bagpipe*-expressing visceral mesoderm, which has moved

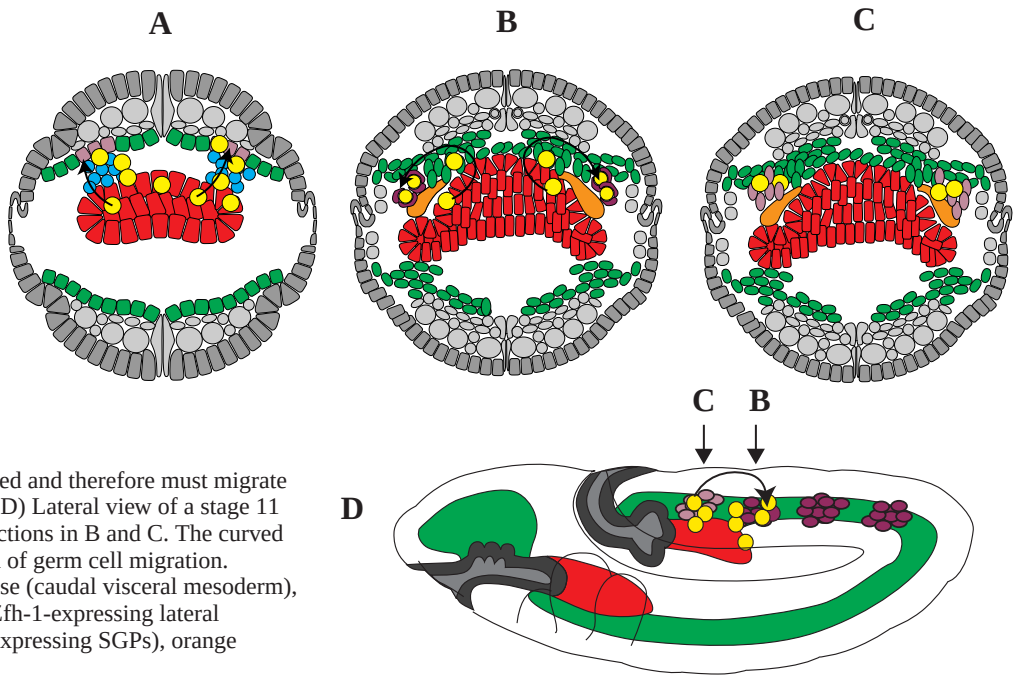

Fig. 8. Germ cells follow different paths to reach SGPs. (A-C) Transverse sections; (D) lateral view. (A) Germ cells that migrate from the PMG to SGPs during late stage 10 follow a direct path along the caudal visceral mesoderm. (B) During stage 11, germ cells migrate more extensively through mesoderm to reach SGPs. (C) Some germ cells migrate into PS13, where SGPs are not specified and therefore must migrate anteriorly through lateral mesoderm. (D) Lateral view of a stage 11 embryo, indicating the plane of the sections in B and C. The curved arrows in A,B,D indicate the direction of germ cell migration. Yellow (germ cells), red (gut), turquoise (caudal visceral mesoderm), green (mesoderm), light purple (low Zfh-1-expressing lateral mesoderm), dark purple (high Zfh-1-expressing SGPs), orange (visceral mesoderm).

inside the SGPs (Azpiazu and Frasch, 1993; Boyle et al., 1997).

Finally, some germ cells migrate anteriorly through lateral mesoderm before contacting SGPs (Fig. 8C,D). These 'lagging' germ cells migrate from the PMG into mesoderm posterior to PS12, where SGPs are not specified. Subsequently, the germ cells navigate anteriorly within the mesoderm until they reach SGPs. Support for the idea that germ cells can retain migratory capabilities late into embryogenesis comes from the analysis of germ cell migration in live embryos bearing GFP-containing germ cells. In these preparations, lagging germ cells eventually join the majority of germ cells already associated with SGPs (M. Starz-Gaiano and R. L., unpublished data).

No matter which path they choose, we do not know how germ cells are attracted to SGPs. Our observations imply that the signal (whether it is a diffusible molecule or a gradient of adhesion molecules) is capable of acting over several cell diameters. In vitro studies suggest that this is the case in mouse, as genital ridge explants attract germ cells from long range (Godin et al., 1990). It will be interesting to determine whether germ cell-germ cell interactions are important in guiding the late arrivers to the developing gonads. Such interactions have been observed in mouse embryos where germ cells send out long processes to form extensive networks with one another (Gomperts et al., 1994). Subsequent studies of germ cell migration in *Drosophila* embryos will further elucidate how germ cell migration to the gonadal mesoderm is mediated.

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