GENETIC DISSECTION OF GERM CELL MIGRATION AND THE DISTINCTION BETWEEN GONADAL MESODERM AND FAT BODY DEVELOPMENT IN DROSOPHILA

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Abstract

Cell migration plays a crucial role in the development of a multicellular organism. The migration of primordial germ cells (PGCs) in Drosophila requires numerous migratory steps and cell-cell interactions in order to form the embryonic gonad, and therefore provides a model system for the study of general cell migration processes. Screens for mutations affecting the migration of PGCs, or germ cells, in Drosophila reveals that this process can be dissected into discrete genetic steps. Each step requires a specific subset of genes including *serpent* (*srp*), *huckebein, columbus, zinc finger homeodomain protein-1* (*zfh-1*), *heartless, tinman (tin), fear-of-intimacy,* the homeotic genes *abdominal A* (*abdA*) and *Abdominal B* (*AbdB*), trithorax group members *trithorax* (*trx*) and *trithoraxgleich,* as well as the loci corresponding to the 9.35 and 22.38 mutations. Closer examination of the role of *trx* in germ cell migration demonstrates that it is required for the function of *AbdB*, but not *abdA* in this process. Many of the genes identified through these screens are necessary for the proper specification and/or differentiation of SGPs into gonadal mesoderm. Analysis of genes required for both embryonic pattern formation and germ cell migration reveals that the origin of gonadal mesoderm lies within the *eve* domain of the mesoderm.

Further investigation into the development of gonadal mesoderm indicates that this tissue is closely related to another *eve* domain mesodermal derivative, the fat body. Both cell types require *tin*, *zfh-1* and *clift* (*cli*) for steps leading to their specification. Although gonadal mesoderm and fat body develop in similar positions along the dorsoventral axis, gonadal mesoderm is only specified within parasegments (PS) 10-12, whereas fat body develops in PS 4-13. The execution of the cell fate decision between fat body and gonadal mesoderm cell identity along the anteroposterior axis is controlled by the *srp* and *abdA* genes. *abdA* acts to repress *srp* expression within PS10-12 and allow development of gonadal mesoderm in place of fat body within these parasegments. Therefore, screens to identify genes required for germ cell migration have laid the groundwork for deciphering many of the developmental steps leading to the proper specification and differentiation of gonadal mesoderm.

Thesis supervisor: Ruth Lehmann
Title: Professor of Cell Biology

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CHAPTER 1

Introduction

Primordial germ cell migration in vertebrates

The role of cell movements in the embryonic development of an organism has been the focus of countless studies. Cell movements are involved in a number of developmental processes ranging from morphogenic events such as gastrulation, to cell migration systems including neural crest cells and primordial germ cells (PGCs). PGCs are those cells that give rise to either sperm or eggs, and in many organisms arise in a position distinct from where they eventually populate the gonad (Dixon, 1994). Moreover, PGCs must migrate through and along different tissues in order to reach their final destination. The process of PGC migration has been well described in two vertebrate species, Xenopus and the mouse. In both organisms, PGCs form near the invaginating endoderm primordium, and are subsequently incorporated into the developing gut. PGCs then migrate through the gut primordium, along its developing mesentery, and toward the genital ridge that will comprise the somatic component of the gonad (reviewed in Chiquoine, 1954; Heasman and Wylie, 1981). Thus, PGCs must adopt numerous migratory characteristics during their travels to the gonad: they posses invasive behavior as they move through the gut wall, they must adhere to and migrate along the gut mesentery, and finally, they must recognize the target genital ridge tissue and halt their migratory cell behavior in order to differentiate along with somatic cells into the gonad. Given their highly stereotyped migratory path, PGCs presumably receive and respond to cues from surrounding tissues in order to be properly guided to the genital ridge. PGC migration therefore provides an excellent model system for the study of general processes of cell migration within a developmental framework.

The migration of PGCs can also provide a paradigm for addressing cell biological questions regarding mechanisms of cell motility and adhesiveness. In vitro studies of Xenopus PGCs reveal that their movement is associated with certain structural characteristics such as filipodia and retraction of the trailing end (Heasman and Wylie, 1978). Ultrastructural analysis has shown that the migratory morphology of PGCs is correlated with particular cytoskeletal arrangements such that PGCs orient their movement and cytoskeletal architecture in the same direction as the somatic cells over which they migrate (Heasman and Wylie, 1981). One extracellular matrix (ECM) component, fibronectin (FN), has been implicated in facilitating the adhesiveness of Xenopus PGCs to somatic cells in culture (Heasman et al., 1981). Moreover, it has been shown that mouse PGCs alter their adhesiveness to FN depending on their developmental stage. PGCs in the hindgut have a stronger affinity to FN than those migrating along the gut mesentery, while PGCs found in the genital ridge are even less adhesive to FN than migratory PGCs (ffrench-Constant et al., 1991). Mouse PGC adhesiveness to another ECM molecule, laminin, also decreases once they emigrate from the gut, but then remains the same until gonadal differentiation (Garcia-Castro et al., 1997). Thus, changes in the affinities of PGCs to ECM components may facililate the migration of these cells over different tissues to reach the genital ridge. Both FN and laminin are found along the tissues over which the PGCs migrate, consistent with the model that these factors may indeed play a role in PGC migration in vivo (Garcia-Castro et al., 1997).

In addition to interactions between PGCs and the somatic cells along which they migrate, PGC migration in the mouse also involves specific interactions between the PGCs themselves. At early developmental stages when PGCs are located within the gut, few PGCs are found to associate with one another. However, once they leave the gut, PGCs are found linked to one another via filipodia-like structures. These networks are lost by the time PGCs reach the genital ridge, where they re-adopt the rounded morphology seen at early stages. Interestingly, the first PGCs to migrate from the gut often move directly to the genital ridge, since the gut mesentery has not developed by this stage. It is thought that these "pioneer" PGCs may guide subsequent PGCs along the developing gut mesentery via their intercellular connections (Gomperts et al., 1994).

In addition to cell biological studies, numerous experiments with mouse PGCs have been aimed at the identification of additional factors that actively guide germ cells along their migratory route. A number of secreted molecules, including stem cell factor (SCF, or *Steel* factor, SF), leukemia inhibitory factor (LIF), interleukin-4 (IL-4), basic fibroblast growth factor (bFGF), retinoic acid (RA), and tumor necrosis factor-alpha (TNF-α) all increase the number of PGCs found in a variety of cell-culture conditions when taken from

embryos at various developmental stages (Cooke et al., 1996; Dolci et al., 1991; Felici and Dolci, 1991; Godin et al., 1991; Kawase et al., 1994; Koshimizu et al., 1995; Matsui et al., 1991; Resnick et al., 1992). SCF and IL-4 appear to function in vitro by promoting PGC survival rather than proliferation, as evidenced by the lack of an increase in bromodeoxyuridine (BrdU) labeling in experimental versus control samples (Cooke et al., 1996; Godin et al., 1991). In fact, addition of SCF or LIF to mouse PGCs in culture suppresses apoptosis that normally occurs in the absence of these factors (Pesce et al., 1993). However, PGC proliferation can be induced in culture by the addition of factors resulting in high intracellular cAMP levels (Felici et al., 1993; Pesce et al., 1996). Another secreted molecule, transforming growth factor-β1 (TGF-β1) has been shown to inhibit PGC proliferation even though it also imparts a chemotropic effect on PGCs in culture (Godin and Wylie, 1991).

Consistent with their effects on PGCs in culture, many of these factors are expressed within the tissues along which the PGCs migrate, or in the PGCs themselves. SCF RNA is expressed around the hindgut and its associated mesentery, as well as in the genital ridge (Matsui et al., 1990), while its cognate receptor, c-kit, is expressed within the PGCs (Manova and Bachvarova, 1991; Orr-Urtreger et al., 1990). IL-4 protein can be detected from genital ridge extracts at the time when PGCs are normally imbedded within this tissue (Cooke et al., 1996) Likewise, a receptor for LIF (LIFR) is expressed on the surface of PGCs isolated from genital ridges (Cheng et al., 1994). RNAs encoding pituitary adenylate cyclase-activating polypeptides (PACAPs), which raise PGC intracellular cAMP levels in culture, are expressed in migrating PGCs and gonadal ridges colonized with PGCs (Pesce et al., 1996). Expression of TGF-β1 is also found within the genital ridge, but its domain of expression expands to outlying regions of the dorsal body wall as well (Godin and Wylie, 1991). This expression outside of the genital ridge is consistent with TGF-β1's in vitro ability to inhibit PGC proliferation, rather than promote survival.

Although the expression patterns of some of these factors combined with their effects on PGCs in culture suggest that these molecules may be involved in PGC migration, a lack of functional data for these factors in vivo has hampered further investigations. Moreover, the majority of these molecules have only been shown to increase PGC number in culture, and therefore the mechanisms by which these factors actually function in guiding PGCs along a stereotyped migratory path remain to be elucidated. An exception to this lack of functional data is provided by genetic analysis of the roles of SCF and c-kit in PGC migration in the mouse. Mutations in the gene encoding SCF, known as *Steel (Sl),* as well as in the gene encoding c-kit, *Dominant White Spotting*

(W), affect a number of tissues in the developing mouse, including the gonads (reviewed in Fleischman, 1993). In homozygous *W* mutants, less than half the normal number of PGCs are found migrating toward the genital ridge. This number continues to decrease as development ensues. In addition to a loss in PGC number, those that do survive are often found in ectopic locations, including ventral to the hindgut from which they emigrate, instead of their normal dorsal position. Virtually no PGCs are successful in reaching the genital ridge, and are often found adhering to one another in large clumps (Buehr et al., 1993). Together with cell culture and expression data, these results suggest a model by which *Sl* and *W* function in PGC migration by promoting the survival of only those PGCs that follow the correct migratory path (Godin et al., 1991). However, given that PGCs are found in ectopic locations in *W* mutants, *W* and *Sl* most likely have an additional, active role in PGC guidance.

PGC migration in Drosophila

Although detailed descriptions of PGC behavior, cell-cell interactions, and growth conditions have been made available through studies in Xenopus and the mouse, with the exception of SCF and c-kit, very little is known regarding the actual molecules that function in vivo to facilitate PGC migration in vertebrates. In order to understand the mechanisms underlying PGC migration, and how particular factors contribute to this process, I have used a system more amenable to genetic analysis, *Drosophila melanogaster*, to address these questions.

Remarkably, PGC migration in Drosophila is strikingly similar to that observed in vertebrates. PGCs form extraembryonically, and are incorporated into the developing gut. They migrate through the gut and into the mesodermal layer where they encounter the cells that will comprise the somatic component of the gonad. Germ cells and somatic cells finally coalesce into the spherical structure of the embryonic gonad (for a detailed description, see Chapter 2; Moore et al., 1998; Warrior, 1994). Therefore, the hallmarks of migratory cell behavior seen in Xenopus and mouse PGCs are also found in the analogous cells of the fly. Drosophila PGCs must possess invasive behavior to penetrate the gut wall, they must move toward and recognize the correct somatic gonadal cells, and presumably must change their states of adhesiveness to form the tightly coalesced gonad. Given that PGC migration in Drosophila can therefore also provide an excellent model system for the study of mechanisms governing general cell migration processes, one can utilize the myriad of genetic and developmental tools available in this system to investigate PGC migration further.

Early Drosophila PGC development

In Drosophila, the PGCs are the first to cellularize within the embryo. Numerous genes have been identified that are required for PGC formation through maternal effect screens (Boswell and Mahowald, 1985; Boswell et al., 1991; Lehmann and Nüsslein-Volhard, 1986; Manseau and Schüpbach, 1989; Schüpbach and Weischaus, 1989; Schüpbach and Wieschaus, 1986). In the absence of these genes' activities, the morphologically distinct "germ plasm", which is thought to harbor the molecules necessary for directing PGC formation, is absent (reviewed in Rongo and Lehmann, 1996). These genes are additionally required for the proper localization of *nanos* (*nos*) RNA (Wang et al., 1994). *nos* is required for development of abdominal structures in the embryo, and cannot exert its normal function unless localized to the posterior pole (Gavis and Lehmann, 1994; Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). Therefore, genes identified through these maternal-effect screens are not specific for PGC development. However, one of these genes, *oskar (osk)*, is sufficient to direct all the steps necessary to form functional PGCs in an ectopic location, demonstrating the pivotal role for this gene in PGC development. Moreover, *osk* gene dosage controls the number of PGC that form in the embryo (Ephrussi and Lehmann, 1992).

More recently, a number of molecules have been identified that may be involved more specifically in PGC formation and/or function, without an associated role in abdomen formation. The mitochondrial large ribosomal RNA (mtlrRNA) was identified as a component of the germ plasm based on its ability to rescue PGC formation in u.v.-irradiated Drosophila embryos (Kobayashi et al., 1993; Kobayashi and Okada, 1989). Ultrastructural analysis of embryos hybridized in situ to detect mtlrRNA demonstrated that this RNA is largely found outside of the mitochondria in germ plasm, consistent with a role for this factor within the germ plasm itself (Kobayashi et al., 1993). Although the PGCs "rescued" by mtlrRNA injection appear to migrate into a gonad, the resulting adult flies are sterile, suggesting that PGC development is impaired. Moreover, the ability of mtlrRNA to induce ectopic PGCs is only observed when it is co-injected with UV-irradiated germ plasm. These results demonstrate that mtlrRNA is not sufficient to act as a PGC determinant. However, recent preliminary evidence suggests that mtlrRNA does play a role in PGC development. When injected into early embryos, a ribozyme that targets this RNA is able to prevent the formation of PGCs (Kobayashi, 1997).

Another factor implicated in the formation of Drosophila PGCs is the product of the *germ cell-less* (*gcl*) gene. *gcl* was fortuitously identified as a transcript localized to the posterior region of the embryo, from where the PGCs form. The RNA and protein products of this gene are incorporated into PGCs, and can be detected within these cells

until they exit the gut (Jongens et al., 1994; Jongens et al., 1992). Immunoelectron microscopy revealed that the Gcl protein is associated with the nuclear pore complex of PGC nuclei on the nucleoplasmic side (Jongens et al., 1994). Expression of antisense-*gcl* results in viable embryos lacking PGCs, suggesting a role for *gcl* in PGC formation (Jongens et al., 1992). Conversely, increased levels of *gcl* expression can initially induce a greater number of PGCs than normally found in wild-type embryos, but these additional PGCs do not survive to develop into functional germ cells. Similarly, ectopic *gcl* expressed at the anterior pole of the embryo results in anterior buds that are reminiscent of early PGCs, but they fail to form functional PGCs capable of migration (Jongens et al., 1994). Taken together, these results suggest a role for *gcl* in PGC formation, but reveal its insufficiency in directing the entire PGC developmental path.

Although not found to play a role in PGC formation, an RNA product called *Polar granule component (Pgc)* was identified as a message specifically found in the posterior region of early Drosophila embryos. Like *gcl, Pgc* RNA is subsequently taken up by the PGCs, persisting until the PGCs begin their migration through the gut. PGCs form in embryos expressing antisense-*Pgc* RNA, but their numbers are drastically reduced in later developmental stages. Although a few PGC are capable of forming gonads in these embryos, the resulting adults are sterile. Sequence analysis of the *Pgc* RNA suggests that it is an untranslatable product. Therefore, *Pgc* RNA appears to be necessary for PGC development and for subsequent differentiation into functional germ cells (Nakamura et al., 1996).

While these three factors appear to play specific roles in PGC formation and/or development in Drosophila, more detailed analyses of their actual functions are unavailable due to the lack of existing mutations in the corresponding genes. Moreover, it is unclear if any of these factors play a direct role in the migration of PGCs during embryogenesis. One gene recently identified to be required in the PGCs for their migration is *nos*. It had been shown previously to function as the posterior determinant in the Drosophila embryo necessary for directing formation of abdominal structures, but not PGCs (Wang and Lehmann, 1991). However, removing *nos* function specifically in the PGCs reveals a primary role for this gene in PGC migration. PGCs lacking *nos* activity are able to migrate out of the developing gut, but instead of moving toward cells that will comprise the somatic component of the gonad, they remain in large clumps outside the gut wall. In addition, PGC morphology is aberrant, suggesting a role for *nos* in PGC migratory cell behavior (Forbes and Lehmann, 1998).

The isolation of additional genes required early in the PGCs through classical genetic screens for zygotically acting factors may prove difficult. Analysis of zygotic gene

expression in early Drosophila embryos through the incorporation of radiolabeled nucleic acid precursors revealed that PGCs appear transcriptionally quiescent until they are incorporated into the developing gut (Zalokar, 1976). A potential explanation for this result has been provided by the observation that a phosphorylated form of RNA polymerase II is specifically absent from early Drosophila as well as *C. elegans* PGCs, while it is found in somatic cells. It is thought that this phosphorylated form of RNA polymerase II is involved in transcriptional elongation; therefore, its absence in PGCs is consistent with their lack of RNA polymerase II transcriptional activity (Seydoux and Dunn, 1997). Additional studies have shown that early Drosophila PGCs are incapable of transcribing Gal4-VP16-dependent target genes, even when this transcriptional activator is artificially provided to these cells. Moreover, germ plasm localized ectopically to the anterior of the embryo is sufficient to repress RNA polymerase II-dependent gene expression in this region (Van Doren et al., 1998). Taken together, these results suggest that a global transcriptional repression mechanism is acting on RNA polymerase II-dependent promoters within the early PGCs, perhaps directly at the level of RNA polymerase II modification. Interestingly, Kobayashi et al. (1996) have observed the premature expression of zygotic PGC-specific markers in PGCs lacking *nos* function. Given that *nos* is required in the PGCs for their migration, these results suggest that repression of zygotically active genes is important for PGC migratory behavior.

Although these results imply that genes acting zygotically in the PGCs may not function at early developmental stages, transcriptional activity resumes in the PGCs before their initial migratory steps. The RNA polymerase II phosphoisoform can be detected as the PGCs begin their invagination into the gut primordium (Seydoux and Dunn, 1997). Moreover, one of the earliest known Drosophila PGC-specific transcripts, *vasa*, is present before the PGCs begin their migration through the gut endoderm (Van Doren et al., 1998). Therefore, it may yet be possible to identify zygotically-acting genes required in PGCs for their migration.

Origins of Drosophila somatic gonadal cells

The migration of PGCs not only involves the PGCs themselves, but also the somatic cells with which they associate to form the gonad. Therefore, many genes required for PGC migration may act within these somatic cells to ensure proper gonadogenesis. Early studies of gonad development through observational analysis revealed that the coalesced gonad contains 26-37 somatic mesodermal cells ensheathing the PGCs (Sonnenblick, 1941). Fate mapping of the Drosophila blastoderm using gynandromorph analysis suggested that the primordium of the somatic component of the gonad arises from either parasegment (PS) 10

or PS11 (Szabad and Nöthiger, 1992). The more recent identification of markers expressed within the somatic gonadal cells suggested that they form within three parasegments of the embryo, PS10-12 (Boyle et al., 1997; Brookman et al., 1992; Warrior, 1994).

One of these markers, the 412 retrotransposon, is expressed within the mesoderm of all parasegments at the time when PGCs are migrating through the gut. After PGCs have left the gut, high levels of 412 retrotransposon expression become specifically found in those cells that appear to encapsulate the PGCs to form the gonad. However, this specific expression is not dependent on the presence of PGCs. Many copies of this retrotransposon are present within the Drosophila genome, and at least more than one insert displays this expression pattern. It is unclear whether the specific gonadal expression is due to an increase in levels within the somatic gonadal cells, in addition to a decrease in other parasegments (Brookman et al., 1992). The 68-77 strain is an enhancer trap line expressing β-galactosidase within somatic gonadal cells, and is thought to be under the regulatory control of a 412 retrotransposon (Boyle and DiNardo, 1995; Simon et al., 1990; Warrior, 1994; M. Boyle and S. DiNardo, personal communication). Consequently, its developmental expression profile mimics that of the 412 retrotransposon (Boyle and DiNardo, 1995). A second enhancer-trap line, bluetail, is expressed only within cells in PS12-14. By following cells expressing β-galactosidase in the bluetail line during PGC migration, it was observed that mesodermal cells in PS12, in addition to PS10 and PS11, most likely contribute to the embryonic gonad, consistent with the conclusions drawn based on expression of the 412 retrotransposon (Boyle and DiNardo, 1995; Brookman et al., 1992; Galloni et al., 1993).

Genes required for somatic gonadal cell development

Although the aforementioned markers have proved valuable in the identification of those cells contributing the somatic component of the gonad, they do not appear to have a function in the development of the resulting tissue, the gonadal mesoderm. Recent efforts to examine somatic gonadal cell development have revealed a number of genes involved in the specification and differentiation of these cells.

The homeotic genes

The homeotic genes are required for specifying the identities of particular parasegments in the development of the fly. Remarkably, they function in assigning the correct identities to multiple tissue-types within a region of the embryo and adult (for reviews see Duncan, 1987 and Peifer et al., 1987). Given that the origins of the somatic

gonadal cells appear to lie within PS10-12, it is not surprising that the homeotic genes *abdominal A* (*abdA*) and *Abdominal B* (*AbdB*) are required for their development. *abdA* is required for specifying identities in PS7-13, whereas *AbdB* is required for PS10-14. *abdA* was first implicated in the development of the gonad through analysis of mutations within its regulatory region (Lewis, 1978). One particular mutation, *iab-4,* is homozygous viable, but results in a transformation of PS10 into the identity of PS9 as well as adult sterility (Karch et al., 1985; Lewis, 1985). More detailed investigations of *iab-4* mutants revealed that this sterility is due to a lack of gonadal development within the embryo. In *iab-4* mutant embryos, PGCs migrate through the gut, but fail to be surrounded by somatic gonadal cells and consequently disperse within the embryo. These studies also determined by nuclear transplantation that wild-type function of *abdA* is required in the soma for gonad formation (Cumberledge et al., 1992). Careful observations of gonadal mesoderm development in *iab-4* mutants revealed that the PGCs and somatic cells initially associate with one another, but fail to coalesce into the gonad (Boyle and DiNardo, 1995).

It has also been shown that *abdA* and *AbdB* are required for the expression of somatic gonadal cell-specific markers. For example, expression of the 412 retrotransposon is markedly reduced in embryos lacking *abdA* function. Loss of *AbdB* function also results in fewer cells specifically expressing the 412 retrotransposon, although not to the same extent as that seen in *abdA* mutants. Associated with the reduction in 412 expression in *AbdB* mutants is a PGC migration defect, whereby a reduced number of PGCs are incorporated into the gonad (Brookman et al., 1992). This effect on 412 expression seems to be due a requirement of AbdB in the specification of a subset of somatic gonadal cells. In wild-type embryos, the bluetail enhancer trap line and the *clift* (*cli,* also known as *eyes-absent*) gene are specifically expressed within the posterior gonad after coalescence. In *AbdB* mutants, expression of these markers is no longer detected, presumably because these posterior cells are never specified (Boyle and DiNardo, 1995).

Consistent with their functions, *abdA* and *AbdB* are expressed within the parasegments that they control (Boulet et al., 1991; Celniker et al., 1989; DeLorenzi and Bienz, 1990; Karch et al., 1990; Macias et al., 1990). Within the mesoderm, AbdA protein is present in PS8-12, and can be detected in gonadal cells surrounding the PGCs (Boyle and DiNardo, 1995; Cumberledge et al., 1992; Karch et al., 1990). In *iab-4* mutants, this gonadal expression is reduced, whereas in other mesodermal tissues, AbdA protein levels appear unchanged (Boyle and DiNardo, 1995). Therefore, the specific gonadal defect seen in *iab-4* mutants can be attributed to a loss in *abdA* expression in this tissue. Whereas AbdA protein is detected in all parasegments from which somatic gonadal cells develop, at

early embryonic stages, AbdB protein is only mesodermally expressed in PS12-14. However, at later stages, this expression expands into PS11 (DeLorenzi and Bienz, 1990).

The initial domains of *abdA* and *AbdB* gene expression are set up by members of the segmentation genes, which are responsible for early patterning of the embryo (Harding and Levine, 1988; Reinitz and Levine, 1990). Maintenance of proper expression borders for all homeotic genes requires both activating and repressive inputs. Genes required for maintaining the active state of homeotic gene expression are collectively known as the trithorax-group (reviewed in Kennison, 1993). The founding member of this group, *trithorax* (*trx*), encodes a large protein with zinc finger-like motifs, suggesting that it may bind directly to DNA (Mazo et al., 1990). The Polycomb group of genes are necessary for preventing ectopic homeotic gene expression after their initial boundaries have been set (reviewed in Kennison, 1995). Sequence analysis of the *Polycomb* (*Pc*) gene, a Polycomb group member, reveals a domain that is homologous with one found in the Drosophila HP1 protein. This protein is associated with heterochromatic regions within the genome and is involved in inhibiting the expression of genes found near these sites as a result of chromosomal rearrangements or transposition (James and Elgin, 1986; Eissenberg et al., 1990; Eissenberg et al., 1992). These results suggested a model by which the Pc protein product may repress homeotic gene expression through its involvement in higher order chromatin structure (Paro, 1990).

It is crucial to the development of an organism that the expression levels and boundaries of the homeotic genes are maintained by the trithorax and Polycomb group members. Mutations in these genes mimic the homeotic mutations themselves, resulting in the mis-specification of proper segment identities. In the embryo, both *trx* and a number of Polycomb group members are required for maintaining the proper levels and boundaries of *abdA* and *AbdB* expression (Breen and Harte, 1993; Sedkov et al., 1994; Simon et al., 1992). It has been shown that proper expression boundaries of both *abdA* and *AbdB* are also required for normal gonad formation. Mutations in one Polycomb group member, *extra sex combs* (*esc*), results in an expansion of 412 expression into more anterior regions of the embryo. This result suggested that ectopic *abdA* and/or *AbdB* is sufficient to specify additional somatic gonadal cells. Surprisingly, in *esc* mutants fewer PGCs coalesce into a gonad, but do so in the correct position along the antero-posterior axis (Brookman et al., 1992).

In order to further explore these results, Boyle and DiNardo (1995) examined gonadal mesoderm development in embryos expressing ectopic *abdA* activity driven by a heat shock promoter. They found that like in *esc* mutants, 412 is expressed in more anterior parasegments. Moreover, PGCs were able to travel more anteriorly to associate

with the ectopic 412 expressing cells, suggesting that they indeed are specified as somatic gonadal cells. This result was corroborated by Greig and Akam (1995) who showed that expression of *abdA* throughout the mesoderm also resulted in an anterior expansion of 412 expression. However, their results did not indicate that PGCs could migrate more anteriorly, although gonad coalescence was disrupted. Boyle and DiNardo suggest that anterior PGCs found in heat shock-*abdA* embryos are a result of migration defects at late stages in gonadogenesis; therefore, it may be that ectopic expression of *abdA* in Greig and Akam's experiments did not persist long enough to cause anterior migration of PGCs. In contrast, ectopic *AbdB* expression is not sufficient to specify anterior somatic gonadal cells. However, this is presumably due to *AbdB*'s ability to repress *abdA* expression when ectopically produced (Greig and Akam, 1995).

cli *function in somatic gonadal cell development*

Previous to our screens, the *cli* gene was the only marker expressed specifically in somatic gonadal cells within the mesoderm that had been shown to actually function in gonadogenesis. Early in embryogenesis, Cli protein is expressed throughout the mesoderm, but then is refined to discrete clusters of cells in PS10-12 that contact PGCs. These cell clusters are most likely somatic gonadal cells, since they associate and coalesce with PGCs and are absent in *abdA* mutants (Boyle and DiNardo, 1997). In *cli* mutants, the number of cells specifically expressing 412 is markedly reduced. Those few remaining somatic gonadal cells are able to associate with PGCs, but never coalesce into a gonad (Boyle et al., 1997; Moore et al., 1998; see Chapter 2). *cli* seems to function in the differentiation of somatic gonadal cells, rather than their specification. In embryos lacking *cli* function, *cli* RNA can still be detected in somatic gonadal cells when they first associate with PGCs. However, this expression is soon lost, and is consistent with the reduction in 412 expressing cells observed in *cli* mutants (Boyle et al., 1997).

Given that PGC migration in Drosophila is similar to that seen in vertebrates, combined with the numerous migratory steps and cell-cell interactions that PGCs experience along their developmental path, we hope that PGC migration in Drosophila can provide an excellent paradigm for the study of migratory cell behavior during development. Although the studies described above have proven useful in identifying factors involved in Drosophila PGC and somatic gonadal cell development, key questions remain as to what additional mechanisms are involved in guiding PGCs along their migratory route. I have therefore undertaken a genetic approach to identify genes required for PGC migration in Drosophila by screening for mutations that disrupt this process during embryonic

development. The goal of this type of screen is to identify those genes playing a role in ensuring that PGCs reach their destination in the embryonic gonad. This type of approach could yield a number of different types of molecules, ranging from those involved in signaling processes between the PGCs and the somatic cells with which they interact, to the factors involved in the proper specification and differentiation of these same cell types.

Chapter 2 describes a screen taken to near saturation that identified genes on the third chromosome necessary for PGC migration and gonad formation. The results from this screen delineate discrete steps occurring during the migration of PGCs, and identify a number of genes required for development of the gonadal mesoderm. Thus, proper specification and differentiation of this tissue is crucial for the PGCs to carry out the necessary migratory steps involved in gonadogenesis.

Chapter 3 provides phenotypic analyses of three mutants obtained from the third chromosome screen, as well as from a screen for second chromosome mutants. The identification of genes required for steps in somatic gonadal cell specification led to a more in-depth analysis of the development of gonadal mesoderm. In Chapter 4, I describe experiments demonstrating that gonadal mesoderm is closely related to another mesodermal tissue, the fat body, as well as provide an outline of the genetic mechanisms deciding between gonadal mesoderm and fat body cell fates. Finally, Chapter 5 discusses the implications of the results from the screens, as well as of the analysis of gonadal mesoderm and its relationship to the fat body. Given what has been learned from the experiments outlined in this thesis, further directed studies aimed at extending our understanding of PGC migration and gonadal mesoderm development are suggested.

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Specific Aims

The aims of this thesis are to describe a screen conducted to identify genes required for germ cell migration and gonad formation in Drosophila, as well as preliminary characterizations of a subset of phenotypes yielded from this screen. In addition, an in-depth analysis of the control of the cell fate decision between gonadal mesoderm and fat body identities during embryogenesis is presented. Chapter 2 describes a saturation mutagenesis of the third chromosome and the genes identified to affect gonad formation. This chapter also includes an analysis of gonadal mesoderm development in mutants isolated from the screen, and how these results demonstrate that gonadal mesoderm originates from the *eve* domain of the mesoderm. A phenotypic analysis of three genes identified through this screen of the third chromosome as well as one carried out on the second chromosme is presented in Chapter 3. Chapter 4 includes studies indicating that gonadal mesoderm is closely related to another mesodermal cell type, fat body, as well as how the cell fate decision between these two tissues is controlled along the anteroposterior axis. Finally, Chapter 5 discusses some of the issues raised from this work, including a comparison to mammalian systems.

PREFACE

This work involved a collaboration of a number of people in the lab. I wrote the manuscript that appears in press, as well as established the fly stocks used for mutagenesis and heat shock protocols used during the screen. Heather Tarczy Broihier, Dr. Mark Van Doren and I contributed equally to the screening and phenotypic characterization of the mutants identified. Heather Tarczy Broihier and I participated equally in determining that the origin of gonadal mesoderm lies within the *eve* domain. Lynn B. Lunsford provided technical assistance throughout the screen.

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*these authors contributed equally to this work

CHAPTER 2

Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila

SUMMARY

Gonadogenesis in the Drosophila embryo is a complex process involving numerous cellular migratory steps and cell-cell interactions. The mechanisms guiding germ cells to move through, recognize, and adhere to specific cell types are poorly understood. In order to identify genes which are required for these processes, we have conducted an extensive mutagenesis of the third chromosome and screened for mutations disrupting germ cell migration at any point in embryonic development. Phenotypic analysis of these mutants demonstrates that germ cell migration can be broken down into discrete developmental steps, with each step requiring a specific set of genes. Many of these genes are involved in the development of gonadal mesoderm, the tissue which associates with germ cells to form the embryonic gonad. Moreover, mutations we isolated that affect embryonic patterning as well as germ cell migration suggest that the origin of gonadal mesoderm lies within the *eve* domain of the developing mesoderm.

INTRODUCTION

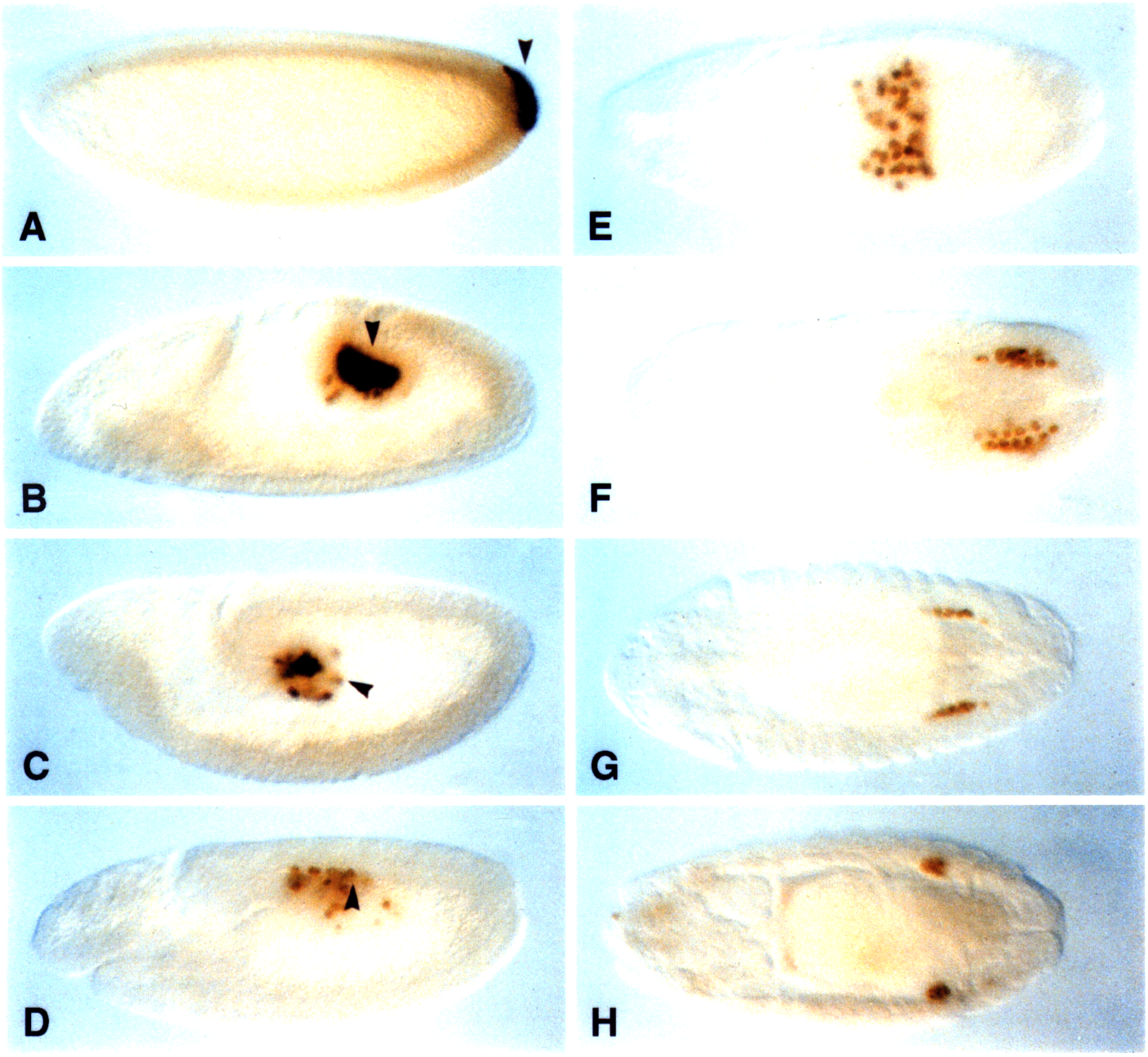
Cellular movements play a crucial role in the development of a multicellular organism. They can serve a variety of functions ranging from creating different tissue layers during gastrulation to the processes of organogenesis. Some of these processes include bringing different cell types into contact with one another in order for their final differentiation to proceed. The migration of primordial germ cells (PGCs) provides a model system for the study of cellular movement and differentiation during development. In many organisms, germ cells form in a position distinct from where they will eventually populate the gonad. The PGCs must locate and adhere to cells that will comprise the somatic component of the gonad, which requires movement through and along different tissue layers. Previous in vitro studies in Xenopus and mouse have identified adhesive molecules such as fibronectin that are involved in some aspects of gonadogenesis (ffrench-Constant et al., 1991; Heasman et al., 1981). Moreover, genetic studies in mouse have shown that the signaling molecule Steel factor and its receptor, c-Kit, are involved in germ cell survival (Fleischman, 1993). Presumably, many other factors required for the migration of PGCs remain to be identified.

PGC migration in Drosophila is similar to that found in vertebrates, including some of the cellular movements and interactions described above (Fig. 2-1). The PGCs, often referred to as pole cells in Drosophila, are the first to cellularize at the posterior pole of the embryo (Fig. 2-1A). During gastrulation, they move along the dorsal surface of the embryo along with the posterior midgut (PMG) primordium, and are incorporated into the invaginating PMG pocket (Fig. 2-1B). The PGCs then migrate through the PMG wall, moving along its basal surface to the dorsal side of the embryo (Fig. 2-1C). From this position they move toward lateral mesodermal cells in parasegments 11-13 (PS 11-13, Fig. 2-1D,E). As the germ band retracts, PGCs associate and align with mesodermal cells in PS 10-12 that will give rise to the somatic component of the gonad (Fig. 2-1F). Finally, the PGCs and gonadal mesoderm coalesce in PS 10 to form the embryonic gonad (Fig. 2-1H). Germ cell migration in Drosophila therefore provides a model system for the study of cellular movements and cell-cell interactions.

Figure 2-1. Germ cell migration in wild-type embryos.

Anterior is to left in all panels. Staging according to Campos-Ortega and Hartenstein (1985). (A-H) Germ cells visualized using an anti-Nanos antibody (arrowheads); (A-D) lateral views; (E-H) dorsal views. (A) Stage 5. Germ cells form at the posterior pole of the embryo. (B) Stage 8. During gastrulation, germ cells adhere to the posterior midgut (PMG) anlage, and are carried into the PMG lumen. (C) Stage 9-10. Germ cells begin their migration through the PMG. (D) Stage 11. Germ cells have migrated to the dorsal side of the PMG, and begin to associate with lateral mesoderm in PS 11-13. (E) Stage 11. In the fully extended embryo, germ cells have migrated into the lateral mesodermal layer and are beginning to separate into two bilaterally symmetric groups. (F) Stage 12. During germ band retraction, germ cells migrate anteriorly and associate with somatic gonadal precursors (SGPs) in PS 10-12. (G) Stage 13. Once the germ band has retracted, all germ cells have aligned with the SGPs. (H) Stage 15. Germ cells and gonadal mesoderm coalesce into the embryonic gonad.

Fig. 2-1: Germ cell migration in wild-type embryos



Recent work has characterized one of the first steps in Drosophila germ cell migration, that of the movement of the germ cells through the PMG. Ultrastructural studies have shown that during this stage, apical junctions dissolve in the PMG, and intercellular gaps form through which the germ cells migrate (Callaini et al., 1995; Jaglarz and Howard, 1995). However, it is not known if these gaps are required for germ cell movement through this tissue. Mutations affecting the development of the PMG suggest that this may be the case. *serpent* (*srp*) and *huckebein* (*hkb*) are both required for the proper differentiation of the midgut, as mutations in them cause a transformation of part of the PMG into a more hindgut-like tissue. In these mutants, the PMG intercellular gaps fail to form, and germ cells are rendered helpless to reach their destination in the mesoderm (Jaglarz and Howard, 1995; Reuter, 1994; Warrior, 1994). Once across the gut wall, the germ cells in a wild-type embryo then migrate along the basal surface of the gut to its most dorsal side. Genetic analysis has revealed that *wunen* (*wun*) is required for this directed migration of the germ cells along the basal surface of the PMG. The expression pattern of this gene within the PMG and hindgut suggests that it acts by repelling germ cells away from other areas of the gut (Zhang et al., 1996; Zhang et al., 1997).

The development of the somatic component of the gonad has also been the focus of numerous studies. It has been known for some time that mutations in the homeotic gene *abdominal A* (*abdA*) abolish gonad function (Karch et al., 1985; Lewis, 1978), and that *abdA* is required in the soma for gonad formation (Cumberledge et al., 1992). Moreover, a regulatory mutation in the *abdA* locus, *iab4,* causes specific defects in gonad coalescence (Boyle and DiNardo, 1995; Cumberledge et al., 1992; Warrior, 1994). More recent work has shown that both *abdA* and *Abdominal B* (*AbdB*) are required for the specification of somatic gonadal precursors (SGPs), those cells which give rise to gonadal mesoderm, in PS 10-12 (Boyle and DiNardo, 1995). Prior to this specification step, the *tinman* (*tin*), and *wingless* (*wg*) genes are involved in establishing domains within the parasegment from which SGPs can develop (Boyle et al., 1997). Subsequent to their specification, SGPs in PS 11-12 migrate anteriorly toward PS 10, and along with germ cells coalesce to form the embryonic gonad (Boyle and DiNardo, 1995). The maintenance of SGP cell fate during this migration requires the function of the *clift* (*cli*, also known as *eyes-absent*) gene. *cli* expression in the mesoderm is restricted to SGPs by stage 11, and depends on *abdA* and *AbdB* function (Boyle et al., 1997).

Although the combined results of this work have lent valuable information toward the understanding of processes required for gonadogenesis in Drosophila, many questions remain unanswered. For instance, very little is known regarding how the germ cells are directed away from the PMG to associate with SGPs in PS 10-12. Moreover, the

mechanisms behind how the germ cells and their somatic partners migrate anteriorly and coalesce to form the embryonic gonad remain to be elucidated. One powerful technique that can be used for the identification of additional genes involved in these developmental processes is mutational analysis. Previous screens of existing mutants have identified genes required for both general patterning and gonad assembly in the Drosophila embryo (Boyle et al., 1997; Warrior, 1994). However, a comprehensive study of all mutations that affect germ cell migration had yet to be accomplished. We describe here a large-scale mutagenesis of the third chromosome identifying zygotic mutations affecting germ cell migration at discrete points during Drosophila embryogenesis. Over 8000 mutagenized lines were screened for defects in gonad formation, yielding more than 300 lines which were kept for further analysis. We present the phenotypic analysis of mutants corresponding to 11 genes which have the most specific effects on gonad formation, and describe how these mutants provide further insight into the mechanisms governing the proper migration of germ cells in Drosophila.

MATERIALS AND METHODS

EMS mutagenesis and establishment of balanced lines

See Fig. 2-2 for an outline of the screen. A *ru st e^s ca* chromosome carrying the *fat facets-lacZ* (*faf-lacZ*) transgene (Fischer-Vize et al., 1992), that had recently been isogenized was used for the target mutagenesis strain. This line had been selected for its low frequency of germ cells found outside the gonad at stage 15. A total of 2100 *ru st P{faf-lacZ} e^s ca* males were mutagenized with EMS (Sigma and ICN: 1500 with a 25 mM solution, and 600 with a 35 mM solution) in 1% sucrose for 24 hours according to standard procedures (Ashburner, 1989), with the modification that they were starved for 6 hours on a Kimwipe saturated with water prior to EMS treatment. These males were then mated to 4200 virgin females of the genotype *Df(3R)H99 P{hs-hid} p^p/ Ubx-lacZ TM3, Sb* [The Df(3R)H99 P{hs-hid} chromosome was used as a dominant temperature sensitive lethal mutation and was a generous gift from Megan Grether and Hermann Steller (Grether et al., 1995)]. The crosses were incubated at 25°C, and after 5 days the males were discarded to prevent clonal mutations. A total of 12,500 single males from the F1 generation of either genotype were each mated to 2 *Df(3R)H99 P{hs-hid} p^p/Ubx-lacZ TM3, Sb* virgin females. These crosses were allowed to lay eggs for 5 days, after which the parents were discarded. The progeny were then subjected to 2 hours of heat shock on days 5 and 6 by placing vials directly in a 37°C water bath, with a 24 hour interval between heat shocks. Induction of ectopic *hid* expression in this manner presumably causes massive cell death, and results in embryonic/larval lethality. We found that about 10% of our isolates contained flies that were not of the mutagenized *ru st P{faf-lacZ} e^s ca/Ubx-lacZ TM3, Sb* genotype. However, these "lines" usually contained only 1-2 "escaper" progeny and did not pose a serious problem to the screening procedure. Lines that were kept for further analysis (see below) were inspected for "escaper" flies and if necessary, virgin females and males of the desired genotype were collected and used to establish balanced stocks. Lethal lines were determined by the absence of *ru st e^s ca* homozygotes.

Screening procedures and detection of β-galactosidase activity

Eggs of approximately 6-16 hours of age were collected from balanced lines using the block method as described in Nüsslein-Volhard et al. (1984). Eggs were collected from apple juice-agar plates and placed into 18-well staining blocks (design by Phillip Zamore), and processed for X-gal staining using the following procedure: Eggs were washed twice in PBT, and then dechorionated by placing in a 50% bleach solution for 5 minutes. After washing twice in PBT, they were fixed in heptane saturated with 2.5% glutaraldehyde for 7

minutes. The embryos were allowed to dry for 4 minutes in a fume hood, and then washed in PBT for 30 minutes. Embryos were then stained for β-galactosidase activity using 10% X-gal in DMSO (Diagnostic Chemicals Limited), 1:50 in a staining buffer containing 10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM K4[FeII(CN)6], 3 mM K3[FeIII(CN)6], and 0.1% Triton X-100. Staining took place at 37°C, and embryos were then screened directly in staining blocks under a dissecting microscope for defects in germ cell migration. Any line producing embryos that failed to form wild-type gonads was propagated an additional generation and subjected to a secondary screening procedure (see below).

Whole-mount antibody staining

Antibody staining was performed with either a rabbit polyclonal anti-Vasa or anti-Nanos antibody (generously provided by Anne Williamson and Charlotte Wang, respectively), and rabbit anti-β-galactosidase (Cappel). Prior to use, the anti-β-galactosidase and secondary antibodies (see below) were diluted 1:10 and preabsorbed against an overnight collection of wild-type embryos.

All antibody detection was done with horseradish peroxidase using a biotinylated secondary antibody (Jackson ImmunoResearch) and the Elite Kit (Vector Labs). For the secondary screen all incubations, including fixation and devitellinization, were conducted in the 18-well staining blocks described above (protocol modified from Royzman, et al. (1997)]. Embryos were dechorionated as above and fixed for 20 minutes with gentle shaking in 4:1 heptane:4% formaldehyde in PBS. Embryos were washed twice in fresh heptane, and an equal volume of methanol was added followed by rigorous shaking for divitellinization. Non-divitellinized embryos were removed from the blocks, and the remaining embryos were rehydrated and subjected to antibody staining as described in Eldon and Pirrotta (1991). Embryos were mounted onto slides in LX112 embedding medium (Ladd Research Industries, Inc.) according to Ephrussi et al. (1991), then analyzed with a Zeiss Axiophot microscope using Nomarski optics.

Cuticle preparations

Cuticle preparations were made of all potential mutant lines in a manner similar to that described by Nüsslein-Volhard et al. (1984), with the following modifications: Embryos were collected on apple juice-agar plates for 12 hours, and allowed to age for 24 hours at 25°C. Unhatched eggs were collected into specialized 18-well staining blocks (design by Philip Zamore), dechorionated, and fixed for 10 minutes in a 3:1 acetic acid:glycerol solution at 65°C. Embryos were washed twice in PBT and placed onto a slide. Excess

PBT was removed with a filter paper (Whatman), replaced with a small drop of Hoyer's medium, and covered by a 22x22 mm coverslip. Embryos were cleared by a 36 hour incubation at 65°C, and analyzed with a Zeiss Axiophot using dark field with a 20x objective.

Complementation tests, mapping, and deficiency analysis

For lines that showed relatively normal patterning (Class 1), complementation tests were conducted between mutants with similar germ cell migration defects. Allelism was determined based on failure to recover transheterozygous viable progeny, as well as the presence of a germ cell migration defect in transheterozygous embryos. Lines which showed obvious defects in pattern formation were crossed to mutants obtained from the Bloomington Stock Collection having similar phenotypes. In addition, once complementation groups were established from the "specific" class of mutants, a representative allele from each group was crossed to three mutants previously known to show defects in gonad formation: *abdA* (Cumberledge et al., 1992), *AbdB* (Brookman et al., 1992), and *tin* (our observations; Boyle et al., 1997).

Fifteen mutants in our "specific" class do not fit into complementation groups, even when tested against each other. Given that these mutants all have relatively weak phenotypes with poor penetrance, we believe them to be the result of synthetic effects caused by more than one mutation. This result is similar to that obtained in the screens for defects in embryonic pattern formation (Nüsslein-Volhard et al., 1984). Moreover, we found 14 lines with defects in dorsal closure that also complemented each other, and could not be attributed to known loci. If we include these "single alleles" in our calculation of allele frequencies, we have induced an average of 3 alleles per locus. However, given that we have identified more than 1 allele for 20 of 22 known loci, we presume it unlikely that these other single alleles represent 29 unknown loci. Therefore we have not included this "single allele" class from our calculations to estimate the degree of saturation for this screen.

Six complementation groups were roughly mapped by meiotic recombination using the *ru st* e^s *ca* markers. Once mapped to an interval, mutants were crossed to deficiency stocks (obtained from the Bloomington Stock Collection) uncovering the interval and tested for complementation based on lethality. Once a non-complementing deficiency was found, mutants of known genes uncovered by the deficiency were tested against our mutants for allelism, again based on lethality. In this way we discovered that 3 of our complementation groups were allelic to the *htl*, *trx,* and *zfh-1* loci.

Whole-mount in situ hybridization

Whole-mount in situ hybridization to embryos with biotinylated and digoxigenin-labeled antisense riboprobes was performed according to the double labeling method as described in Lehmann and Tautz (1994).

Antisense riboprobes were prepared for detection of the 412 retrotransposon using the pSK2.4 #3 plasmid (Brookman et al., 1992), and synthesized using T7 RNA polymerase and biotin-21-UTP (Clontech) according to the method of Lehmann and Tautz (1994). Antisense RNA probes were prepared for the detection of lacZ using the pC4 β-galactosidase plasmid (Thummel et al., 1988), and synthesized using T7 RNA polymerase and the Boehringer Mannheim 'Genius' 4 Kit according to the method of Gavis and Lehmann (1992). Embryos were mounted as described above.

Fly Stocks

The following alleles were used for the complementation analyses described above, and all further phenotypic analyses: *abdA*^{mx1}, *AbdB* $^{D101.3}$ (both gifts from Welcome Bender), *cno*^{2}, Df(3R)crbS87-5, *Dl*^{9D}, *fkh*^{E200}, *ftz*^{7B}, *htl*^{AB42} (a gift from James Skeath), *hh*^{1J}, *hkb*^{2}, *kni*^{FC}, *opa*^{1IP}, *srp*^{9L}, *srw*^{1}, *tll*^{L10}, *tin*$^{\Delta GC14}$ (a gift from Manfred Frasch), *tld*^{9Q}, *trx*^{B11} (a gift from Jim Kennison). All alleles not designated above were obtained from either the Bloomington or Tübingen stock collections.

RESULTS

A screen for mutations affecting germ cell migration

In order to identify genes required for germ cell migration and gonad formation, we conducted a systematic screen of the third chromosome for EMS mutations that disrupt this process at any point during embryonic development. In theory, one could approach this task by assaying for gonad function through a screen for sterility of homozygous male and female animals. However, previous screens based on this assay have mainly identified genes involved in oogenesis, spermatogenesis, and embryonic pattern formation, and not those required for germ cell migration or gonad formation itself (Castrillon et al., 1993; Schupbach and Wieschaus, 1991; Broihier and Lehmann, unpublished results). One possible explanation for this result could be that mutations in genes required for germ cell migration may also be lethal, thereby rendering it impossible to isolate homozygous mutant adults. Moreover, it is known through experiments involving germ cell transplants that only a small number of germ cells is required to form a fertile gonad. Therefore, a mutation would have to prevent all germ cells from becoming incorporated into a gonad in order to result in sterility. In light of these arguments, we chose to directly screen mutant embryos for defects in gonad formation by labeling germ cells. We reasoned that this would allow the identification of genes required for germ cell migration that are involved in other processes necessary for viability of the fly. This direct visualization was made possible by the use of a *fat facets-lacZ* transgene (*faf-lacZ*; Fischer-Vize et al., 1992). The protein product of this transgene is maternally provided and localized to the posterior pole of embryos where it is incorporated into germ cells. β-galactosidase activity is maintained in these cells throughout embryonic development. In order to facilitate rapid screening of mutant lines, we utilized a "blue balancer" (*Ubx-lacZ* TM3) to distinguish homozygous mutant embryos from their siblings. The crossing scheme used to generate the single balanced mutant lines is shown in Fig. 2-2. For our target mutagenesis strain, we created an isogenic, multiply marked chromosome (including the *faf-lacZ* transgene) that showed a very low background loss of germ cells. Establishment of single balanced mutant stocks was facilitated by the use of a *hs-hid* transgene (Grether et al., 1995). Any fly carrying this transgene dies when subjected to heat shock during embryonic or larval development. In this way, only the desired genotype was able to survive in these crosses.

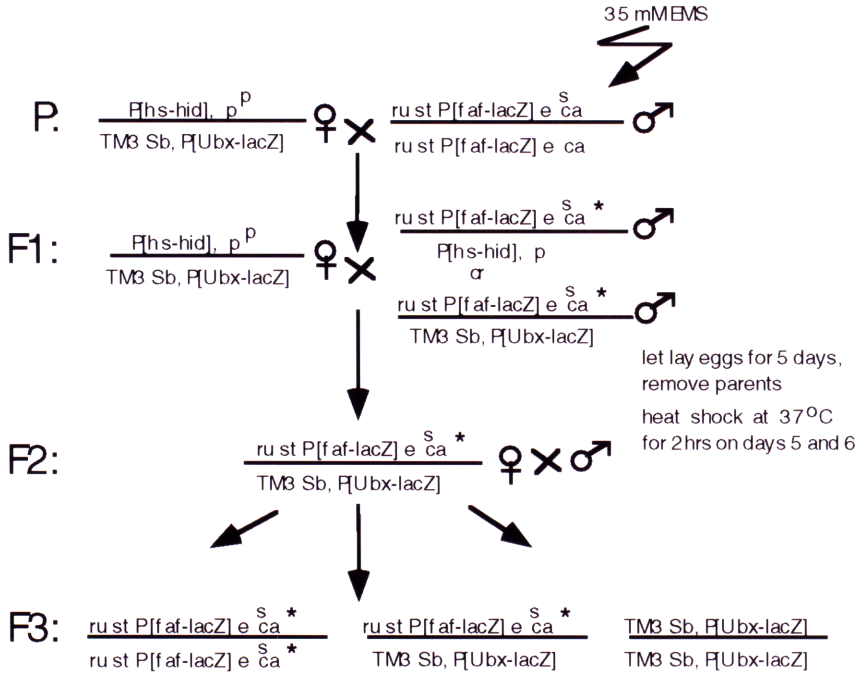
Embryos of 6-16 hours of age were collected from mutant lines, and stained for β-galactosidase activity. Thus, one staining procedure allowed us to visualize the germ cells as well as identify homozygous embryos by their absence of the characteristic striped staining pattern (an example is shown in Fig. 2-2). Any mutant line that produced embryos

lacking wild-type gonads, or that showed a significant number of germ cells outside the coalesced gonad was kept for further analysis.

Figure 2-2. Crossing scheme to establish lines isogenic for a mutagenized third chromosome

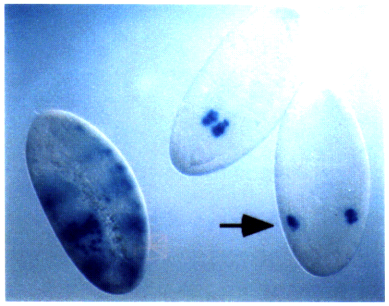
For an explanation of stocks used, see Materials and Methods). Markers as described in Lindsley and Zimm (1992). * designates mutagenized chromosome. Below the crosses is shown an example of embryos from a wild-type line containing the *faf-lacZ* chromosome over a "blue balancer," stained for β-galactosidase activity. Homozygous embryos are at stage 14, showing germ cells in coalesced gonads (arrow). "Blue balancer" embryo is at stage 11.

Fig. 2-2: Mutagenesis scheme for identification of genes affecting germ cell migration



collect embryos, stain with X-gal; test for lines with aberrant pole cell migration under stereomicroscope

* represents mutagenized chromosome



The results of our screen of the third chromosome are summarized in Table 2-1. We analyzed 8854 independent lines, 86% of which are homozygous lethal. Using the Poisson distribution, we calculate an average frequency of 1.9 lethal hits per chromosome, and therefore estimate to have screened a total of 17,000 lethal hits. We chose 327 lines to keep for further study, and subjected them to a secondary screen consisting of two procedures. In order to analyze the overall developmental state of mutant embryos as well as to inspect in more detail the germ cell migration defect, we immunolabeled embryos to highlight the germ cells using an anti-Vasa antibody (see Materials and Methods). In addition, we assayed for defects in embryonic patterning by preparing cuticles of unhatched larvae. All lines which failed to show a germ cell migration defect in this analysis were discarded.

Classification of mutant phenotypes

The results of the secondary screen enabled us to categorize the mutants into classes based on phenotypic similarity (Table 2-1).

Class 1: Mutations that most specifically affect germ cell migration and gonad formation. We found that 70 lines, or 21% of the mutants selected from the secondary screen, consist of mutants where overall embryonic morphology and patterning of the embryo appear relatively normal. However, many mutants in this class have subtle developmental defects in addition to those affecting germ cell migration (see below). Although the majority of these mutants show strong, highly penetrant germ cell migration defects, 15 mutants in this class show a relatively weak germ cell phenotype with variable penetrance. We have chosen not to study these mutants further given that they fail to fit into complementation groups (see Materials and Methods), and their phenotype overlaps with variability found in wild-type strains.

Class 2: Mutations affecting embryonic patterning. A significant proportion (34%) of our mutants show defects in embryonic patterning as well as in germ cell migration. This was an expected result, given that previous studies as well as our own analysis demonstrate that a majority of existing patterning mutants have defects in germ cell migration (Warrior, 1994; Broihier, Moore, and Lehmann, unpublished results).

Class 3: Dominant maternal/synthetic effects/multiple mutations. A small fraction (2%) of the selected mutants do not fit into the classes described above. Two of our mutant lines show dominant maternal dorso-ventral polarity effects, given that heterozygous females lay mutant eggs when outcrossed to wild-type males. These mutations are variable in penetrance, which allowed the stocks to survive in order to be analyzed in our screen. In four mutants, >25% of the total embryos laid have severe developmental defects, including

faulty patterning of the larval cuticle. These phenotypes could be explained as the result of multiple lesions on more than one chromosome.

Class 4: Lost stocks. 9% of the mutants kept did not survive long enough to be placed into the above categories. This includes stocks that either died or lost the balancer chromosome, and therefore, the original mutation(s).

Class 5: False positive. This class (34%) includes mutants that either could not be attributed to third chromosome lesions, or that failed to show a phenotype in the secondary screen.

Table 2-1. Screen for genes required for germ cell migration and gonad formation on chromosome 3.

(n) represents the number of mutant lines in each category. ([a]) A small percentage of our lines could not be placed into complementation groups. Two show dominant effects with variable penetrance. Four lines showed grossly abnormal cuticle phenotypes, and could not be categorized into a particular class of patterning mutants. We assume these are the result of multiple lesions on one or more chromosomes. ([b]) 28 lines were not included in the secondary screen either due to death of the stock, or loss of the balancer chromosome. ([c]) The phenotypes of many of our mutant lines do not segregate with embryos homozygous for the third chromosome, and therefore are probably the result of mutations on another chromosome. In addition, some of the mutants kept from the primary screen did not show a germ cell migration defect when subsequently analyzed using an anti-Vasa antibody.

Table 2-1: Screen for genes required for germ cell migration and gonad formation on chromosome 3

lines scored	8842
lines selected	327
lethal hits/chromosome	1.9

Phenotypic Classes	n	%of selected lines
specific effect on germ cells	70	21
pattern formation	110	34
dominant/ multiple [a]	6	2
lost [b]	28	9
false positive [c]	113	34

Complementation analysis suggests a high degree of saturation

In order to sort our mutant lines into complementation groups, we selected Class 1 mutants with similar phenotypes and crossed them to each other, scoring both for lethality and defects in germ cell migration (all of our selected mutant lines are homozygous lethal). For the Class 2 mutants, we crossed our alleles to previously identified mutations with similar cuticle defects. These complementation tests were also scored for lethality.

Seventy Class 1 mutant lines that displayed a strong, highly penetrant germ cell migration defect fall into 9 complementation groups (Table 2-2). Prior to our screen, it had been shown that the *abdA*, *AbdB*, and *tin* genes are required for gonad formation (Boyle et al., 1997; Cumberledge et al., 1992; Warrior, 1994). Complementation tests between our mutants with germ cell migration defects similar to those reported for *abdA*, *AbdB*, and *tin* mutants revealed that we isolated alleles of all loci, demonstrating our screen's success in identifying genes required for the process.

We also conducted complementation tests between Class 2 alleles and many of the mutants identified in previous screens for defects in pattern formation (Table 2-2; Jürgens et al., 1984). This analysis illustrates two important results from our screen. First, when comparing our data to previous studies analyzing pattern mutants and their effects on germ cell migration (Warrior, 1994; Broihier, Moore, and Lehmann, unpublished results), we find that our screen was successful in isolating alleles of all genes required for embryonic patterning that are also necessary for germ cell migration. Secondly, we obtained multiple alleles for the majority of loci identified by our screen (Table 2-2). When combining the results for the allele frequencies of genes in both the Class 1 and Class 2 mutants, we have isolated an average of 5.8 alleles per locus (see also Materials and Methods). This allele frequency is similar to that obtained in the saturation screens for defects in embryonic patterning (Jürgens et al., 1984). Given the results of our complementation analysis of both Class 1 and Class 2 mutants, we are confident to have thoroughly screened the third chromosome for zygotic mutations affecting germ cell migration and gonad formation. Although it is possible that genes required for overall embryonic patterning could also play a role in germ cell migration, we have chosen to focus the remainder of our phenotypic analysis on those complementation groups having relatively specific effects on germ cell migration and gonad formation.

Table 2-2. Complementation analysis of Class 1 and Class 2 mutants.

([a]) 6 of our lines fail to complement alleles of the previously identified *thread* (*th*) locus, which has been recently found to be required for cellularization of the early embryo (Eric Wieschaus, personal communication).

Table 2-2: Complementation Analysis

	#alleles
Class 1: specific germ cell migration defect	
abdominal A (abdA)	3
Abdominal B (AbdB)	3
columbus (clb)	15
heartless (htl)	4
fear-of-intimacy (foi)	3
tinman (tin)	1
trithorax (trx)	17
trithoraxgleich (trg)	4
zinc finger homeodomain-1 (zfh-1)	5
Class 2: pattern formation mutants	
gut development	
huckebein (hkb)	4
serpent (srp)	11
dorsal/ventral polarity	
shrew (srw)	8
tolloid (tld)	19
gap	
hunchback (hb)	4
forkhead (fkh)	2
knirps (kni)	5
tailless (tll)	3
pair-rule	
fushi-tarazu (ftz)	2
odd-paired (opa)	8
segment polarity	
hedgehog (hh)	7
neurogenic	
Delta (Dl)	4
Delta-like (Dl-l)	3
dorsal open	
canoe (cno)	1
cellular differentiation	
crumbs (crb)	2
cellularization	
thread (th)[a]	6

Placing genes on the chromosomal map

Rough mapping of 2 representative alleles from each of the 6 remaining complementation groups in Class 1 placed the genes between the intervals of either *ru* and *st* [*fear-of-intimacy* (*foi*)], *st* and *e* [*trithorax* (*trx*), *trithoraxgleich* (*trg*), and *heartless* (*htl*)], *e* and *ca* [*columbus* (*clb*)], or distal to ca [*zinc finger homeodomain protein-1* (*zfh-1*)]. Mutants were then crossed to deletions spanning their respective intervals, and again scored for lethality. Deletion analysis and further complementation tests revealed that 3 of our groups were allelic to the *htl*, *trx*, and *zfh-1* genes (see Materials and Methods). For the *clb*, *foi*, and *trg* loci, all deletion strains obtained from the Bloomington stock center deficiency kit complemented our alleles. The approximate meiotic map positions for these loci are as follows: *clb*, 3-80.0; *foi*, 3-25.2; *trg*, 3-55.1.

Phenotypic analysis of mutants reveals discrete steps in germ cell migration

Closer inspection of the germ cell migration defects in each of the mutant groups revealed that most could be categorized into discrete classes according to the earliest step of germ cell migration they disrupt (Fig. 2-3):

-Migration of germ cells through the PMG.

Previous work has shown that mutations in *srp* and *hkb* disrupt the ability of the germ cells to invade the gut wall and pass through to the interior of the embryo (Brönner et al., 1994; Jaglarz and Howard, 1995; Warrior, 1994). Our phenotypic analysis has demonstrated that they were the only mutants we identified that affect this particular step of migration (Fig. 2-3A). Given that we have thoroughly screened the third chromosome for defects in germ cell migration, it is likely that these are the only genes on this chromosome required zygotically for the migration of the germ cells through the PMG.

-Movement of germ cells from endoderm toward mesoderm.

Mutations in *clb, htl,* and *zfh-1* result in many germ cells remaining associated with the basal surface of the gut, instead of moving into lateral mesoderm (Fig. 2-3C). Those germ cells that do leave the PMG often appear disorganized within the mesoderm, and do not correctly navigate toward SGPs (see Broihier et al., 1998). *zfh-1* mutants have an additional defect in that those germ cells that do detach from the gut will often continue to migrate past lateral mesoderm and into the ectoderm (for a detailed description, see Broihier et al., 1998). It is interesting to note that although the majority of germ cells do not migrate correctly in *clb*, *htl,* and *zfh-1* mutants, there is always a small number of germ cells in each mutant that are able to associate correctly with SGPs (see Discussion).

We have begun an analysis of the cause of these defects by assessing the development of the gonadal mesoderm using specific markers. One of these markers, the

412 retrotransposon, specifically recognizes SGPs after the germ band has retracted (Brookman et al., 1992). *zfh-1* mutants show a drastic reduction in the number of cells expressing 412 (Fig. 2-4B). Combined with the severe germ cell migration defect seen in *zfh-1* mutants, these data suggest a pivotal role for this gene in the development of the gonadal mesoderm. Mutations in the *htl* gene also reduce the number of gonadal mesoderm cells found in stage 14 embryos, but not to the same degree as that found in *zfh-1* mutants. Moreover, gonadal mesoderm cells in *htl* mutants are irregularly shaped, suggesting an additional defect in gonadal mesoderm differentiation (Fig. 2-4C). In contrast, 412 expression appears normal in *clb* mutant embryos, implying that this gene is not required for the specification of SGPs (M. Van Doren and R. Lehmann, unpublished results). Results consistent with those described above are seen using a variety of markers, including anti-Cli, Dwnt-2 RNA, and anti-Zfh-1, which recognize gonadal mesoderm at various points in development.

-Maintenance of association with gonadal mesoderm.

Previous work has shown that the homeotic genes *abdA* and *AbdB* are required for gonad assembly (Boyle and DiNardo, 1995; Cumberledge et al., 1992; Warrior, 1994). Comparing these mutant phenotypes with those of other genes identified in our screen allows us to place the requirement for *abdA* and *AbdB* at a discrete point during germ cell migration.

Our phenotypic analysis demonstrates that mutants lacking *abdA* function show an earlier germ cell migration defect than had been seen in previous studies (Fig. 2-3E). For the present analysis, we have used the $abdA^{mx1}$ allele (see Materials and Methods), which is a translocation breaking within the coding region, and fails to express a protein that is detectable by existing anti-AbdA antibodies (Karch et al., 1990). Earlier studies focused on a mutation in the *abdA* regulatory region, *iab4*, that affects *abdA* function in a subset of abdominal segments, and perhaps, in a subset of tissues (Cumberledge et al., 1992; Warrior, 1994). In mutants lacking most or all *abdA* function, germ cells are able to move through the PMG and initially find lateral mesoderm. However, germ cells fail to maintain their specific association with the mesoderm, and disperse in the posterior of the embryo. Earlier work has shown that *abdA* is required in the soma for gonad formation (Boyle and DiNardo, 1995; Cumberledge et al., 1992). This defect appears to be the result of a failure of gonadal mesoderm development, since the expression of 412 is severely reduced in these mutants in a manner similar to that seen in embryos lacking the Bithorax-Complex (Brookman et al., 1992).

Whereas *abdA* is required for the development of all gonadal mesoderm cells, mutations in *AbdB* appear to only affect the posterior component of these cells. In these

mutants, many germ cells are able to coalesce along with gonadal mesoderm to form a gonad; however, some germ cells are excluded from this gonad, presumably due to the reduction in number of SGPs (Boyle and DiNardo, 1995; Brookman et al., 1992; our observations). We also identified mutations in a regulator of homeotic gene expression, *trx*, that has a germ cell migration phenotype very similar to that seen in *AbdB* mutants (Fig. 2-3G). The "lost" germ cells in *trx* and *AbdB* mutants remain in an area ventral and posterior to the gonad until after coalescence. The *trx* gene is known to be required for maintaining the expression of homeotic genes including *abdA* and *AbdB* (Breen and Harte, 1993; Mazo et al., 1990). Several lines of evidence suggest that the defect seen in *trx* mutants is due to reduced function of *AbdB*, including the result that a *hs-AbdB* construct can partially rescue the *trx*⁻ germ cell migration defect (see Chapter 3). Surprisingly, initial results from our analysis of gonadal mesoderm development appeared to be inconsistent with this theory. In embryos lacking zygotic *trx*, 412 appears to be expressed at normal levels (see Chapter 3), whereas in *AbdB* mutants, fewer SGPs express high levels of 412 than the number seen in wild type (Brookman et al., 1992). However, embryos which lack both maternal and zygotic *trx* show 412 expression levels identical to those seen in *AbdB* mutants (see Chapter 3). These results suggest that *trx*, like *AbdB*, is required for a subset of SGPs to maintain their identity and as a result, to maintain their association with germ cells (see Chapter 3; Boyle and DiNardo, 1995).

In addition, we have identified another complementation group, *trg,* which has a germ cell migration defect identical to that seen in *AbdB* and *trx* mutants. Mutations in *trg* show genetic interactions with homeotic genes including *Ultrabithorax (Ubx)*, *abdA*, and *AbdB*. Flies that are transheterozygous for *trg* and any of the aforementioned homeotic genes are only semi-viable and often show thoracic abnormalities, suggesting that *trg* is a new member of the trx-group of genes (Moore and Lehmann, unpublished results).

Mutations in the *tin* locus have a unique effect on germ cell migration. Germ cells are able to migrate through the gut epithelium to find their target mesodermal cells, and remain associated with SGPs throughout germ band retraction. The germ cells attempt to line up, but do not achieve the organized nature they attain in wild-type embryos (Fig. 2-3I). The alignment of germ cells continues to deteriorate as development ensues, resulting in the dispersion of germ cells at stage 14. It has been shown that *tin* is required for proper development of gonadal mesoderm (Boyle et al., 1997). We have found that expression of 412 is virtually abolished in embryos lacking *tin* function (Fig. 2-4D). This result is consistent with previous studies demonstrating expression of another SGP marker, *cli*, is drastically reduced in *tin* mutants (Boyle et al., 1997). It is unclear why *tin* mutants show

such a relatively late germ cell migration defect, given *tin's* striking effect on expression of gonadal mesoderm markers (Boyle et al., 1997; Broihier et al., 1998; see DISCUSSION).

-Gonad coalescence

Mutations in a novel gene, *foi*, specifically affect the ability of the germ cells and gonadal mesoderm to coalesce into the embryonic gonad. The hallmark of this phenotype is the appearance of very late stage embryos with germ cells and SGPs remaining in a line, instead of the characteristic round shape normally found in gonads by stage 14 (Fig. 2-3K). Once again, the fault appears to lie with gonadal mesoderm as highlighted by 412 expression (Fig. 2-4E). Although 412 is expressed in an apparently normal number of cells, their morphology and shape is aberrant in a way very similar to that found in *htl* mutants (compare Fig. 2-4C with Fig. 2-4E). In wild-type embryonic gonads, gonadal mesodermal cells are tightly associated with one another and with the encapsulated germ cells. This is in sharp contrast to that seen in *foi* mutants, where the SGPs appear as if they are incapable of making close contacts with one another.

Figure 2-3. Genes required for germ cell migration act during discrete steps in development.

Anterior left in all panels. (A-L) Germ cells visualized using an anti-Vasa antibody. (A-H) lateral views; (I-L) dorsal views. (A,C,E,G,I,K) Mutant embryos displaying their characteristic phenotypes. (B,D,F,H,J,L) Wild-type embryos of comparative stages. (A) *srp*$^-$ (stage 12). Many germ cells fail to exit the PMG, due to its transformation into a more hindgut-like structure (arrowhead). (C) *clb*$^-$ (stage 11). A subset of the germ cells associates with lateral mesoderm (arrow), but many remain behind on the basal surface of the PMG (arrowhead). (E) *abdA*$^-$ (stage 13). Germ cells fail to remain associated with mesodermal cells (arrowhead). (G) *trx*$^-$ (stage 13). A subset of the germ cells (arrowhead) is found ventral and posterior to the gonad. (I) *tin*$^-$ (stage 14). Germ cells lose their attachment to mesodermal cells once the germ band has retracted (arrowheads). (K) *foi*$^-$ (stage 15). Germ cells fail to coalesce into the embryonic gonad (arrowhead), but remain aligned with SGPs.

Fig. 2-3: Genes required for germ cell migration act during discrete stages in development

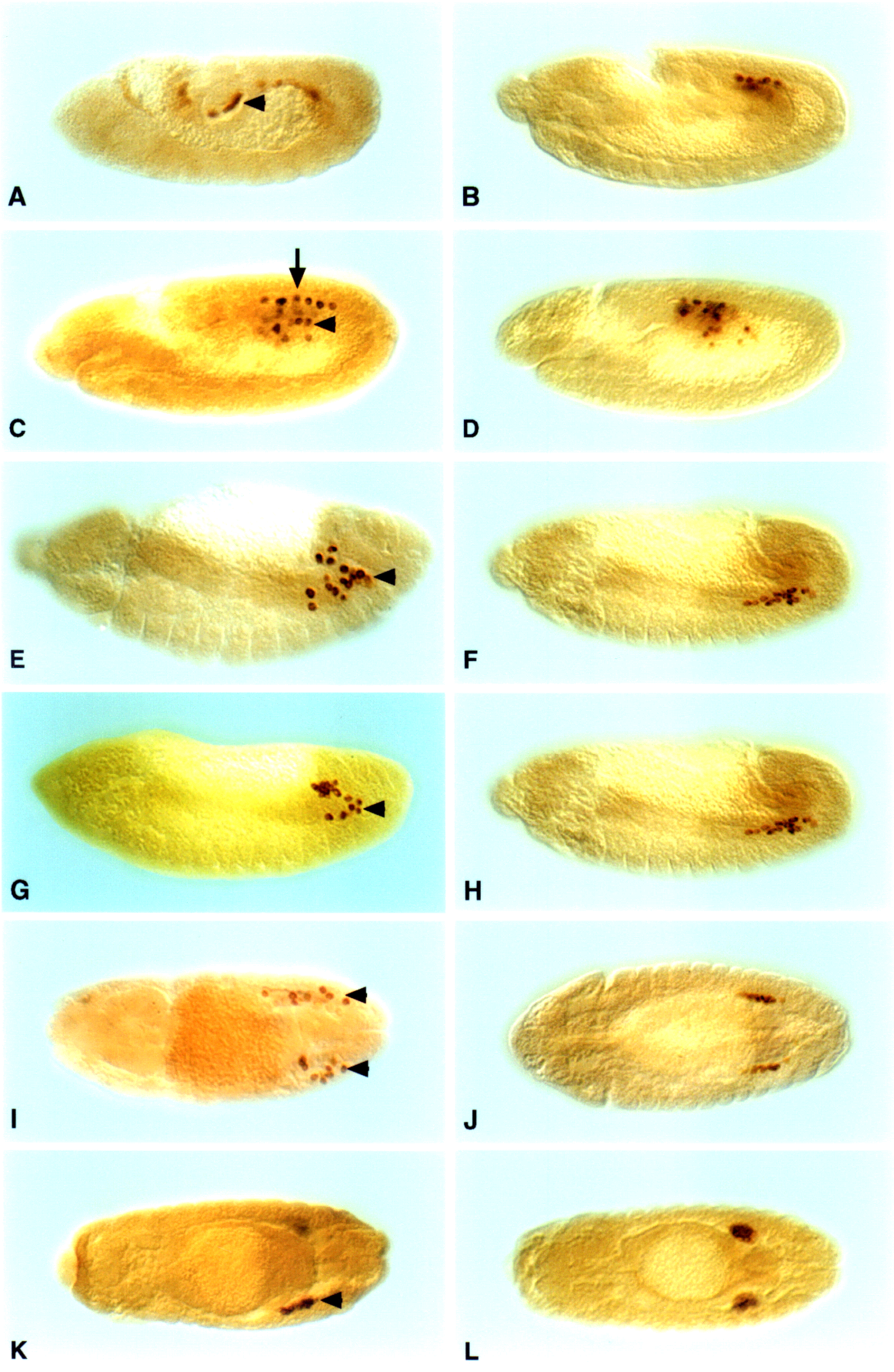
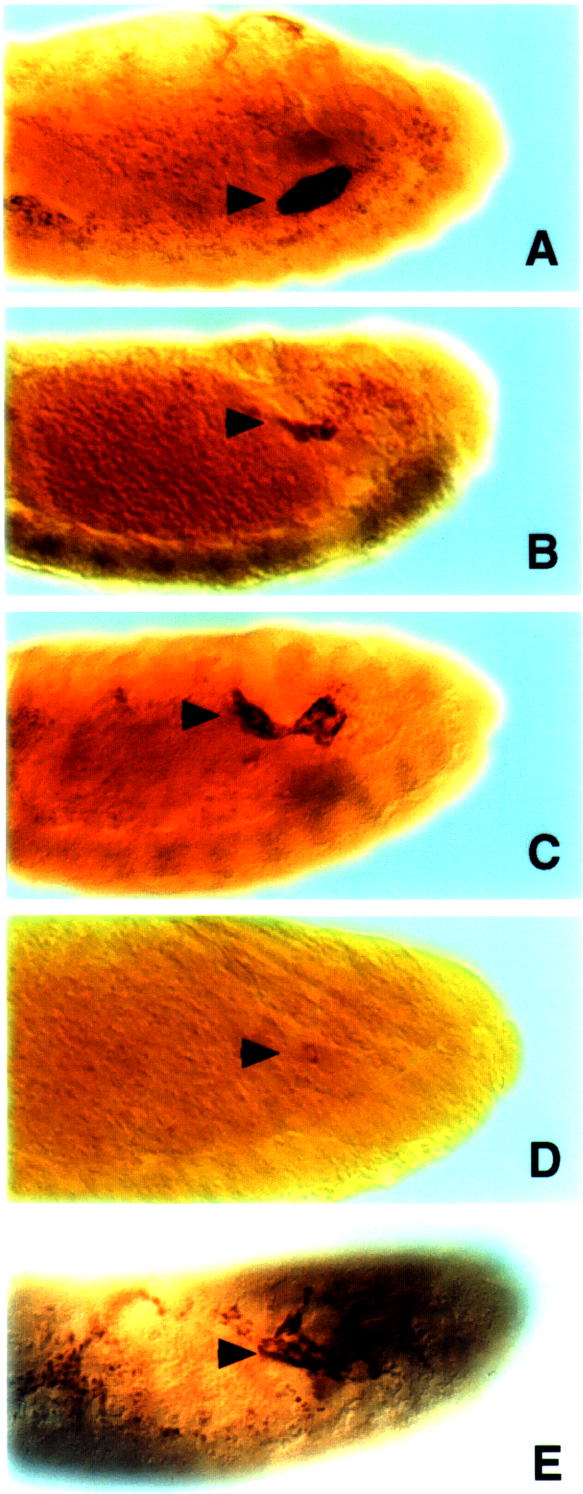


Figure 2-4. Mutations affecting gonadal mesoderm development.

Anterior left in all panels; lateral views. (A-E) Gonadal mesoderm development assayed by expression of the 412 retrotransposon (arrowheads). All embryos are at approximately stage 14 (Campos-Ortega and Hartenstein (1985)]. (A) Wild type; (B-E) Mutants. (B) *zfh-1*$^{-}$. The number of gonadal mesoderm cells is drastically reduced compared to wild type. (C) *htl*$^{-}$. Both the number and morphology of gonadal mesoderm cells is affected. However, more cells are present than in *zfh-1* mutants (compare with panel B). (D) *tin*$^{-}$. Gonadal mesoderm cells are virtually abolished by this stage in development. (E) *foi*$^{-}$. SGPs show aberrant morphology. Finger-like protrusions are seen, and they fail to show the tight cell-cell interactions characteristic of a coalesced gonad. However, SGP number appears normal. This embryo has been stained longer than the embryos in A-D, revealing low levels of 412 expression in the fat body.

Fig. 2-4: Mutations affecting gonadal mesoderm development



The segmental origin of gonadal mesoderm is within the *eve* domain

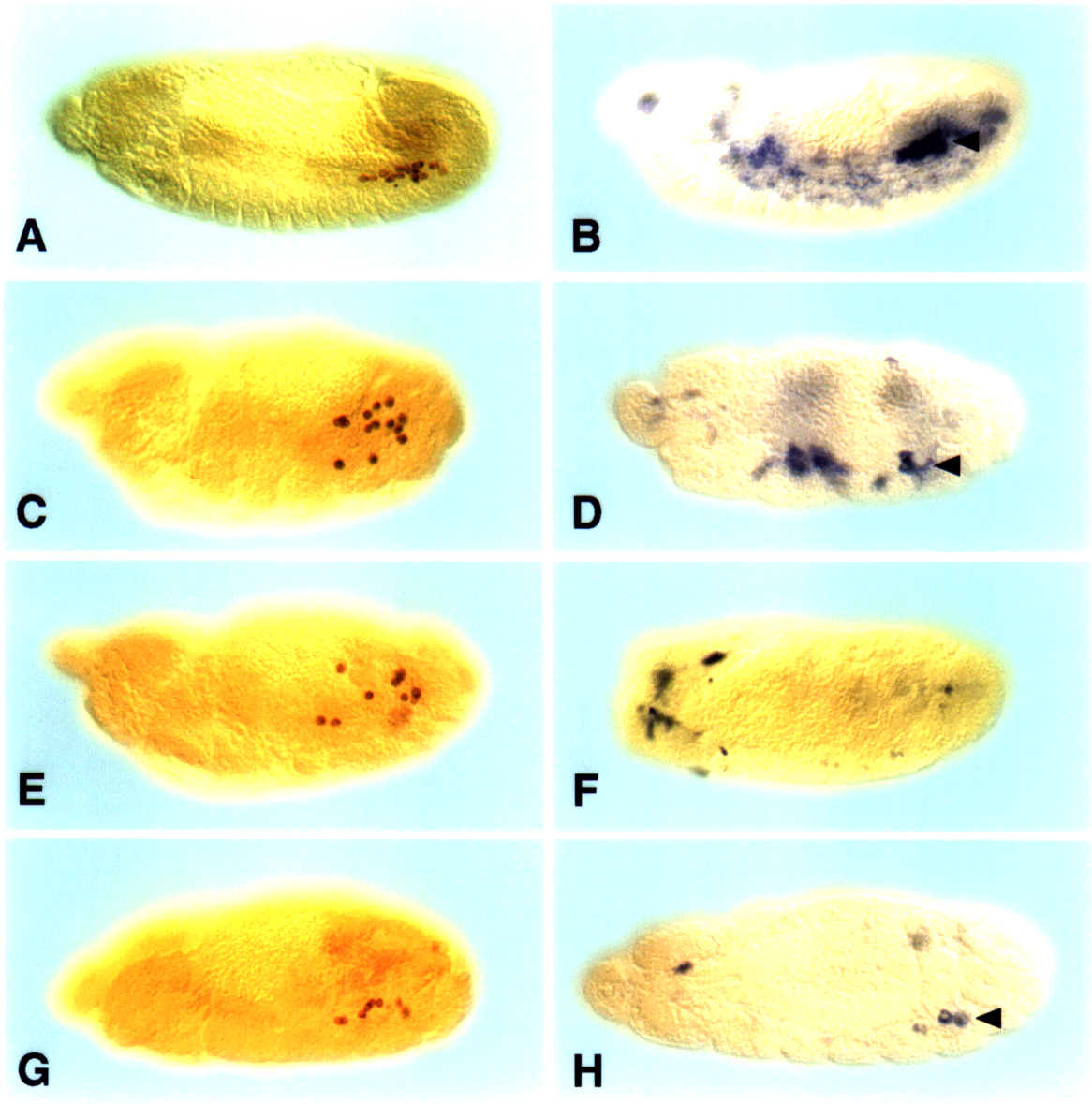
Recent work has analyzed the role of pair-rule and segment polarity genes in the specification of certain mesodermal cell types (Azpiazu et al., 1996). Of those genes located on the third chromosome, these studies found that *fushi-tarazu (ftz), odd-paired (opa),* and *hedgehog (hh)* are required for development of the midgut visceral mesoderm and fat body. Moreover, these results place the origin of the midgut visceral mesoderm and fat body within the "*eve* domain" of each parasegment. The results from our screen demonstrate that genes required for the development of these tissues are also required for germ cell migration (Table 2-2). We therefore reasoned that this requirement may be attributable to the function of *ftz, hh* and *opa* in the development of the gonadal mesoderm. Mutations in *ftz, opa,* and *hh* all result in embryos showing significant reductions in the number of cells expressing 412 (Fig. 2-5). Thus, the germ cell migration defect in these mutants is most likely due to their effect on gonadal mesoderm development.

It is interesting to note that, while we identified alleles of *ftz, opa,* and *hh* in our screen, we did not identify alleles of *hairy (h),* another pair-rule gene on the third chromosome. This result is consistent with the fact that loss of *h* function does not result in a failure of midgut visceral mesoderm development (Azpiazu et al., 1996). In fact, we find that the gonadal mesoderm appears to develop correctly in *h* mutants. These results suggest that the origins of the gonadal mesoderm, like midgut visceral mesoderm and fat body, lie within the *eve* domain of the mesoderm.

Figure 2-5. Pair-rule and segmentation genes affecting germ cell migration and gonadal mesoderm development.

Anterior left in all panels; lateral views. Embryos are at approximately stage 13-14. (A,C,E,G) Germ cells visualized using an anti-Vasa antibody; (B,D,F,H) gonadal mesoderm development assayed by expression of the 412 retrotransposon (arrowheads). (A,B) Wild type; (C,D) *ftz*$^{-}$; (E,F) *opa*$^{-}$; (G,H) *hh*$^{-}$. (C,E,G) Mutations in *ftz*, *opa*, and *hh* all result in the failure of germ cells to associate with mesodermal cells; (D,F,H) The number of gonadal mesoderm cells is severely reduced in all mutants shown.

Fig. 2-5: Segmentation genes required for gonadal mesoderm development



DISCUSSION

A comprehensive screen of the third chromosome

We have conducted an exhaustive screen of the third chromosome to identify genes required for germ cell migration and gonad formation in the Drosophila embryo. This screen was made possible by the use of a set of tools that allowed us to establish close to 9000 independent mutagenized lines, and screen them directly by utilizing a histological marker for germ cells and balancer-bearing embryos. We isolated 186 mutant lines with a strong germ cell migration defect, and have categorized them according to their phenotypes. Based on our isolation of multiple alleles for most loci, combined with the fact that we identified mutations in all loci on the third chromosome previously known to be required for gonad formation, we are confident to have come close to saturation in this screen. Thus, the genes and phenotypes we identified represent nearly all zygotic factors affecting germ cell migration and gonad formation on the third chromosome.

Before undertaking this screen, we predicted that most mutants affecting germ cell migration would be lethal. This was not an obvious assumption, since mutants lacking fertile gonads are perfectly viable (Lehmann and Nüsslein-Volhard, 1986). However, previous studies screening for adult sterility failed to isolate mutations causing aberrant migration of embryonic germ cells (Castrillon et al., 1993; Schüpbach and Wieschaus, 1991). Indeed, our assumption proved correct; all mutants that showed a strong, highly penetrant germ cell migration defect are also lethal. Although studies to determine the cause of lethality for some of these mutants are still underway, one simple explanation is that the mutations are pleiotropic. If this is the case, then one can argue that most genes required zygotically for the proper migration of germ cells are also necessary for other developmental processes in the embryo. We have already found that many of these genes are required for the development of a number of different cell types during embryogenesis (see below).

An additional problem with assaying for sterility is that it was unclear if mutations affecting the migration of germ cells would necessarily result in sterile adults, since the results of pole cell transplantation experiments demonstrate that only a small number of germ cells is sufficient for gonad function. In order to prevent this inherent bias, we chose to screen embryos directly, and kept any mutants which showed even the most subtle defects in gonad formation. Interestingly, many of our newly identified mutants do not abolish the ability of some germ cells to associate with SGPs, but nevertheless exert severe effects on the process as a whole. Moreover, we have identified additional genes required

for both patterning of the embryo and gonad formation that were missed in earlier studies due to our more stringent screening assay (Table 2-2).

Identification of genes required for discrete steps during the migration of germ cells

The results of our phenotypic analyses of mutants identified in this screen show that the process of gonad assembly can be broken down into discrete steps (Fig. 2-6): 1) Migration of germ cells through the PMG, 2) Migration away from the PMG and into lateral mesoderm, 3) Alignment and maintenance of germ cell association with somatic gonadal precursors (SGPs), and 4) Gonad coalescence.

Although detailed studies have analyzed the process of migration through the PMG (Callaini et al., 1995; Jaglarz and Howard, 1995), little was known before this screen about the ability of the germ cells to detach from the endoderm and move into the mesodermal layer. Previous work had shown that mesoderm was required for this process, given that germ cells failed to move away from the endoderm in mutants lacking *twist (twi)* and *snail (sna)* activity (Warrior, 1994). However, since *twi* and *sna* are required for the development of all mesoderm, it was unclear what, if any, more specific mesodermal factors played a role in this step. We have found that *clb*, *htl* and *zfh-1* all are necessary in directing the germ cells away from the endoderm and into the mesodermal region. Moreover, these genes appear to function within the developing mesoderm (see below). Since some mesodermal cell types do develop in embryos lacking *clb*, *htl*, and *zfh-1* function (Lai et al., 1993; Beiman et al., 1996; Gisselbrecht et al., 1996; M. Van Doren and R. Lehmann, unpublished), their phenotypes suggest a role for these genes beyond general mesoderm formation. Furthermore, germ cells in these mutants do not find the correct mesodermal target cells in PS 10-12, and some continue to migrate into other parasegments, as well as other tissues. This result suggests that in wild-type embryos, at the time the germ cells migrate away from the PMG, the mesoderm to which they adhere has become somewhat specialized, requiring the function of *clb*, *htl*, and *zfh-1*. Experiments are underway to determine how the genes identified in our screen function in this regional specialization (see Broihier et. al., 1998).

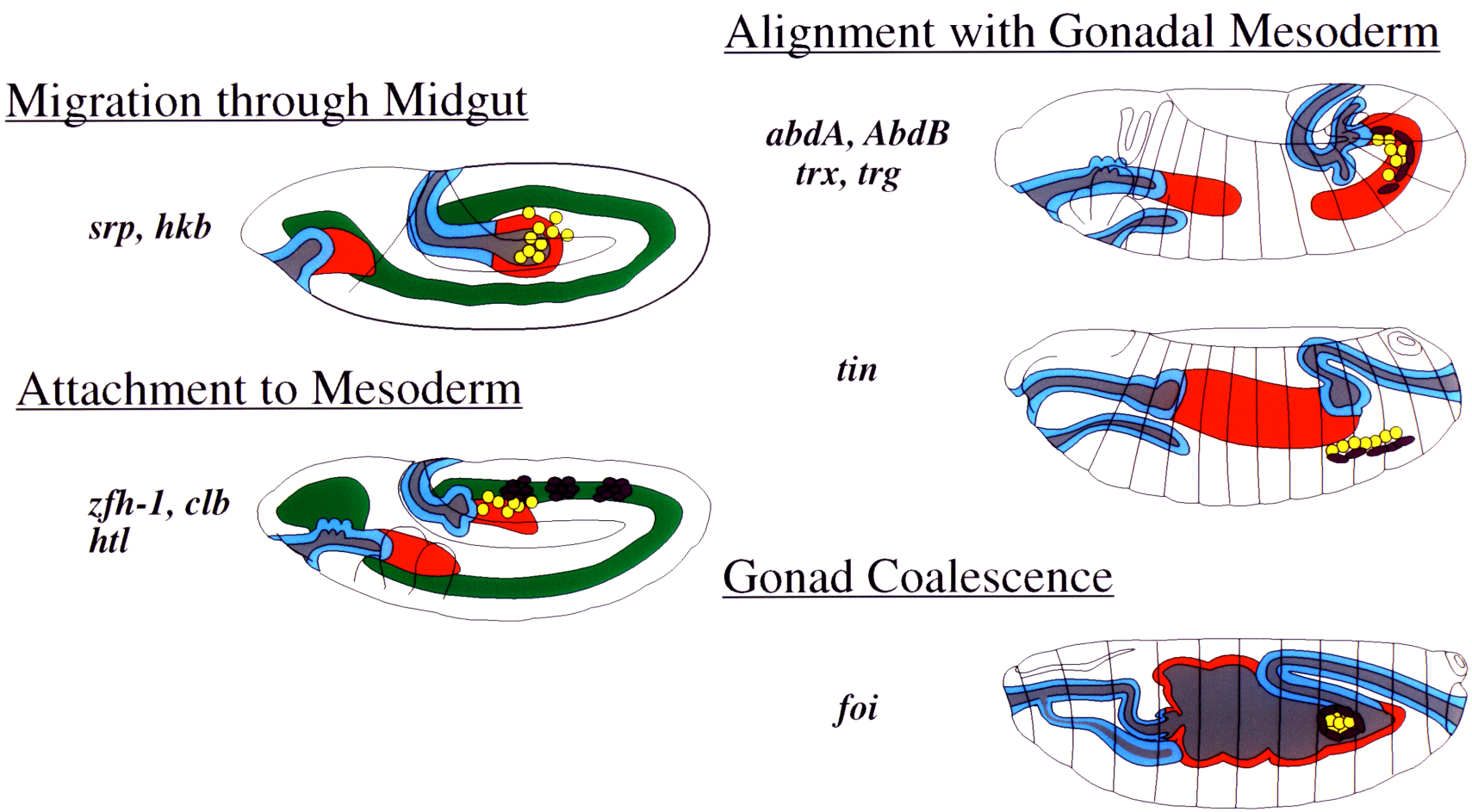
The majority of mutants that had been analyzed previous to our screen display a phenotypic onset during the alignment of germ cells with SGPs (Boyle and DiNardo, 1995; Cumberledge et al., 1992). We identified mutations in a gene, *foi*, that is required at an even later stage in embryogenesis: gonad coalescence. This gene provides a missing link between the tight association of germ cells with their somatic partners, and their cooperative movement into the spherical structure of the gonad. Given the nature of the defect within gonadal mesoderm, *foi* provides our most promising candidate for an

adhesive factor involved in preferential cell-cell interactions between the gonadal mesoderm cells themselves. This theory is especially tantalizing, given that *foi* has an additional requirement in late embryonic tracheal branch fusion, a process requiring cell-cell interactions (Van Doren and Lehmann, unpublished results).

Figure 2-6. Genetic summary of germ cell migration: third chromosome.

Embryo drawings after V. Hartenstein. Blue: foregut and hindgut; red: anterior and posterior midgut; gray: gut lumen; green: mesoderm; purple, SGPs; yellow: germ cells. Phenotypic analysis of mutants identified in our screen shows that germ cell migration in Drosophila can be broken down into discrete developmental steps. Genes identified in our screen are shown beside the first embryonic stage at which germ cell migration is disrupted in corresponding mutants.

Fig. 2-6: Genetics of germ cell migration: third chromosome



Mutations affecting gonadal mesoderm development

Many of the genes identified in our screen appear to be required for germ cell migration via their role in the development of the somatic tissue involved in gonadogenesis. Previous studies have analyzed the specification of the SGPs, and have also found *abdA*, *AbdB*, *cli*, and *tin* necessary for the development of these cells (Boyle et al., 1997; Boyle and DiNardo, 1995). Our results suggest that initiation of the developmental pathway toward the specification of SGPs occurs at an earlier step than previously identified. *zfh-1* and *htl* both are required for the development of gonadal mesoderm, but exert their effect on the interaction with germ cells at an earlier stage than that seen for *abdA, AbdB, cli,* and *tin.*

We and others have found the *tin* gene to be required for development of gonadal mesoderm, as exemplified by the lack of expression of gonadal mesoderm specific markers in *tin* mutants (Boyle et al., 1997; Fig. 2-4D). Although the drastically reduced expression of *cli* in *tin* mutants can be seen as early as stage 11 (Boyle et al., 1997), the resulting germ cell migration defect cannot be detected until stage 13 (Fig. 2-3I). More recent work has shown that most SGPs are at least partially specified in *tin* mutants, but fail to maintain this specification during later developmental stages (Broihier et al., 1998). This may explain how germ cells are initially able to associate with SGPs, but lose this association as SGPs fail to maintain their identity.

It has been recently shown that the *htl* gene, which encodes a Drosophila fibroblast growth factor receptor (DFR1/DFGF-R2), is involved in the dorsolateral migration of the invaginating mesodermal layer along the overlying ectoderm. Loss of function mutations in this locus affect the development of a number of dorsal mesodermal cell types, including midgut visceral mesoderm, cardiac mesoderm, and some somatic mesodermal derivatives. These studies further indicate that the number of precursors corresponding to the affected mesodermal cell types is significantly reduced in *htl* mutant embryos (Beiman et al., 1996; Gisselbrecht et al., 1996). Our phenotypic analysis of both germ cell migration and gonadal mesoderm defects in *htl* embryos demonstrates that this gene is required at an early stage in the development of yet another mesodermal cell type, the gonadal mesoderm. *htl* is also necessary for the Dpp-dependent maintenance of *tin* expression in dorsal regions of the mesoderm (Gisselbrecht et al., 1996). Therefore, *htl* could be acting through *tin* to specify SGPs, since *tin* is required for gonadal mesoderm development (see above). Conversely, *htl* could be required for a signaling process that is independent of its role in maintaining *tin*'s dorsal expression pattern. Further experiments are necessary to distinguish between these two possibilities, but the finding that *htl* has an additional requirement in gonadal mesoderm morphological differentiation suggests that the latter theory could prove correct.

Moreover, recent studies demonstrate that *tin*'s role in gonadal mesoderm development is independent of Dpp signaling (Broihier et al., 1998).

We have also identified genes involved in the differentiation of gonadal mesoderm in addition to those required for its initial specification. Given that gonadal mesoderm morphology, but not cell number, is affected in *foi* mutants, it likely represents a downstream target of genes such as *zfh-1* and *tin*. Molecular characterization of *foi* will allow a better understanding of its role in the differentiation of gonadal mesoderm.

Origin of the gonadal mesoderm

We have found that the segmentation genes *ftz*, *hh*, and *opa* are all required for germ cell migration and gonadal mesoderm development. Furthermore, in a preliminary screen to identify patterning genes required for germ cell migration, we have found that mutations in *even-skipped (eve)* and *engrailed* (*en*) also have a drastic effect on the development of gonadal mesoderm (Broihier, Moore, and Lehmann, unpublished results). These segmentation genes identified in our screens have been previously shown to play a role in the patterning of a component of mesoderm which gives rise to midgut visceral mesoderm and fat body (Azpiazu et al., 1996), termed the "*eve*-domain". Taken together, these results show that genes required for patterning of the mesoderm affect gonadal mesoderm in the same way in which they affect midgut visceral mesoderm and fat body.

Recent studies suggest that each parasegment of the mesoderm is initially subdivided into two domains. *hh* and *en* are positively regulated by pair-rule gene action in the *eve*-domain of the mesoderm, whereas *wg* is a target in the "*slp* domain" (Azpiazu et al., 1996; Riechmann et al., 1997). Our observations of the loss of gonadal mesoderm in *hh* and *en* mutants support the model that SGP origin lies within the *eve* domain of the mesoderm (Fig. 2-5). This conclusion is further supported by the observation that more gonadal mesoderm cells form in *slp* mutants (Broihier, Moore, and Lehmann, unpublished results). Because *wg* is positively regulated by *slp,* this model conflicts with the finding that loss of *wg* function causes a reduction in the number of SGPs, while ectopic expression of *wg* leads to an increase in the number of SGPs (Boyle et al., 1997; Broihier, Moore, and Lehmann, unpublished results). We therefore propose that the effects of loss and gain of Wg activity reflect a function for this gene which occurs at a later time than initial mesodermal A-P patterning. Indeed, the model described above concerning mesodermal *slp* and *eve* domains proposes that the segmentation genes, including *hh* and *wg*, have an additional requirement beyond A-P specification of the mesoderm involving the resolution of sharp borders between the *slp* and *eve* domains. Moreover, *hh* and *wg* show numerous regulatory interactions between one another (Hidalgo, 1991; Ingham and

Hidalgo, 1993; Lee et al., 1992), therefore implying that *wg* may function indirectly in the development of gonadal mesoderm. Further studies are required to determine whether the roles described above, or other as yet uncharacterized functions of these segmentation genes, are involved in gonadal mesoderm development.

Germ cell specific genes?

Current evidence suggests that genes required zygotically for germ cell migration act in the soma rather than in the germ cells. It has been previously shown that *abdA* is required in the soma for gonad assembly (Cumberledge et al., 1992). The phenotypes of both *srp* and *hkb* mutants, in which the germ cells are unable to migrate through the PMG, can most likely be attributed to the genes' requirements for the development of the PMG (Brönner et al., 1994; Jaglarz and Howard, 1995; Reuter, 1994). Moreover, with the exception of *clb*, all of our remaining Class 1 genes are required for the development of gonadal mesoderm, which can presumably explain their roles in germ cell migration. Although *clb* is not required for SGP specification, recent studies have found it to be expressed in gonadal mesoderm, but not in the germ cells, suggesting that it too acts in the soma (Van Doren and Lehmann, unpublished results). Given that we have thoroughly screened the third chromosome for genes required zygotically for germ cell migration, it is curious that we have no compelling candidates for genes that function in the germ cells for the many processes they must execute to form a coalesced gonad. Presumably, there are factors expressed in the germ cells that allow them to move through tissue layers and guide them to recognize their target mesodermal cells. It is possible that these factors may be maternally provided to the embryo, and thus could not be identified in a zygotic screen. Indeed, two molecules known to act in the germ cells for proper gonad formation, *nanos* (*nos*), and *Polar granule component-1* (*Pgc-1*), are both contributed by the mother to the oocyte (Kobayashi et al., 1996; Nakamura et al., 1996; Forbes and Lehmann, 1998). Thus, maternal-effect screens may be key in identifying the missing germ-line cues which act in concert with genes we have identified that are essential for germ cell migration, gonadal mesoderm development, and gonad coalescence.

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CHAPTER 3

Phenotypic analyses of three mutants affecting gonad formation in Drosophila

SUMMARY

The combined results from screens of the second and third chromosomes reveal that germ cell migration in Drosophila occurs in discrete developmental steps with each step requiring a specific subset of genes. Phenotypic analyses of three mutants that affect different steps of this process are presented. First, I describe the role of the *trx* gene in germ cell migration and show that *trx* most likely acts to ensure proper function of *AbdB*, but not *abdA*, in this process. Futhermore, germ-line clonal analysis demonstrates that *trx* is required throughout embryogenesis to maintain gonad integrity. A screen of the second chromosome revealed two mutants, 9.35 and 22.38, which affect different steps in gonad formation. In 9.35 mutants, germ cells fail to migrate properly away from the midgut and into lateral mesoderm. The 22.38 lesion affects a later step involving germ cell-somatic gonadal precursor associations. Both mutants appear to specifically affect germ cell migration, given that many other embryonic developmental processes are normal. Preliminary mapping experiments place them within broad regions of chromsome 2L.

INTRODUCTION

Our analysis of genes on the third chromosome required for germ cell migration in Drosophila led to the model that this developmental process can be broken down into discrete genetic steps, with each step requiring a different subset of genes (see Chapter 2; Moore et al., 1998). In addition to conducting a saturation mutagenesis of the third chromosome for genes required for germ cell migration, Heather Broihier and I also carried out a screen for mutations affecting this same process on the second chromosome. The screen methodology was very similar to that used in the third chromosome screen, with the exception that a dominant temperature-sensitive (DTS) mutation was used to establish balanced lines instead of the *hs-hid* construct. We screened 3194 mutant lines for defects in germ cell migration, and identified 7 complementation groups that had strong, highly penetrant, and relatively specific effects on this process. We also identified 8 genes that were previously known to be required for pattern formation in the embryo, and determined that they were also necessary for germ cell migration (Table 3-1). Although this screen of the second chromosome was not carried to saturation, our results from this analysis are consistent with those described in the screen of the third chromosome (see Chapter 2; Moore et al., 1998). For example, we found that the developmental steps required for germ cells to properly migrate and form a gonad that we disrupted through mutations on the second chromosome were the same as those characterized in the third chromosome screen. Moreover, those segmentation genes on the second chromosome required for patterning of the *eve*-domain of the mesoderm were identified in our screen. We have since determined that these genes in fact affect the development of the gonadal mesoderm (see Chapter 2; Moore et al., 1998).

Given that the combined results of both screens identified similar steps in germ cell migration that can be genetically disrupted, I chose to conduct further studies aimed at elucidating the mechanisms underlying some of these particular steps. I report here phenotypic analyses of three mutants identified through our screens that affect various steps of germ cell migration: one was recovered from the third chromosome mutagenesis, and two were isolated during the second chromosome screen. Two mutants interfere with germ cell-SGP interactions, while the other disrupts the migration of germ cells away from the PMG and into lateral mesoderm.

Of the complementation groups on the third chromosome that we identified to play a role in gonad formation, I conducted further analyses on two that showed nearly identical phenotypes affecting the association of germ cells with SGPs. Mapping and complementation analysis revealed that one group was allelic to the *trithorax (trx)* gene,

while the other group has not been ascribed to a previously identifed locus (see Chapter 2; Moore et al., 1998). Although much is known regarding the requirements for *trx* in the regulation of homeotic gene expression (see Introduction), a specific role for *trx* in the process of germ cell migration had not been previously described. I therefore chose to pursue studies of this gene focusing on its function in formation of the embryonic gonad.

Mutations in the *trx* gene were initially identified as a result of their phenotypic similarities to loss-of-function mutations in a number of genes in the Antennapedia and bithorax complexes including *Antennapedia, Sex combs reduced, Ultrabithorax, abdominal A (abdA),* and *Abdominal B (AbdB)* (Capdevila and Garcia-Bellido, 1981; Ingham and Whittle, 1980; Lewis, 1968). Further genetic studies combined with molecular analyses revealed that these phenotypes were due to a requirement for the *trx* gene in positively maintaining the expression of the homeotic genes (Breen and Harte, 1991; Breen and Harte, 1993; Capdevila and Garcia-Bellido, 1981; Duncan and Lewis, 1982). Consistent with these observations, *trx* encodes a large protein containing regions of homology with zinc finger-like DNA-binding domains, suggesting that it may regulate gene expression at the transcriptional level (Mazo et al., 1990). Genetic screens aimed at the isolation of additional mutants showing dosage interactions with homeotic mutations demonstrated that *trx* is a member of a large group of genes, called the *trx*-group, which is required for positively regulating homeotic gene function (Kennison and Tamkun, 1988). I have tested mutations in the *trx*-group of genes that are located on the third chromosome for their ability to complement mutations from our screen with similar phenotypes, and have found none to be allelic (see Chapter 2; Moore et al., 1998). Although it was known previous to our screens that the *trx*-group of genes were required for positively regulating homeotic genes including *abdA* and *AbdB*, members of the *trx*-group had not been shown to play a role *per se* in germ cell migration. Moreover, the mechanism by which *trx* functions to ensure proper gonad formation had not been investigated.

I present here a phenotypic analysis of the germ cell migration defect associated with the loss of *trx* function. Given that both *abdA* and *AbdB* play active roles in gonad formation, it follows that *trx* could be required for maintaining the expression of these genes in the tissues in which they act to affect germ cell migration. Surprisingly, my results strongly suggest that while *trx* is necessary for *AbdB* function in this process, it is not required for the initial role of *abdA* in germ cell migration. Furthermore, germ-line clonal analysis demonstrates that *trx* function is required throughout embryogenesis to maintain gonad integrity.

Of those previously unidentified mutants on the second chromosome that affect germ cell migration, I chose to study further two that had strong, highly penetrant

phenotypes that showed relatively specific effects on the process. I present here a phenotypic analysis of these two mutants, 9.35 and 22.38, as well as preliminary efforts to determine their map positions. These results show that the two mutants affect two different steps during germ cell migration, and do not show obvious defects in other embryonic developmental process. Furthermore, I have demonstrated that for one of these mutants, 22.38, the phenotype appears to be caused by a defect in germ cell-SGP interactions.

MATERIALS AND METHODS

Fly Stocks

The majority of deletion and P-element strains were obtained from the Bloomington Stock collection except for the following: *Df(2L)TE80x1, Df(2L)TE128x11, Df(2L)TE128x36, Df(2L)TE128x33, Df(2L)TE128x45* were all generous gifts from G. Reuter, and *Df(2L)N22-14* and *Df(2L)N22-5* were both kindly provided by T. Schüpbach. R. Mottus graciously provided the *Df(2L)J2* stock. P-element stocks with the designation *P{lacW}kxxxxx* were kindly provided by the Berkeley Drosophila Genome Project through T. Laverty.

The trx^{B11} allele, a generous gift from J. Kennison, contains a small deletion within the coding sequence producing a truncated protein, and therefore is likely to represent the null phenotype (Breen and Harte, 1993; Sedkov et al., 1994).

Hs-AbdB and *Hs-abdA* were both generously provided by G. Morata through M. Boyle. *Hs-Wg/TM3* was also a gift from M. Boyle and is described in Noordermeer et al. (1992). In the *Hs-AbdB* and *Hs-abdA* "rescue" experiments, the following strains were constructed: *Hs-AbdB/ftz lacZ CyO*; trx^{B11}*/Ubx-lacZ TM3*, and *Hs-abdA/ftz-lacZ CyO*; trx^{B11}*/Ubx-lacZ TM3*. Ectopic expression of the heat-shock constructs was induced according to the method described in Boyle et al. (1997). Embryos produced from these lines were scored as phenotypically trx^{-} by assaying for lost germ cells found ventral to the gonad, and this number was compared to that obtained from counting embryos laid by the same strains but without heat-shock.

Whole-mount antibody staining

Antibody stainings were performed with rabbit polyclonal anti-Vasa and mouse monoclonal anti-Cli antibodies (generously provided by A. Williamson and N. Bonini, respectively).

Antibody detection for anti-Vasa was done with horseradish peroxidase using a biotinylated anti-rabbit secondary antibody (Jackson ImmunoResearch) and the Elite Kit (Vector Labs), and for anti-Cli using an alkaline phosphatase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch). Both secondary antibodies were pre-absorbed against an overnight collection of wild-type embryos prior to use. Embryos were fixed and devitellinized according to the method of Gavis and Lehmann (1992), with the modification that embryos used for the anti-Cli detection were fixed in 1XPBS with 50mM EDTA. Embryos were rehydrated and subjected to antibody staining as described in Eldon

and Pirrotta (1991). Double antibody detection was performed according to the method described in Patel (1994). Embryos were mounted onto slides in PolyBed 812 (Polysciences) according to Ephrussi et al. (1991), then analyzed with a Zeiss Axiophot microscope using Nomarski optics.

Germ-line clones

Germ line clones of *trx* mutant cells were induced according to the method described in Chou and Perrimon (1992). The *P[ry$^+$, hs-neo, FRT]*82B chromosome was kindly provided by J. Treisman and recombined with the trx^{B11} allele according to the method described in Xu and Rubin (1993). The *P[ry$^+$, ovo^{D1}]/TM3* and *P[ry$^+$, hs-FLP]* stocks were also gifts from J. Treisman. Flies of the appropriate cross were allowed to lay for 2 days at 25°C. The resulting embryos were aged for two more days, and then subjected to a 2 hour heat-shock at 37°C on days 4 and 5.

DAPI staining of ovaries

DAPI staining was carried out as described by Lin et al., 1994. Samples were mounted in 50:50 PBS:Glycerol containing 2.5% DABCO (sigma).

Whole-mount in situ hybridization/antibody double labeling

Whole mount in situ hybridizations and subsequent antibody stainings were performed according to Lehmann and Tautz (1994).

An antisense-412 retrotransposon digoxigenin-labeled riboprobe was synthesized using the Boehringer-Mannheim 'Genius' 4 kit as described in Gavis and Lehmann (1992) and the pSK2.4#3 plasmid (Brookman et al., 1992) with T7 RNA polymerase. Embryos were mounted and analyzed as described above.

Recombination frequencies

The following values were obtained from mapping experiments described for 9.35, with x representing the number of recombinant flies, and n the total number of progeny scored: k10210, x=2, n=185; k07602, x=4, n=138; k09030, x=15, n=181; k00206, x=6, n=142; k07118, x=11, n=124; k07704, x=10, n=157. Statistical significance between values was determined using a standard *t*-test.

RESULTS AND DISCUSSION

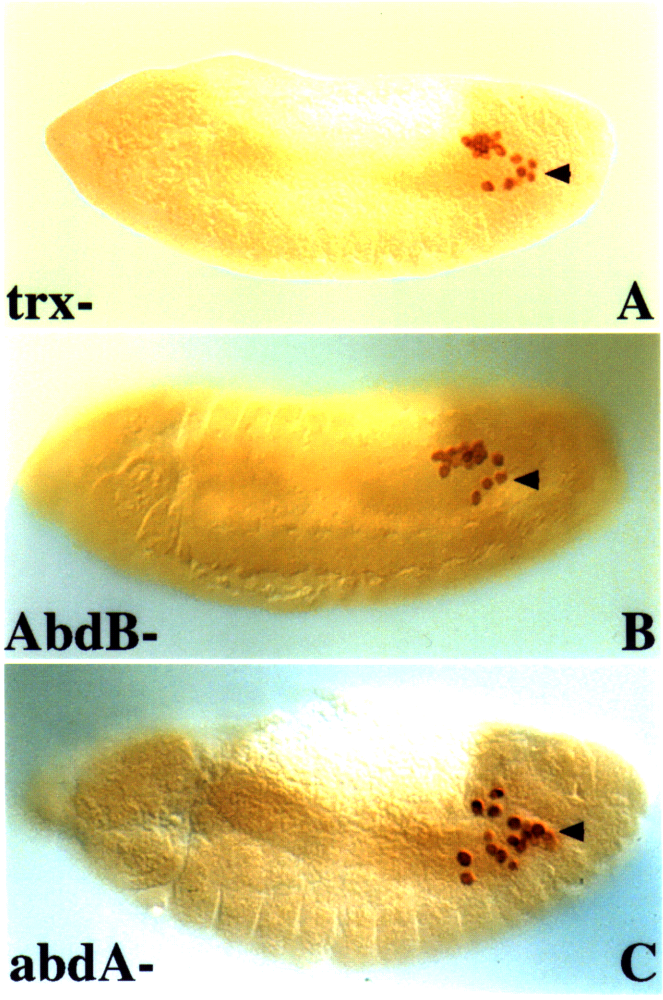
Gonadogenesis defects in *trx* mutants closely resemble those seen in *AbdB*, but not *abdA* mutants.

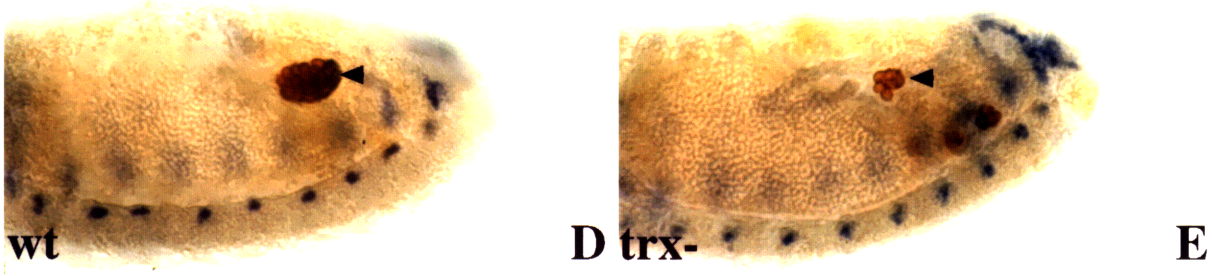
In a screen to identify genes required for germ cell migration on the third chromosome (see Chapter 2; Moore et al., 1998), we isolated 17 alleles of the *trx* locus. It has been shown that *trx* is required for maintaining the expression of homeotic genes, including *abdA* and *AbdB*, which are known to play a role in germ cell migration (Breen and Harte, 1993; Mazo et al., 1990). It therefore seemed possible that the germ cell migration phenotype seen in *trx* mutants was due to loss of *abdA* and/or *AbdB* activity. In an effort to determine if this is the case, I directly compared the *trx*$^{-}$ germ cell migration defect to that seen in embryos lacking *abdA* or *AbdB* function (Fig. 3-1). Germ cell migration in *trx* mutants is virtually identical to that seen in *AbdB* mutants, but differs from that observed in embryos lacking *abdA* activity (compare Fig. 3-1A with 3-1B,C). In *trx* mutants, like *AbdB* mutants, the posterior component of the germ cells fail to maintain their association with SGPs, and remain in an area ventral to where the gonad is forming (Fig. 3-1A,B, arrowheads). This contrasts to embryos lacking *abdA* function, where all germ cells disassociate from SGPs (Fig. 3-1C, arrowhead). These results suggest that the germ cell migration defect in *trx* mutants is due to a loss of *AbdB* activity, whereas *abdA* function in this process is uncompromised. In order to further test the phenotypic relationship between *trx* and *AbdB* mutants, I analyzed expression of *AbdB*-dependent gonadal mesoderm markers in *trx* mutant embryos. An example of one marker, Cli, is shown in Fig. 3-1D,E. In wild-type coalesced gonads, Cli protein expression in SGPs is maintained only in the posterior component of gonadal mesoderm (Fig. 3-1D, arrowhead). This maintenance of posterior gonadal Cli expression is dependent on *AbdB* activity (Boyle and Dinardo, 1995). I found that *trx* is also required for maintaining Cli expression after gonad coalescence. In embryos lacking *trx* function, fewer germ cells are incorporated into the gonad, and the surrounding gonadal mesoderm fails to express Cli protein (Fig. 3-1E, arrowhead). This phenotype is virtually identical to that observed in *AbdB* mutants (Boyle, 1997). A similar result was observed for expression of Dwnt-2, another marker maintained in the posterior gonad in an *AbdB*-dependent manner. These results support the hypothesis that *trx* is required for *AbdB*'s function in germ cell migration.

Figure 3-1: Gonadogenesis defects in *trx* mutants closely resemble those seen in *AbdB*, but not *abdA* mutants

Anterior is to left in all panels. Embryos in A-C are approximately stage 13; embryos in D,E are stage 14 (Campos-Ortega and Hartenstein, 1985). (A-E) Germ cells visualized using an anti-Vasa antibody in brown. (D,E) Cli expression is in blue. (A) *trx*$^{-}$. The posterior-most germ cells fail to associate with SGPs and are instead found ventral to the forming gonad (arrowhead). (B) *AbdB*$^{-}$. The germ cell migration phenotype is virtually identical to that seen in *trx* mutants. (C) *abdA*$^{-}$. In contrast to *trx* and *AbdB* mutants, all germ cells fail to incorporate into a gonad (arrowhead). (D) Wild type. After gonad coalescence, Cli expression is only maintained in the posterior region of the gonad (arrowhead). (E) *trx*$^{-}$. No Cli expression is detected after gonad coalescence (arrowhead). This result is identical to that seen in *AbdB* mutants (Boyle and Dinardo, 1995).

Fig. 3-1: Gonadogenesis defects in *trx* mutants closely resemble those seen in *AbdB*, but not *abdA*, mutants





Ectopic *AbdB* expression can partially rescue the *trx*⁻ germ cell migration phenotype

If the germ cell migration defect seen in *trx* mutants is due to loss of *AbdB* function, then ectopic expression of *AbdB* may "rescue" the *trx*⁻ germ cell migration phenotype. In order to test this hypothesis, ectopic *AbdB* activity was induced via a heat shock promoter in a *trx* mutant background (see Materials and Methods). The resulting embryos were scored for the presence of a *trx*⁻ germ cell migration defect. This number was then compared to the number of phenotypically *trx*⁻ embryos laid from the same line with no heat-shock administered. In order to determine if rescue of the *trx*⁻ phenotype is *AbdB*-dependent, a similar experiment was conducted by ectopically inducing *abdA* expression in a *trx* mutant background. The combined results from three independent experiments for ectopic *AbdB* expression as well as the accompanying controls are shown in Table 3-1. In the absence of heat-shock, roughly one-half of the embryos laid from the *HsAbdB;trx*⁻ line showed a *trx*⁻ germ cell migration defect. This number is consistent with that seen in the *trx* mutant line without the heat-shock construct. However, when *AbdB* activity is ectopically induced, only approximately one in seven embryos has the *trx* mutant germ cell migration defect. This is slightly more than the one to eight ratio expected for complete rescue (one-quarter of the embryos will not harbor the *Hs-AbdB* construct). In contrast, the ratios of wt:phenotypically *trx*⁻ embryos are identical in the *Hs-abdA;trx*⁻ experiment, with or without heat shock (Table 3-1). The reduced penetrance of the *trx*⁻ phenotype in the *Hs-abdA; trx*⁻ experiments is presumably due to the *Hs-abdA* insert and not *abdA* activity, since the values are identical with or without heat shock. However, it is still possible that this result is due to low levels of *abdA* expression. Nevertheless, the most likely explanation for these results is that ectopic expression of *abdA* cannot rescue the *trx*⁻ germ cell migration defect. Taken together, these results strongly suggest that ectopic *AbdB* activity, but not *abdA*, can partially compensate for the loss of *trx* function, indicating that *trx's* requirement for germ cell migration in wild-type embryos is to ensure proper *AbdB* function.

Table 3-1: *hs-AbdB* can partially rescue the *trx-* germ cell migration defect

	wt	*trx*⁻ phenotype	wt: *trx*⁻ phenotype
hs-AbdB; trx- [a]	154.3 ±22.14	21.3 ±7.4	7.2:1
no heat-shock	81	43	2:1
hs-abdA; trx-	180	45	4:1
no heat-shock	59	15	4:1

Embryos were scored as either phenotypically *trx*⁻ or wt based on the germ cell migration phenotype. Although half the *hs-AbdB;trx-* embryos had a *trx-* germ cell migration defect (See text) without heat-shock, only approximately one in seven had the same phenotype when heat-shock was administered. In contrast, the ratios of wt:*trx-* embryos in the *hs-abdA;trx-* experiment remain the same, with or without heat-shock.

[a] For *hs-AbdB;trx*⁻, three independent experiments were scored. The average number of embryos with each phenotype is presented, and the ratio calculated using these averages.

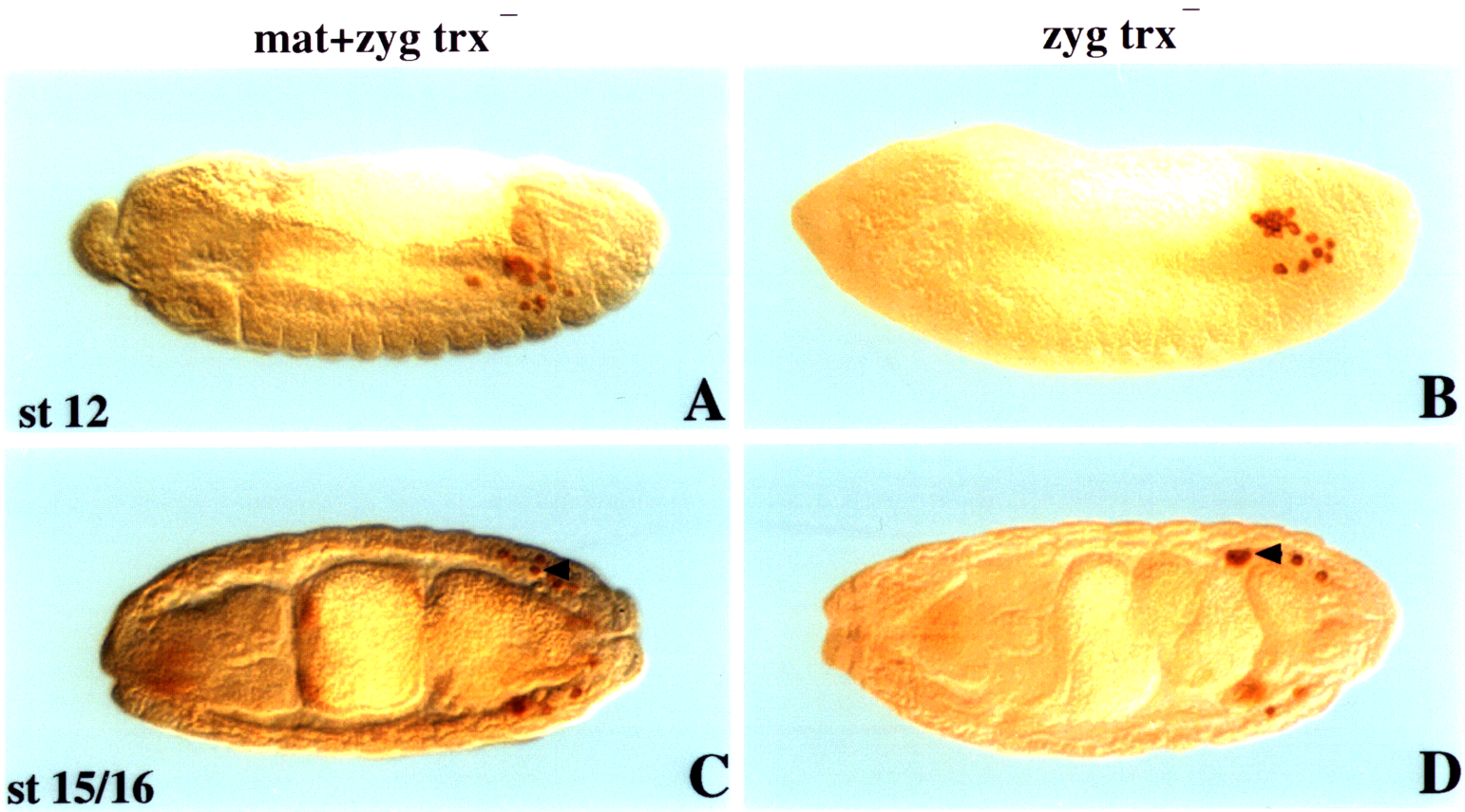
Maternally contributed *trx* activity is necessary for maintaining gonad integrity

The finding that *trx* regulates *AbdB*, but not *abdA,* function in germ cell migration is surprising, given that trx is known to be required for *abdA* activity in other developmental processes (Capdevila and Garcia-Bellido, 1981; Ingham and Whittle, 1980; Lewis, 1968). One possible explanation for why zygotic *trx* activity is not required for *abdA* function in germ cell migration is that the maternal contribution of *trx* plays this role. In order to test this hypothesis, I removed maternal *trx* function by inducing germ-line clones of *trx* mutant cells using the FLP/FRT*ovo*^{D} recombination system (see Materials and Methods; Chou and Perrimon, 1992; Xu and Rubin, 1993). The germ cell migration phenotype seen at stage 12 in embryos lacking both maternal and zygotic *trx* activity is identical to that seen in embryos lacking zygotic *trx* function alone (Fig. 3-2A,B). This result indicates that even in the complete absence of *trx* activity, the initial function of *abdA* in germ cell migration is not affected. However, in *trx*$^{-}$ embryos derived from *trx* mutant germ-line clones, the gonad fails to remain coalesced at late embryonic stages, and germ cells are consequently scattered in the posterior region of the embryo (Fig. 3-2C, arrowhead). This is in contrast to *trx* zygotic mutants, in which the gonad, albeit small, retains its characteristic spherical structure (compare arrowhead in Fig. 3-2D with that in Fig. 3-2C). This result demonstrates that *trx* plays a continued role in maintaining gonad integrity throughout embryogenesis. It has not been determined if this late activity of *trx* is necessary for proper *abdA* or *AbdB* function, or plays an as yet uncharacterized role.

Figure 3-2: *trx* is required throughout embryogenesis to maintain gonad integrity

Anterior is to left in all panels. Embryos in A,B are stage 13; embryos in C,D are stage 16 (Campos-Ortega and Hartenstein, 1985). (A-D) Germ cells visualized using an anti-Vasa antibody; (A,B) lateral views; (C,D) dorsal views. (A,C) Embryos lacking both maternal and zygotic *trx* function. (B,D) Embryos lacking zygotic trx function alone. (A,B) The germ cell migration phenotype seen at this stage in embryos lacking both maternal and zygotic *trx* function is identical to that observed in embryos mutant for *trx* zygotic activity alone. (C) When both maternal and zygotic *trx* functions are removed, germ cells that were originally in gonads disperse throughout the posterior region of the embryo (arrowhead). (D) When only zygotic *trx* activity is removed, those germ cells that are incorporated into a gonad remain there even in very late embryonic stages.

Fig. 3-2: *trx* is required throughout embryogenesis for maintaining gonad integrity



It is still unclear why *trx* mutants do not show an *abdA*-like germ cell migration phenotype. One possibility is that *abdA* function in germ cell migration occurs at an early stage when *trx*-mediated maintenance of its expression has not yet occurred. However, it has been shown that a later function of *abdA*, mediated through the *iab-4* regulatory region, is required for gonad coalescence (Cumberledge et al., 1992; Warrior, 1994). This *iab-4* mutant phenotype is distinct from that observed in *trx* and *AbdB* mutant embryos. An alternative explanation is that *trx* has different roles in maintaining the expression of homeotic genes in different tissues. For instance, it is possible that *trx* plays a significant role in maintaining *AbdB* lateral-mesodermal expression, whereas the requirement for *trx* in regulating *abdA* expression in this region may not be as stringent. In fact, it has been shown that in *trx* mutants, the reduction in levels of *abdA* expression is more pronounced within heart mesoderm than in somatic mesoderm (Breen and Harte, 1993). This result demonstrates that there are at least different requirements for *trx* function in different aspects of a particular homeotic genes's expression, and therefore, implies that there may be different requirements for *trx* function in maintaining the expression of multiple homeotic genes. Further analysis of *abdA* and *AbdB* expression in the lateral mesoderm of *trx* mutant embryos may help to elucidate the role of *trx* in *abdA* gonadal function.

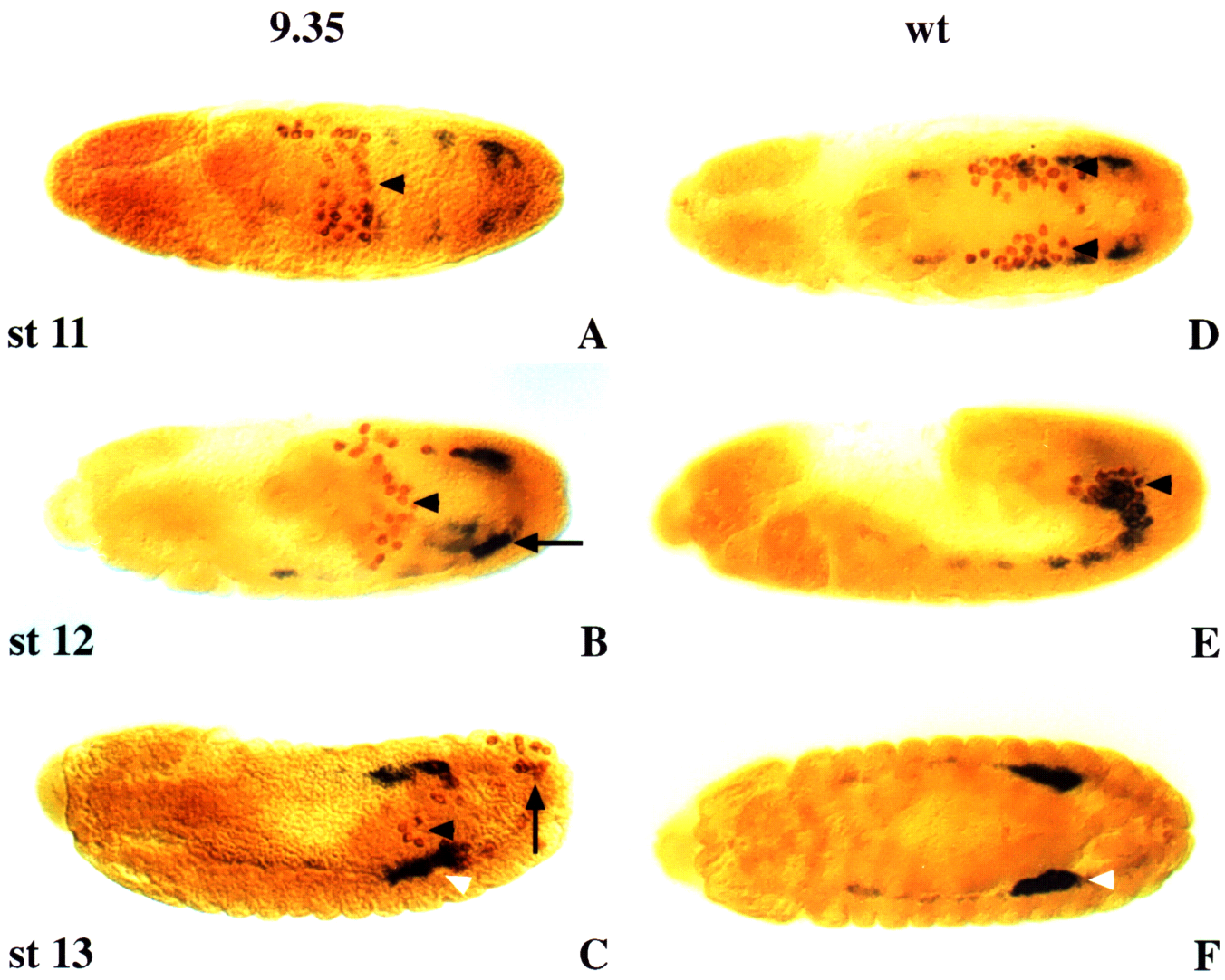
Germ cells fail to move correctly into lateral mesoderm in 9.35 mutant embryos

In a screen to identify genes required for germ cell migration on the second chromosome, we isolated a mutant, 9.35, in which germ cells fail to move correctly away from the gut endoderm and into the lateral mesodermal layer (Fig. 3-3). Although we only isolated one allele of this complementation group, given its strong, highly penetrant and specific phenotype, I decided to characterize this mutant further. Defects in germ cell migration in 9.35 mutants can be detected as early as stage 11. At this stage, instead of migrating into lateral mesoderm, many germ cells remain in one group outside the PMG (Fig. 3-3A, arrowhead). At this stage in wild type, germ cells have normally split into two bilaterally symmetric groups within the mesodermal layer (Fig. 3-3D, arrowheads). In the mutant, as the germ band retracts during stage 12, SGPs migrate anteriorly (Fig. 3-3B, arrow), whereas many germ cells remain in a more posterior location close to the PMG (Fig. 3-3B, arrowhead). In wild-type embryos at this stage, germ cells migrate anteriorly along with SGPs, resulting in the close association of these two cell types (Fig. 3-3E, arrowhead). Germ cells finally do move away from the basal surface of the PMG at later stages in the mutant, but do not contact SGPs (Fig. 3-3C, white arrowhead) and are either found in extreme posterior regions of the embryo (Fig. 3-3C, arrow), or in the yolk near the developing PMG (Fig. 3-3C, black arrowhead). Thus, in 9.35 mutant embryos germ cells appear stalled in their migration away from the basal surface of the PMG and into the mesodermal layer. Germ cells eventually disengage from the PMG, but apparently at a stage beyond the time during which they are able to contact SGPs.

Figure 3-3: Germ cells are delayed in their movement away from the PMG in 9.35 mutant embryos

Anterior left in all panels. (A-F) Germ cells visualized using an anti-Vasa antibody in brown; SGPs are recognized in blue by the 412 retrotransposon. (A,B,C,D,F) dorsal views; (E) lateral view. (A-C) 9.35 mutant embryos; (D-F) Wild-type embryos of comparative stages. (A,D) stage 11; (B,E) stage 12; (C,F) stage 13 (Campos-Ortega and Hartenstein, 1985). (A) In 9.35 mutants, germ cells fail to migrate away from the PMG (arrowhead) at a time that in wild type (D), germ cells have split into two bilaterally symmetric groups within lateral mesoderm (arrowheads). (B) As SGPs migrate anteriorly in 9.35 mutants (arrow), the germ cells remain behind, closely associated with the PMG (arrowhead). (E) In wild type at this stage, germ cells and SGPs have moved together and are closely associated with one another (arrowhead). (C) Germ cells eventually leave the PMG in 9.35 mutants and are either found in extreme posterior regions (arrow), or between the bilaterally symmetric gonads (black arrowhead). Rarely are germ cells associated with SGPs, which appear to develop normally (white arrowhead; compare with white arrowhead in panel F).

Fig. 3-3: Germ cells are delayed in their movement away from the PMG in 9.35 mutant embryos



Many embryonic tissues develop normally in 9.35 mutants

As with all previously unidentified germ cell migration mutants, it is not known whether the 9.35 phenotype is caused by a defect in the germ cells or in one (or more) of the somatic tissues with which the germ cells must interact during their development. In an effort to distinguish between these possibilities, I analyzed the development of a variety of somatic tissues in 9.35 mutants using tissue-specific markers. Since most germ cells fail to contact SGPs in the mutant, one obvious possible explanation is that there is a defect in specification of SGPs or in the development of gonadal mesoderm. We tested this hypothesis by analyzing expression of the 412 retrotransposon, a gonadal mesoderm-specific marker, in 9.35 mutants. As in wild type, 412 is initially expressed in a segmental pattern in the mutant (Fig. 3-3A), and then becomes restricted to developing SGPs at later stages (Fig. 3-3B, arrow; 3-3C, white arrowhead). Therefore, gonadal mesoderm development does not appear to be affected in 9.35 mutants. It has been shown that some germ cells must move around the developing visceral mesodermal layer in order to contact SGPs (Broihier et al., 1998). It seemed therefore possible that a problem with visceral mesoderm development could inhibit germ cells from properly associating with SGPs. However, as assayed by expression of the visceral mesodermal markers *tinman, bagpipe* and Fasciclin III, (Azpiazu and Frasch, 1993; Azpiazu et al., 1996), development of this tissue in 9.35 mutant embryos is normal. Since germ cells in 9.35 mutants are delayed in their movement away from the PMG, it seemed plausible that defects in PMG development could contribute to the phenotype. I therefore examined the expression of a gene, *Race*, which is specifically transcribed in PMG cells at the stage when the germ cells are moving through and away from this tissue (Tatei et al., 1995). I found *Race* expression to be unaltered in 9.35 mutant embryos. In order to determine if the 9.35 mutation affects other migratory cell-systems, I analyzed the expression patterns of the 2A12, BP102, and 22.C10 markers, which are expressed in tracheal system, central nervous system, and peripheral nervous system, respectively (Samakovlis et al., 1996; Seeger et al., 1993; Zipursky et al., 1985). I found no obvious defects in any of the three tissues examined. It is still possible that subtle problems occur during the development of these cell-types, but are not apparent using these particular markers. Taken together, these results show that many embryonic tissues, including those intimately associated with the migrating germ cells, appear to develop normally in 9.35 mutants. However, this mutation is homozygous semi-lethal (see below), suggesting that some aspect(s) of embryonic development are abnormal. Nevertheless, the 9.35 lesion could potentially lie in a gene affecting germ cells specifically, which we did not identify in our screen of the third chromosome. Mosaic analysis of the 9.35 mutant phenotype should determine if this is the case.

9.35 maps to an uncharacterized region between the *dumpy* and *black* markers

Standard recombination experiments placed the 9.35 lesion between the *dumpy (dp)* and *black (b)* markers. In order to further refine the region in which the 9.35 mutation is located, as well as to identify additional alleles, I searched for deletions in this region that failed to complement the 9.35 germ cell migration defect. A list of the deletions tested is shown in Table 3-2. Of all deletions tested, which comprise nearly all deletions available that uncover this region, all complemented the 9.35 germ cell migration defect. As mentioned above, the 9.35 mutation is homozygous semi-lethal. Those rare females that do develop into adults are nearly infertile, as a result of oogenesis being blocked by stage 8. The deletions in Table 3-2 were also tested for their ability to complement the 9.35 oogenesis phenotype. Two overlapping deletions, *Df(2L)TW50* and *Df(2L)pr-A14* fail to complement the 9.35 oogenesis defect, demonstrating that the germ cell migration and oogenesis defects associated with the 9.35 mutant chromosome are most likely due to independent lesions.

Table 3-2. Deficiencies tested against 9.35

	Deficiency breakpoints
Df(2L)dp-h25	21E2-4;25B2-5
Df(2L)tkv3	25A2-3;25D5-E1
Df(2L)cl-h3	25D2-4;26B2-5
Df(2L)2802	25F2-3;25F4-26A1
Df(2L)E110	25F3-26A1;26D3-11
Df(2L)J136-H52	27C2-9;28B3-4
Df(2L)spd	27D-E;28C
Df(2L)TE80x1	28D4;28E1
Df(2L)TE128x11	28E4-7;29B2-C1
Df(2L)TE128x36	29A2-B1;29C2-5
Df(2L)TE128x33	29A1-2;29E1-2
Df(2L)TE128x45	29A2-B1;29E2-F1
Df(2L)N22-14	29C1-2;30C8-9;30D1-2;31A1-2
Df(2L)N22-5	29D1-2;30C4-D1
Df(2L)30A;C	30A;30C
Df(2L)Mdh	30D-F;31F
Df(2L)TE196x1	29E2-F1;30C2-4
Df(2L)J233	31A-B;31F-32A
Df(2L)J39	31C-D;32D-E
Df(2L)J2	31B-32A
Df(2L)esc10	33A8-B1;33B2-3
Df(2L)prd1.7	33B2-3;34A1-2
Df(2L)b87e25	34B12-C1;35B10-C1
Df(2L)osp29	35B1-3;35E6
Df(2L)r10	35E1-2;36A6-7
Df(2L)H20	36A8-9;36E1-2
Df(2L)TW137	36C2-4;37B9-C1
Df(2L)TW50	36E4-F1;38A6-7
Df(2L)pr-A14	37D2-7;39A4-7
Df(2L)E55	37D2-E1;37F5-38A1
Df(2L)TW84	37F5-38A1;39D3-E1
Df(2L)TW161	38A6-B1;40A4-B1

Although the deletions tested uncover a large portion of the region between the *dp* and *b* markers, "gaps" remain within this region that have not been analyzed with respect to the 9.35 germ cell migration defect. In order to determine if the 9.35 lesion affecting germ cell migration maps to one of these gaps, I tested existing lethal P-element-mediated mutants for their ability to complement the 9.35 germ cell migration phenotype. A list of P-element-induced mutant lines used in this study is shown in Table 3-3. Of all mutants tested which map between 27F and 30A7-8, all complement the 9.35 germ cell migration defect. The results from this analysis do not preclude the possibility that the 9.35 germ cell migration lesion maps within the 27F-30A7-8 region, but demonstrate that it is not allelic to previously identified P-element-induced mutations found in this same region.

In an effort to more finely map the mutation responsible for the 9.35 germ cell migration defect, I analyzed recombination frequencies between this lesion and a subset of the P-elements listed in Table 3-3. The results from this analysis are shown in Table 3-4. The recombination frequencies (rf) obtained between 9.35 and various P-element mutations are shown, starting on the left from P-alleles mapping more distally to those located more proximaly in linear order. Of those P-elements tested, k10210 and k07602 map more closely to the 9.35 mutation than the other inserts (rf for k07602 vs. k09030: p=0.97; see Materials and Methods). It is not possible to determine from this data where 9.35 maps with respect to these two P-elements, since the recombinaiton frequencies obtained are not significantly different (p=0.88). Although the recombination frequencies for the more proximal elements appear to be inconsistent with their linear order, these values are also not significantly different (rf for k09030 vs. k00206: p=0.94). A more precise location for the 9.35 lesion will require further mapping with a larger number of progeny.

Table 3-3. P-elements tested against 9.35

	Cytological position
P{PZ}wg[r0727]	27F1-2
J9.86	28C
P{lacW}l(2)k10316	28C1-2
P{PZ}l(2)rL220	28C4-6
P{PZ}gel[1]	28D
P{lacW}l(2)k06009	28D1-2
P{PZ}mts[02496]	28D1-2
P{PZ}l(2)06243	28D3-4
P{lacW}l(2)k10210	28D7-9
P{PZ}l(2)05836	28E1-2
P{lacW}l(2)k07602	28E3-4
P{lacW}l(2)k09030	28E4-5
J9.156	28E7-8
P{lacW}l(2)k15916	28F1-2
P{lacW}l(2)k00206	29A1-2
P{lacW}l(2)k07118	29C1-3
P{PZ}l(2)01482	29C3-5
P{lacW}l(2)k07704	29D1-2
P{PZ}lmg[03424]	29D4-5
P{PZ}l(2)06825	29F1-2
J21.246	30A7-8

Table 3-4: Fine mapping of the 9.35 germ cell migration phenotype

	k10316	k06009	k10210	k07602	k09030	k00206	k07118	k07704	k16002
rf	.178	.183	.011	.029	.083	.040	.089	.064	.094

Recombination frequencies between the 9.35 germ cell migration lesion and various lethal P-element alleles are shown (For map positions of the P-elements, see Table 3-4). P-alleles are designated as kxxxxx. Recombination frequencies are determined by calculating two times the number of recombinants scored (the other half are lethal), divided by the total number of flies. Approximately 150 flies were scored for each P-element tested (see Materials and Methods).

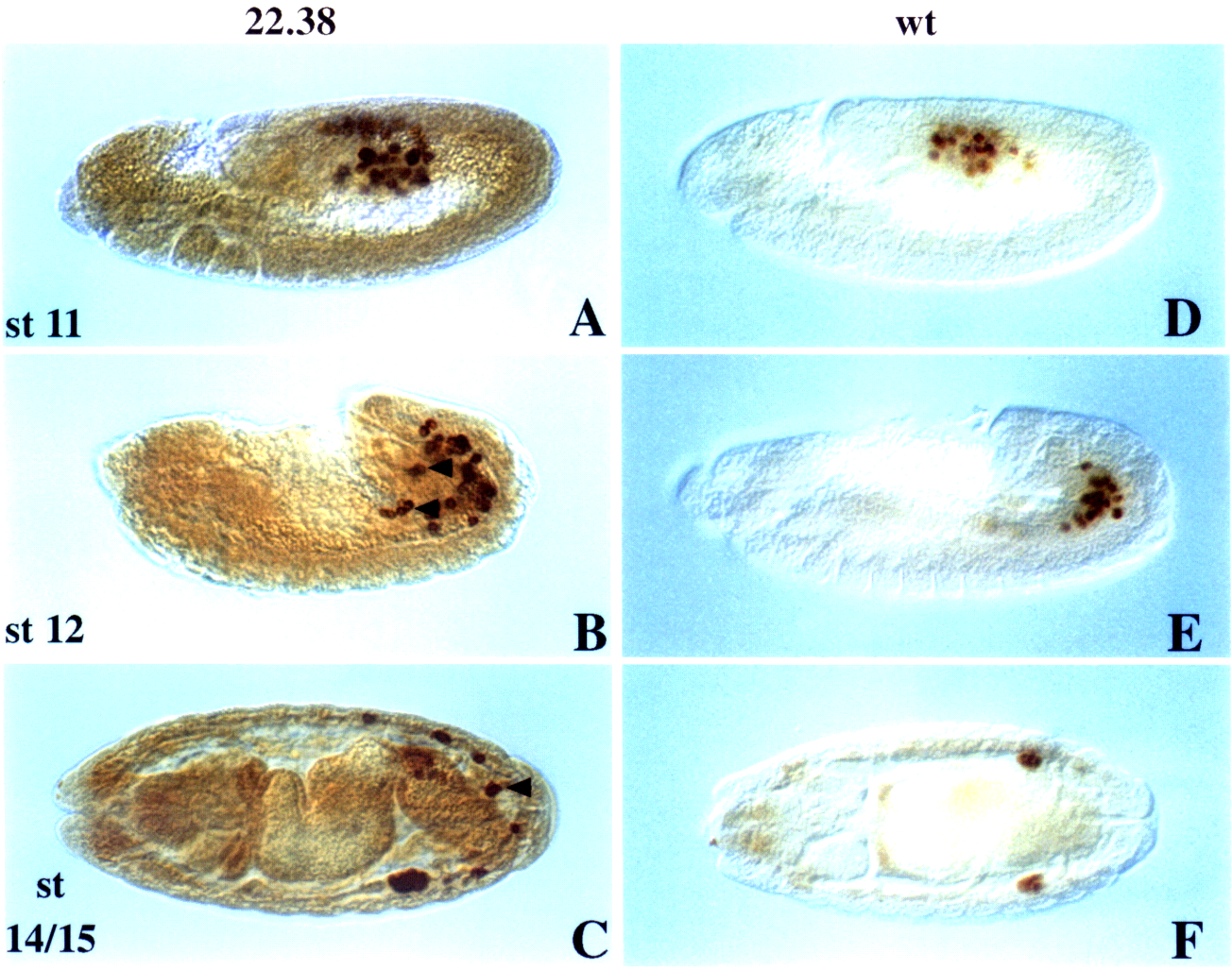
22.38 is a dominant maternal mutation affecting germ cell-SGP association

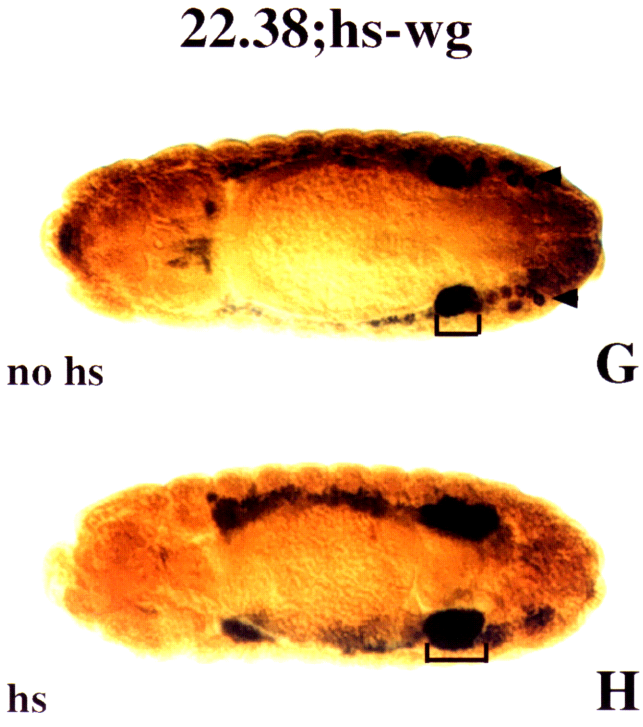
We identified another mutant in our screen of the second chromosome, 22.38, which also displays a strong, highly penetrant germ cell migration defect (Fig. 3-4). However, the onset of the phenotype associated with this mutant occurs at a later stage than that seen in 9.35 mutant embryos. In 22.38 mutants, germ cells successfully move away from the PMG and into the lateral mesodermal layer (Fig. 3-4A). However, many germ cells fail to maintain their association with SGPs and as a result are excluded from the gonad (Fig. 3-4C, arrowhead). Nevertheless, gonads do form in the mutant, and appear to have a similar number of germ cells when compared to wild type (compare Fig. 3-4C with Fig. 3-4F). The defect seen in 22.38 mutants does not appear to be due to a problem with gonadal mesoderm development, since expression of the 412 retrotransposon is normal (Fig. 3-4G).

Figure 3-4. 22.38 affects germ cell-SGP interactions

Anterior left in all panels. (A-H) Germ cells visualized in brown using an anti-Vasa antibody. (G,H) SGPs recognized in blue using the 412 retrotransposon. (A,B,D,E) lateral views; (C,F,G,H) dorsal views. (A-C) 22.38 mutant embryos; (D-F) Wild-type embryos of comparative stages. (G,H) 22.38;*hs-wg*. (A,D) stage 11; (B,E) stage 12; (C,F-H) stage 14/15 (Campos-Ortega and Hartenstein, 1985). (A) Germ cells correctly migrate away from the PMG and into lateral mesoderm in 22.38 mutants (compare with panel D). (B) Some germ cells properly associate with SGPs, but others are found in locations between the developing gonads (arrowheads; compare with panel E). (C) Although some germ cells are incorporated into gonads, others are excluded (arrowhead; compare with panel F). (H) When *wg* is ectopically induced in a 22.38 mutant background, more SGPs are specified (compare size of bracket with that in panel G), and all germ cells are incorporated into the gonad. (G) Germ cells are excluded from gonads in 22.38;*hs-wg* embryos that have not been subjected to heat-shock (arrowheads).

Fig. 3-4: Germ cells fail to properly associate with SGPs in 22.38 mutants





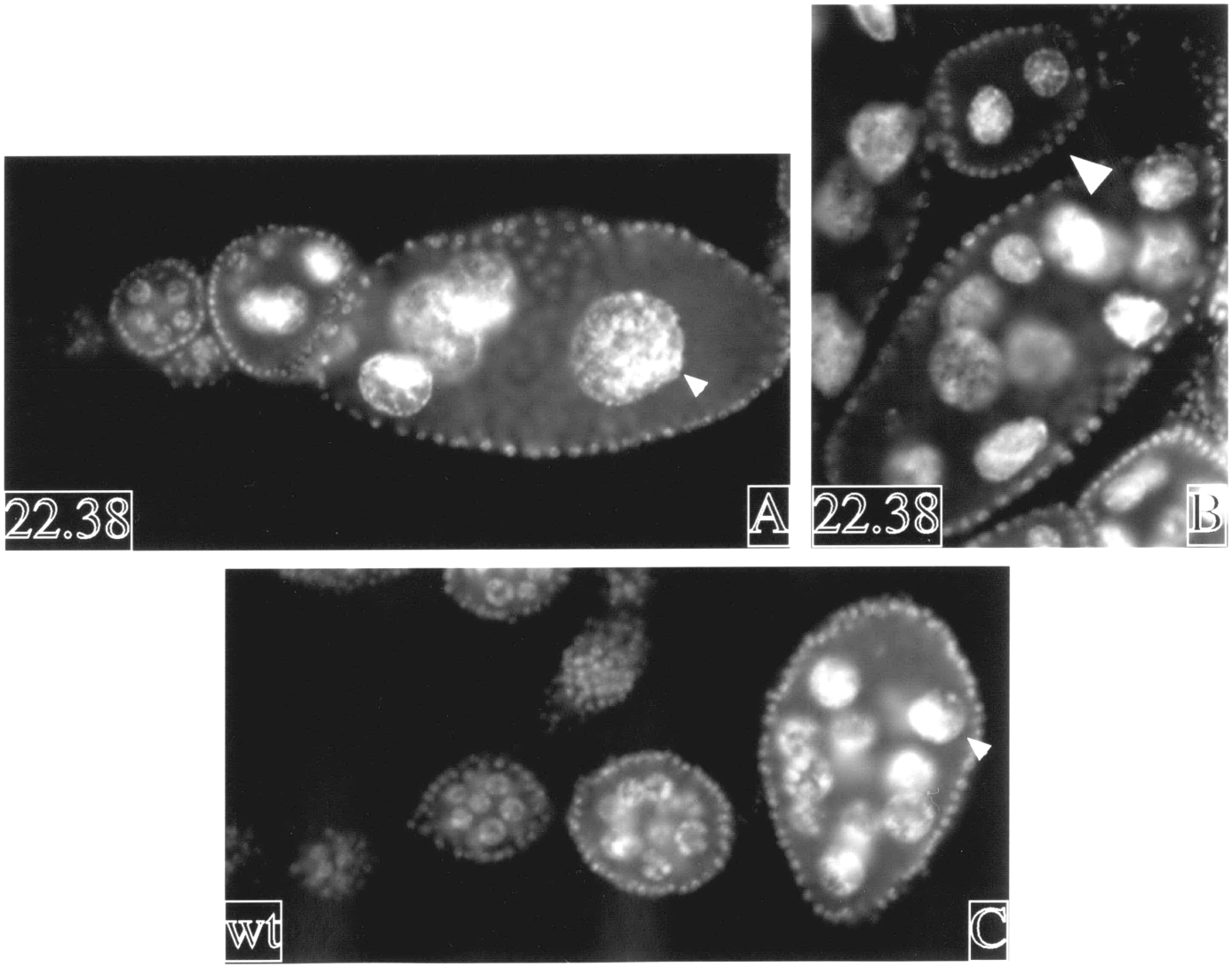
The 22.38 mutant line is unique among our collection in that it is homozygous viable. It therefore is one of our most compelling candidates for a mutation that specifically affects germ cell migration, since all developmental processes required for viability of the fly are normal. This line has an additional intriguing feature in that the mutation(s) responsible for the germ cell migration defect behaves in a dominant, maternal fashion: female flies heterozygous for the 22.38 lesion lay eggs with the characteristic germ cell migration phenotype, regardless of the genotype of the parental male. The 22.38 line is also associated with a zygotic defect. Homozygous mutant adult females lay fewer eggs than wild type. Closer inspection of mutant ovaries reveals that the number of nuclei per egg chamber is often much less than the normal sixteen found in wild type (Fig. 3-5). In extreme cases, as few as two nuclei are found within an egg chamber (Fig. 3-5B, arrowhead). Moreover, the remaining nuclei are often of abnormal size (compare arrowhead in Fig. 3-5A with Fig. 3-5C). Presumably, many of these egg chambers containing an abnormal array of nuclei degenerate, thereby causing the lower fecundity observed for 22.38 homozygous mutants. This zygotic defect appears to be female-specific, since homozygous mutant male flies carry out spermatogenesis normally.

A defect in DNA replication within developing egg chambers has been observed in females homozygous for a weak allele of the *cyclin E* (*cycE*) gene (Lilly and Spradling, 1996). The 22.38 oogenesis defect, however, is most likely not allelic to *cycE*, since females transheterozygous for 22.38 and either a weak or null allele of *cycE* carry out oogenesis normally. Moreover, *cycE* alleles on their own do not show a germ cell migration defect.

Figure 3-5. 22.38 mutant egg chambers contain an abnormal number of nuclei of aberrant shape.

(A-C) DAPI-stained ovaries from 4- to 5- day-old females to highlight nuclei. (A,B) Ovaries from 22.38/22.83 females. (C) Wild type. (A,B) Egg chambers often contain much fewer than sixteen nuclei. An extreme example is shown in (B), where an egg chamber only contains two visible nuclei (arrowhead). Many nuclei are also larger than found in wild type [compare arrowhead in (A) with that in (C)].

Figure 3-5: Nurse cell number and size are altered in 22.38 mutant ovaries



In order to learn more about the nature of the dominant maternal mutation associated with the 22.38 germ cell migration defect, I searched for chromosomal deletions that would either enhance or suppress the phenotype. In addition, I examined the deletion strains themselves for a similar germ cell migration defect, in order to determine if the 22.38 locus is haploinsufficient. Recombination mapping of the lesion associated with the 22.38 germ cell migration phenotype placed it between the *black (b)* and *purple (pr)* markers on chromosome 2L. A list of deletion strains tested that uncover areas between these two markers is provided in Table 3-5. None of the deletions tested show a haploinsufficient germ cell migration defect; however, embryos homozygous for the deletions often showed aberrant germ cell migration. This is presumably a result of the loss of multiple gene functions associated with the deletions. In addition, none of the deletions enhanced or suppressed the 22.38 germ cell migration defect. However, two deletion strains, *Df(2L)r10* and *Df(2L)H20*, fail to complement the zygotic 22.38 oogenesis defect. Interestingly, the *cycE* gene is found in the region deleted in the *Df(2L)r10* strain (Lilly and Spradling, 1996). Although these two deletions fail to enhance or supress the 22.38 germ cell migration phenotype, it is still possible that the mutation responsible for the germ cell migration phenotype is the same as that causing the oogenesis defect. For example, if 22.38 were a neomorphic mutation, lowering the dosage of the wild-type 22.38 gene product would not necessarily enhance or suppress the germ cell migration phenotype. Likewise, it is still possible that 22.38 represents a neomorphic mutation in the *cycE* gene that behaves recessively for oogenesis, and would not show a phenotype when transheterozygous to loss of function *cycE* alleles (see above). Further attempts to separate the 22.38 germ cell migration defect from the oogenesis phenotype may determine if the two phenotypes are due to different, or the same, mutation(s).

Table 3-5: Deficiencies tested against 22.38

	Deficiency breakpoints
Df(2L)b87e25	34B12-C1;35B10-C1
Df(2L)osp29	35B1-3;35E6
Df(2L)r10	35E1-2;36A6-7
Df(2L)H20	36A8-9;36E1-2
Df(2L)TW137	36C2-4;37B9-C1
Df(2L)TW50	36E4-F1;38A6-7
Df(2L)pr-A14	37D2-7;39A4-7
Df(2L)E55	37D2-E1;37F5-38A1
Df(2L)TW84	37F5-38A1;39D3-E1
Df(2L)TW161	38A6-B1;40A4-B1
Df(2L)TW1	38A7-B1;39C2-3
Df(2L)DS6	38F5;39E7-F1
Df(2R)M41A4	41A
Df(2R)M41A8	41A
Df(2R)M41A10	41A
Df(2R)r110a	41A

Ectopic *wingless* expression can rescue the 22.38 germ cell migration defect

Although gonads form in 22.38 embryos, many germ cells fail to be incorporated into them. One possible explanation for this problem is that the SGPs are "saturated" for germ cells in mutant embryos. In other words, the number of SGPs may be the limiting factor to enable all the germ cells to occupy the gonad. This situation is reminiscent of that observed in embryos where the number of germ cells specified is increased due to a higher copy number of the *oskar (osk)* gene. Embryos derived from females carrying four copies of the *osk* gene develop twice the number of germ cells as found in wild-type controls (Ephrussi and Lehmann, 1992), and show a germ cell migration phenotype similar to that seen in 22.38 mutants (data not shown). This suggests that in 4x *osk* embryos, SGP number is not sufficient to incorporate the additional germ cells. There is no significant difference in germ cell number in 22.38 mutants compared to wild type (22.38: x=38; wt: x=40). In order to determine if SGP number is a limiting factor in 22.38 mutants, and as a result causes germ cells to be excluded from the gonad, I attempted to rescue the 22.38 germ cell migration defect by specifying additional SGPs through genetic means. It has been shown that ectopic expression of the *wingless (wg)* gene via a heat shock promoter can induce additional SGPs to be specified within PS10-12 of lateral mesoderm (Boyle et al., 1997). Ectopic wg expression in 22.38 mutant embryos also causes additional SGPs to develop (compare size of brackets in Fig. 3-4H with Fig. 3-4G). Moreover, all germ cells appear to assemble into the gonad, as evident by the lack of germ cells located outside this organ. In contrast, germ cells are excluded from the gonad in 22.38 embryos lacking ectopic *wg* activity but that are subjected to the same heat shock regimen (Fig. 3-4G, arrowhead). Thus, the germ cell migration defect associated with the 22.38 lesion can be diminished in the presence of additional SGPs. Although expression of the 412 retrotransposon appears normal in 22.38 mutants, it is possible that a subtle decrease in SGP number has not been detected. Alternatively, SGP number could be normal in 22.38 mutants, but the function of these cells could be compromised. For example, if SGPs express a reduced number of putative "germ cell-receptors" in 22.38 mutants, then increasing SGP number will effectively increase the concentration of receptor, and could result in the recruitment of all germ cells. Likewise, if the 22.38 migration defect were a result of a reduction in "SGP-ligand" expression in the germ cells, then an effective increase in germ cell receptor through additional SGPs could also rescue the germ cell migration phenotype. Accurate SGP counts as well as mosaic analysis may be necessary for determining in which cell-type the 22.38 defect occurs.

The fact that only one allele has been identified for both the 9.35 and 22.38 germ cell migration defects, combined with the lack of accurate mapping data makes further

genetic and molecular analysis of these loci challenging. Especially in the case of 22.38, it is difficult to determine the wild-type function of the gene associated with the mutation, given that it behaves as a dominant allele. Further screens in the lab are underway in an attempt to saturate the second chromosome for mutations affecting gonad formation: presumably, additional alleles of 9.35, and perhaps 22.38 will be isolated and will facilitate additional phenotypic and molecular characterizations of these loci.

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AUTHOR'S NOTE

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CHAPTER 4

Gonadal mesoderm and fat body initially follow a common developmental path in Drosophila

SUMMARY

During gastrulation, the Drosophila mesoderm invaginates and forms a single cell layer in close juxtaposition to the overlying ectoderm. Subsequently, particular cell types within the mesoderm are specified along the antero-posterior and dorso-ventral axes. The exact developmental pathways that guide the specification of different cell types within the mesoderm are not well understood. We have analyzed the developmental relationship between two mesodermal tissues in the Drosophila embryo, the gonadal mesoderm and the fat body. Both tissues arise from lateral mesoderm within the *eve* domain. Whereas in the *eve* domain of parasegments 10-12 gonadal mesoderm develops from dorsolateral mesoderm and fat body from ventrolateral mesoderm, in parasegments 4-9 only fat body is specified. Our results demonstrate that the cell fate decision between gonadal mesoderm and fat body identity within dorsolateral mesoderm along the antero-posterior axis is determined by the combined actions of genes including *abdA*, *AbdB*, and *srp*: while *srp* promotes fat body development, *abdA* allows gonadal mesoderm to develop by repressing *srp* function. Finally, we present evidence from genetic analysis suggesting that before stage 10 of embryogenesis, gonadal mesoderm and the fat body have not yet been specified as different cell types, but exist as a common pool of precursor cells requiring the functions of the *tin*, *zfh-1*, and *cli* genes for their development.

INTRODUCTION

Developmental programs leading to the specification and differentiation of particular cell types often involve a combination of cell-autonomous factors, specific patterns of cell division, and cell-cell interactions. How these combined elements lead to the specification of cell types within the developing Drosophila mesoderm has recently been the focus of intense study. During stage 8 and 9 of embryogenesis, the pair-rule genes *even-skipped (eve)* and *sloppy-paired (slp)* act in the mesoderm to allocate cells into two domains within each parasegment (PS) along the antero-posterior (A-P) axis (Azpiazu et al., 1996; Riechmann et al., 1997). Cells within each domain will give rise to a specific set of mesodermal tissues, such that midgut visceral mesoderm (gut musculature), fat body (see below), and gonadal mesoderm (somatic component of the gonad) are derived from the *eve* domain, whereas cardiac mesoderm (heart precursors) and somatic mesoderm (muscles) are specified within the *slp* domain. Concomitant with this early patterning of the mesoderm are two consecutive waves of cell division, as well as the migration of the mesoderm dorsally along the ectoderm (reviewed in Bate, 1993). This movement places the mesoderm in close contact with the overlying ectoderm, allowing signaling processes to occur between the two layers. Two such signals are the products of the segment polarity genes *hedgehog (hh)* and *wingless (wg)*, which function downstream of *eve* and *slp*, respectively, to further define mesodermal sub-domains along the A-P axis (Azpiazu et al., 1996).

Patterning of the mesoderm along the dorso-ventral (D-V) axis also requires signaling events between the two germ layers. During stage 10 of embryogenesis, *decapentaplegic (dpp)* signaling is required in the ectoderm to maintain expression of the *tinman (tin)* gene exclusively in the dorsal region of the mesoderm (Frasch, 1995; Staehling-Hampton et al., 1994). Previous to this stage, *tin* is expressed throughout the mesoderm (Azpiazu and Frasch, 1993). Dorsally-restricted *tin* expression is necessary for the specification of dorsal mesodermal derivatives, including the midgut visceral mesoderm, cardiac mesoderm, and dorsal muscles (Azpiazu and Frasch, 1993; Bodmer, 1993). Another wave of cell division occurs during this stage, along with the segregation of the mesoderm into an inner and outer layer. The inner layer in part becomes visceral mesoderm, whereas the outer layer gives rise to the somatic musculature (Bate, 1993). By this time, specification of different mesodermal cell types has commenced, as is evident by the cell-type specific expression of the *bagpipe (bap)* gene in the visceral mesoderm (Azpiazu et al., 1996). Although a developmental and genetic pathway including *eve, hh, dpp, tin*, and *bap* has been described for the specification of the midgut visceral mesoderm,

similar pathways for other mesodermal derivatives such as gonadal mesoderm and the fat body have yet to be elucidated.

Some of the genes required for specification and differentiation of gonadal mesoderm have been recently identified. It has been shown that the homeotic genes *abdominal A (abdA)* and *Abdominal B (AbdB)* are required for specifying somatic gonadal precursors (SGPs), those cells which become gonadal mesoderm, within PS10-12 (Boyle and DiNardo, 1995). The *clift (cli,* also known as *eyes-absent)* gene is required for gonadal mesoderm differentiation, and is specifically expressed in SGPs beginning at stage 11 (Boyle and DiNardo, 1995). This restricted expression requires *abdA* and *AbdB* function (Boyle and DiNardo, 1995; our observations). Additional genes required for the development of gonadal mesoderm including *eve* and *hh* have been identified through screens for mutants affecting germ cell migration in Drosophila (Moore et al., 1998). These studies showed that gonadal mesoderm is virtually abolished in *eve* mutants, but present in *slp* mutants, demonstrating that gonadal mesoderm, like visceral mesoderm and fat body, is derived from the *eve* domain of the mesoderm (Azpiazu et al., 1996; Moore et al., 1998). It has been shown that gonadal mesoderm lies immediately ventral to the visceral mesoderm that requires *tin* function for its specification (Boyle et al., 1997). These and other studies found that *tin* is also required for gonadal mesoderm development (Boyle et al., 1997; Moore et al., 1998). However, this function for *tin* does not depend on a regulator of late *tin* expression, *dpp*, suggesting that the early, uniform expression of *tin* throughout the mesoderm is critical for the development of this tissue (Broihier et al., 1998). The *zinc-finger homeodomain protein-1 (zfh-1)*, has been identified as another regulator of gonadal mesoderm development (Broihier et al., 1998; Moore et al., 1998). It has been demonstrated that when both *tin* and *zfh-1* function are removed from embryos, gonadal mesoderm is abolished, and virtually no fat body cells develop. This suggests a model by which *tin* and *zfh-1* function together in the determination of lateral mesoderm, from which gonadal mesoderm and fat body are derived (Broihier et al., 1998).

The Drosophila fat body is an organ composed of adipose tissue that is thought to function as the fly equivalent of the mammalian liver (Rizki, 1978). It is a mesodermally-derived structure, which like gonadal mesoderm, arises from the *eve* domain of the mesoderm (Azpiazu et al., 1996; Riechmann et al., 1997). Although a number of markers have been identified that are expressed in fat body precursors at various embryonic stages (Abel et al., 1993; Hoshizaki et al., 1994; Rehorn et al., 1996), little is known about the developmental and genetic steps leading toward the specification of this cell type. One fat body marker with a known developmental function is the *serpent (srp)* gene. *srp* encodes a GATA family member transcription factor that is expressed in fat body precursors from

stage 10 throughout embryogenesis (Abel et al., 1993; Rehorn et al., 1996). In *srp* mutants, fat body precursors form, but fail to proliferate and differentiate (Rehorn et al., 1996).

Although these combined studies have provided valuable information toward understanding the developmental programs required for both gonadal mesoderm and fat body, many questions regarding the origin and specification of these two cell-types remain unanswered. We present here an analysis of gonadal mesoderm development as it relates to the development of the embryonic fat body. Our studies indicate that both tissues are found at identical D-V positions within different parasegments, and initially follow a common developmental path relying on the same subset of genes. In addition, we show that the *abdA*, *AbdB*, and *srp* genes determine the cell fate decision between gonadal mesoderm and fat body cell fates along the A-P axis. Our results show that *srp* promotes fat body development, while *abdA* allows gonadal mesoderm to develop by negatively regulating *srp* function.

MATERIALS AND METHODS

Fly stocks

The following alleles were used for all phenotypic analyses: *abdA*^{MX1} and *AbdB*$^{D101.3}$ were both provided by Welcome Bender. There is no detectable protein in *abdA*^{MX1} mutants, and therefore is thought to approximate the null phenotype (Karch et al., 1990). *AbdB*$^{D101.3}$ is a point mutation that is phenotypically null (I. Duncan, personal communication). *cli*^{2D} is a strong allele obtained from Bloomington stock center (Boyle et al., 1997). *dpp*^{H46} was provided by Vern Twombly and Bill Gelbart. This allele contains a deletion within the locus and is thought to approximate the null phenotype (St. Johnston et al., 1990). *srp*^{9L} was obtained from the Bloomington stock center and behaves as a phenotypic null (Reuter, 1994). *tin*$^{\Delta GC14}$ [Df(3R)GC14] was obtained from Manfred Frasch and is a deletion removing the entire locus (Azpiazu and Frasch, 1993). For analysis of lack of *zfh-1* function, embryos transheterozygous for *zfh-1*$^{75.26/65.34}$ were used, and in the *tin,zfh-1* and *cli;zfh-1* double mutant strains, *zfh-1*$^{75.26}$ was used. Both *zfh-1* alleles show no detectable protein in embryos, and behave as phenotypic nulls (Broihier et al., 1998). The *abdA srp* double mutant was constructed using the *abdA*$^{D100.24}$ and *srp*^{9L} alleles. The *abdA*$^{D100.24}$ allele behaves as a phenotypic null (I. Duncan, personal communication.)

The *hsp70-abdA* line was obtained from Gines Morata through Monica Boyle. Ectopic *abdA* function was induced by heat shocking embryos at 4 and 6 hours of development according to the method of Boyle and DiNardo (1995). Embryos were fixed and antibody stained as described below.

Antibody staining

The following antibodies were used in immunostaining of embryos: rabbit polyclonal anti-β-galactosidase (Cappel), mouse monoclonal anti-Cli (provided by Nancy Bonini), rabbit polyclonal anti-Srp (provided by Rolf Reuter), and mouse polyclonal anti-Zfh-1 (provided by Zichun Lai). Prior to use, the anti-β–galactosidase and secondary antibodies (see below) were pre-absorbed against an overnight collection of wild-type embryos.

Antibody detection was performed with either horseradish peroxidase using a biotinylated secondary antibody (Jackson ImmunoResearch) and the Elite Kit (Vector Labs), or with a directly conjugated alkaline-phosphatase secondary antibody (Jackson ImmunoResearch). Embryos were fixed and devitellinized according to the method described in Gavis and Lehmann (1992), with the modification that 1XPBS and 50 mM EDTA were used in place of PEMS during the fixation. Embryos were rehydrated and subjected to antibody staining as described in Eldon and Pirrotta (1991). For whole-mount

analysis, embryos were mounted onto slides in PolyBed812 (Polysciences) according to Ephrussi et al. (1991), then analyzed with a Zeiss Axiophot microscope using Nomarski optics.

RESULTS

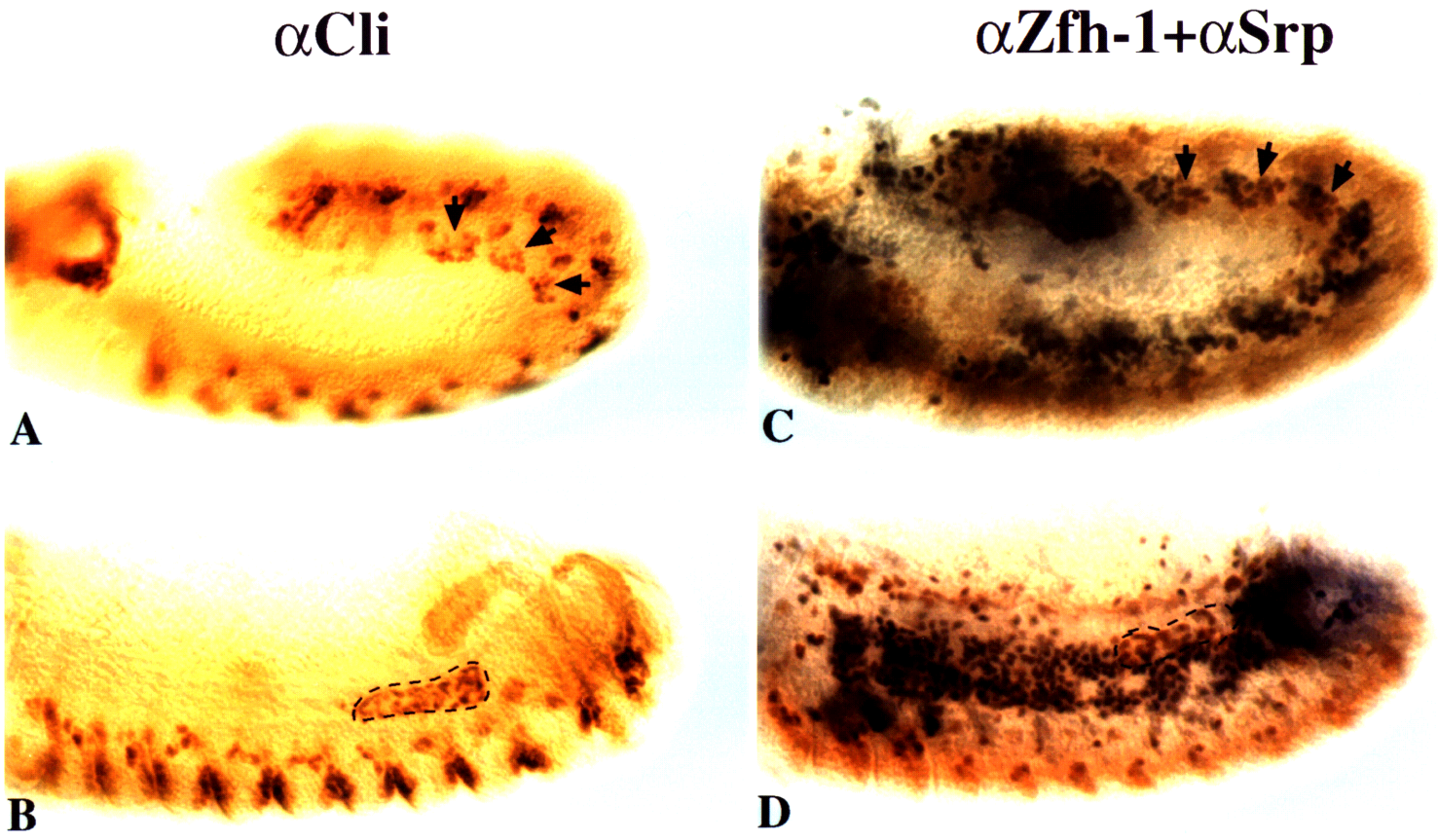
Gonadal mesoderm and fat body precursors share identical positions in different parasegments

It has been recently shown that both gonadal mesoderm and fat body are derived from within the *eve* domain of the mesoderm (Moore et al., 1998; Riechmann et al., 1997). Moreover, in this domain, both tissues arise from clusters of lateral-mesodermal cells as defined by expression of Zfh-1 protein at stage 10 of embryogenesis (Broihier et al., 1998). Since gonadal mesoderm is derived only from PS10-12 (Boyle et al., 1997 and Fig. 4-1A,B), we wanted to investigate in more detail the spatial relationship between gonadal mesoderm and fat body within different parasegments. We found that in PS4-9 and PS13, precursors of the embryonic fat body, as visualized by expression of the Srp protein (Abel et al., 1993; Rehorn et al., 1996), are found in the identical D-V position as SGPs, visualized by high levels of Zfh-1 protein, in PS10-12 (Fig. 4-1). We refer to this region of the fat body and gonadal mesoderm collectively as dorsolateral mesoderm. In all parasegments, additional fat body precursors arise in an area ventral to where the SGPs form in PS10-12 (Fig. 4-1C,D). These cells we have collectively termed ventrolateral mesoderm. As a consequence, while ventral fat body is specified in all parasegments, there is a dorsal gap within the developing fat body in PS10-12 in which SGPs are specified (Fig. 4-1C,D). Therefore, in PS4-9 and PS13, only fat body develops from lateral mesoderm, whereas in PS10-12, both gonadal mesoderm and fat body are specified. We confirmed these results by sectioning embryos stained with markers recognizing both gonadal mesoderm and fat body.

Figure 4-1: Gonadal mesoderm and fat body precursors occupy identical positions within different parasegments.

Anterior left in all panels; lateral views. Embryos in panels A and C are at stage 11; embryos in panels B and D are at stage 13 (stages according to Campos-Ortega and Hartenstein, 1997). (A,B) Somatic gonadal precursors (SGPs) highlighted using an anti-Cli antibody (A, arrows; B, stippled circle). (C,D) SGPs highlighted in brown using an anti-Zfh-1 antibody (C, arrows; D, stippled circle); fat body precursors visualized in blue using an anti-Srp antibody. In PS10-12, SGPs occupy the most dorsal region of staining, whereas fat body precursors are found in more ventral and in (C), posterior areas. The ventral and posterior fat body precursors in (C) most likely give rise to the ventral fat body cells in (D). In PS4-9 and PS13, fat body precursors span the entire region highlighted by staining.

Fig. 4-1. Gonadal mesoderm and fat body develop in similar positions in different parasegments



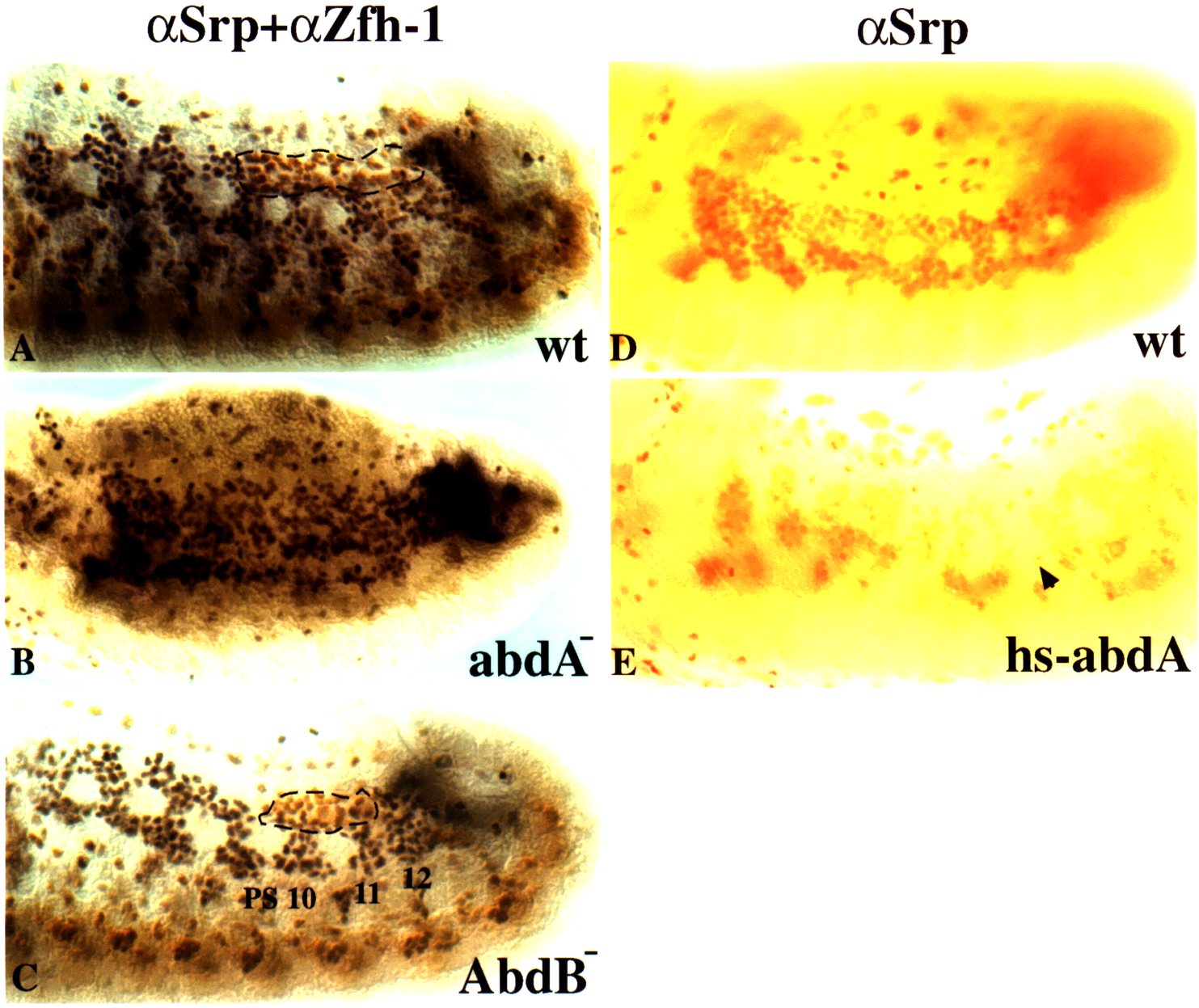
Control of gonadal mesoderm versus fat body cell fate along the A-P axis

The observations that gonadal mesoderm is only specified from dorsolateral mesoderm in PS10-12, and that fat body develops in the same D-V position in PS4-9 and PS13 led us to investigate what controls the cell fate decision between gonadal mesoderm and fat body along the A-P axis. It has been shown that the homeotic genes *abdA* and *AbdB* are required for the specification of gonadal mesoderm, with *abdA* required for gonadal mesoderm in PS10-12, and *AbdB* required only in PS12 (Boyle and DiNardo, 1995). We find that in *abdA* mutants, Srp-expressing cells are found in the region normally occupied by gonadal mesoderm (Fig. 4-2B; compare with 4-2A). Moreover, in embryos lacking *AbdB* function, Srp-expressing cells are now observed in PS12 (Fig. 4-2C; compare with 4-2A). This suggests that in wild-type embryos, *abdA* and *AbdB* function to repress *srp* expression in PS10-12. The *srp* gene has been shown to be required for the proliferation and morphogenesis of fat body precursors (Rehorn et al., 1996). Therefore, *abdA* and *AbdB* result in the inhibition of fat body development within these parasegments. Previous work has shown that ectopic expression of *abdA* in parasegments anterior to PS10-12 results in an expansion of gonadal mesoderm into these parasegments (Boyle and DiNardo, 1995). We find that ectopic *abdA* also represses Srp expression in these same anterior parasegments, suggesting that *abdA* promotes gonadal mesoderm at the expense of fat body (Fig. 4-2E; compare with 4-2D). We also find that ectopic *abdA* activity represses Srp expression even in ventrolateral regions of PS10-12 (Fig. 4-2E, arrowhead; see Discussion). Taken together, these results demonstrate that *abdA* and *AbdB* play key roles in directing the developmental decision between gonadal mesoderm and fat body cell fates along the A-P axis.

Figure 4-2. *abdA* and *AbdB* promote gonadal mesoderm development at the expense of fat body.

Anterior left in all panels; lateral views. (A-C) SGPs highlighted using an anti-Zfh-1 antibody (brown, stippled circles); fat body precursors visualized using an anti-Srp antibody (blue). (D,E) Fat body precursors identified using an anti-Srp antibody (brown). (A,D) Wildtype. (B) *abdA*$^-$. SGPs are absent, and have been replaced by fat body precursors. (C) *AbdB*$^-$. SGPs have been replaced by fat body precursors in PS12 (see designations under stained cells), where *AbdB* is known to function (Boyle and DiNardo, 1995). (E) *hs-abdA*. Fewer fat body precursors are found in PS8-9 than in wildtype. This is precisely where ectopic gonadal mesoderm has been found to develop in *hs-abdA* embryos (Boyle and DiNardo, 1995). Inhibition of *srp* expression extends into ventral regions of the fat body tissue in PS10 (arrow). This area has not been shown to be occupied by SGPs in *hs-abdA* embryos (Boyle and DiNardo, 1995; see Discussion).

Fig. 4-2. *abdA* and *AbdB* determine gonadal mesoderm versus fat body identity in PS10-12



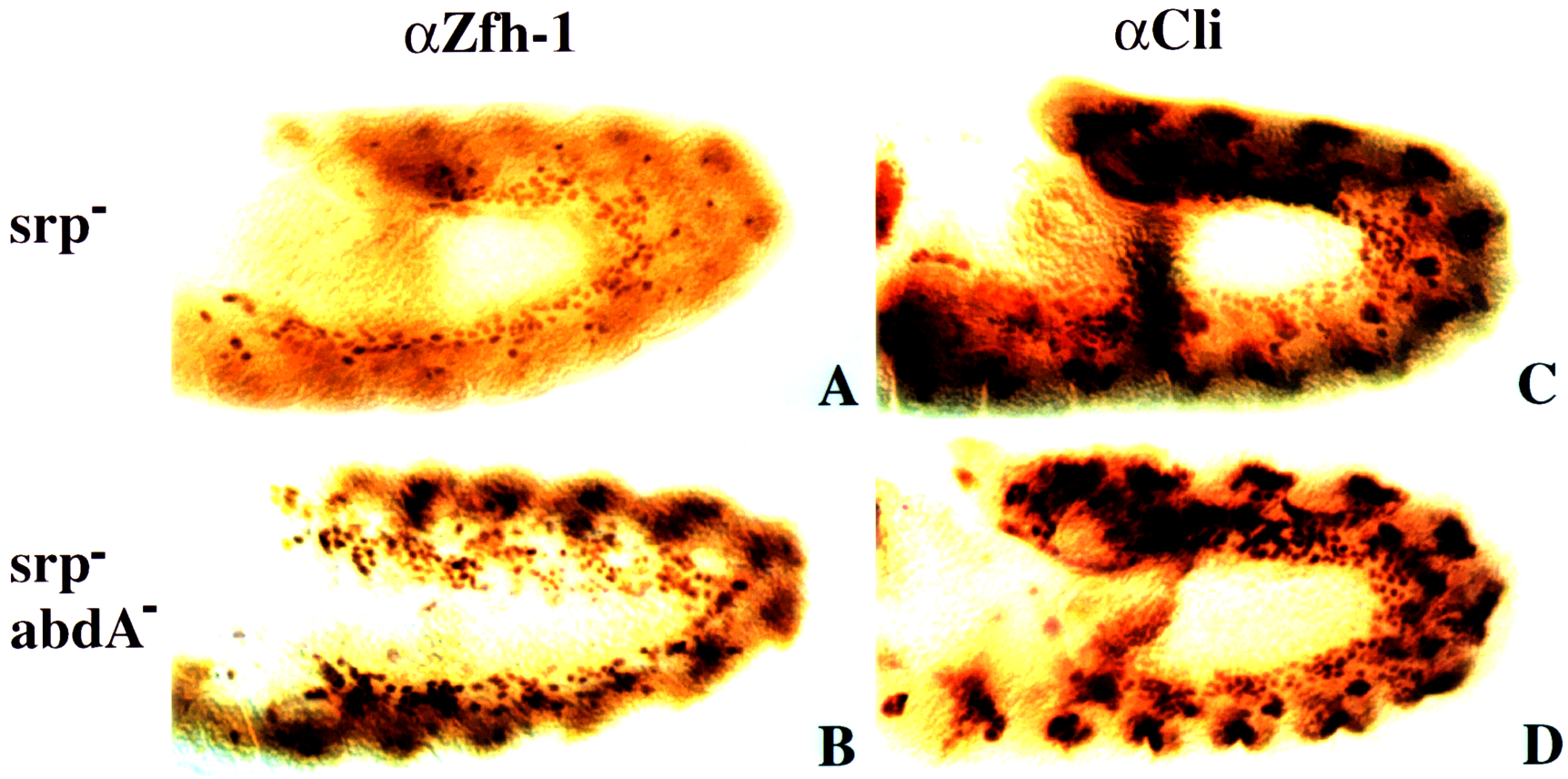
We next wanted to determine if the reciprocal cell fate transformation of fat body into gonadal mesoderm could occur by removing a gene activity required for the development of fat body. One gene known to be required for fat body development is the *srp* gene (Rehorn et al., 1996). We therefore analyzed the expression of two gonadal mesoderm cell markers, Zfh-1 and Cli proteins, in *srp* mutant embryos. By stage 11 in wild-type embryos, high levels of Zfh-1 protein are found in gonadal mesoderm (PS10-12), whereas low levels are expressed in other parasegments (Broihier et al., 1998 and Fig. 4-1C). However, we find that in *srp* mutant embryos, high levels of Zfh-1 are expressed in every parasegment (Fig. 4-3A), suggesting that aspects of gonadal mesoderm development are occurring in place of fat body development. Like Zfh-1, Cli protein expression in lateral mesoderm is only found in the gonadal mesoderm within PS10-12 in wild-type embryos (Fig. 4-1A). In embryos lacking *srp* function, Cli expression is expanded anteriorly and posteriorly, indicating that gonadal mesoderm cell types now develop in these parasegments (Fig. 4-3C; compare with Fig. 4-1A). Taken together, these results demonstrate that *srp* activity results in the repression of gonadal mesoderm development outside of PS10-12 and therefore, like *abdA*, plays a role in the decision between gonadal mesoderm and fat body cell fates. Thus, the combined results of the effect of *abdA, AbdB*, and *srp* on the development of fat body and gonadal mesoderm suggest that a switch mechanism is involved in specifying gonadal mesoderm versus fat body cell fates along the A-P axis. *abdA* and *AbdB* switch "off" fat body cell fate, thereby allowing gonadal mesoderm development, whereas *srp* is involved in a mechanism switching "off" gonadal mesoderm identity and "on" the developmental program toward fat body differentiation.

Our results demonstrating that *srp* is expressed in dorsolateral mesoderm within PS10-12 in *abdA* mutants suggests that *abdA* normally acts upstream of *srp* to negatively affect its expression within this region. In order to directly test the epistatic relationship between *abdA* and *srp*, we investigated the effect of removing the activities of both genes on the development of dorsolateral mesoderm. We found that like embryos lacking *srp* function alone, embryos mutant for both *abdA* and *srp* express gonadal mesoderm-specific markers in PS4-13 (Fig. 4-3B,D). Thus, *abdA* acts upstream of *srp* to negatively regulate its function, thereby allowing the development of gonadal mesoderm. This result further demonstrates that in the absence of fat body development, *abdA* is no longer necessary for the specification of SGPs.

Figure 4-3. Expression domains of gonadal mesoderm markers are expanded in *srp* and *abdA srp* mutants.

Anterior left in all panels; lateral views. All embryos are at approximately stage 11. (A,C) *srp*$^{-}$. (B,D) *abdA*$^{-}$ *srp*$^{-}$. (A,B) Expression of *zfh-1* (brown) using an anti-Zfh-1 antibody. In wild type, high levels of Zfh-1 mesodermal protein are only found in PS10-12 (see Fig. 4-1C, arrows). In *srp* and *abdA srp* mutants, high levels are detected in all parasegments. (C,D) Cli expression (brown) using an anti-Cli antibody. Cli mesodermal protein expression is only detected in PS10-12 in wild-type embryos (see Fig. 4-1A, arrows). In *srp* and *abdA srp* mutants, Cli expression expands anteriorly and posteriorly.

Fig. 4-3. *abdA* inhibits *srp* function to allow gonadal mesoderm development



Gonadal mesoderm and fat body share common genes for their development

The observation that at stage 13 the dorsal fat body and gonadal mesoderm occupy the same D-V position within different parasegments suggests that they may be developmentally closely-related tissues. In principle, these two tissues could develop by two different mechanisms. On the one hand, at the time that *zfh-1* and *tin* define lateral mesoderm, gonadal mesoderm and fat body could be already specified as distinct cell types. This might be manifested in the requirement of different genes for early steps in the development of each tissue. Conversely, precursors of the gonadal mesoderm and fat body could initially follow the same developmental pathway, therefore requiring the same genes, and only later would follow alternate paths toward gonadal mesoderm or fat body development.

In an effort to distinguish between these two possibilities, we analyzed fat body development in embryos lacking the functions of genes required for gonadal mesoderm development that were identified in a screen for mutations affecting germ cell migration in Drosophila (Moore et al., 1998). We find that the *zfh-1*, *tin*, and *cli* genes are all required for fat body development (Fig. 4-4). In *zfh-1* mutants, fewer fat body precursors develop, often resulting in gaps within the developing tissue (Fig. 4-4A). *tin* is also required for the proper number of fat body cells to develop correctly. In *tin* mutant embryos, the bridges of fat body cells that normally span the parasegments at stage 13 fail to form (Fig. 4-4B, arrow). At this stage, fat body tissue instead remains in a state that morphologically resembles a structure normally seen at stage 12. Previous work has shown that *tin* does not depend on *dpp* for its function in gonadal mesoderm development, suggesting that it is the early, *dpp*-independent expression of *tin* throughout the mesoderm that is necessary for gonadogenesis (Broihier et al., 1998). We have found that in *dpp* mutants, fat body cells do develop, although the morphology of the fat body structure is difficult to assess given the severe developmental defects associated with this genetic background (Fig. 4-4C). However, a larger number of Srp-expressing cells are present in *dpp* mutants than in embryos lacking *tin* function (compare Fig. 4-4C with Fig. 4-4B), implicating the early function of *tin* in the pathway leading toward the specification of fat body as well as gonadal mesoderm. Whereas *tin* and *zfh-1* act at an early stage in gonadal mesoderm development, the *cli* gene is later required for the differentiation of this tissue (Boyle et al., 1997; Broihier et al., 1998; our observations). We find that *cli* also affects differentiation of the fat body. In *cli* mutants, fat body precursors form, but do not differentiate into the characteristic "ladder" structure found at this stage in wild-type embryos (Fig. 4-4D).

It has been shown that the above genes can be placed into a genetic hierarchy based on epistasis experiments. We have shown that gonadal mesoderm is completely absent, and

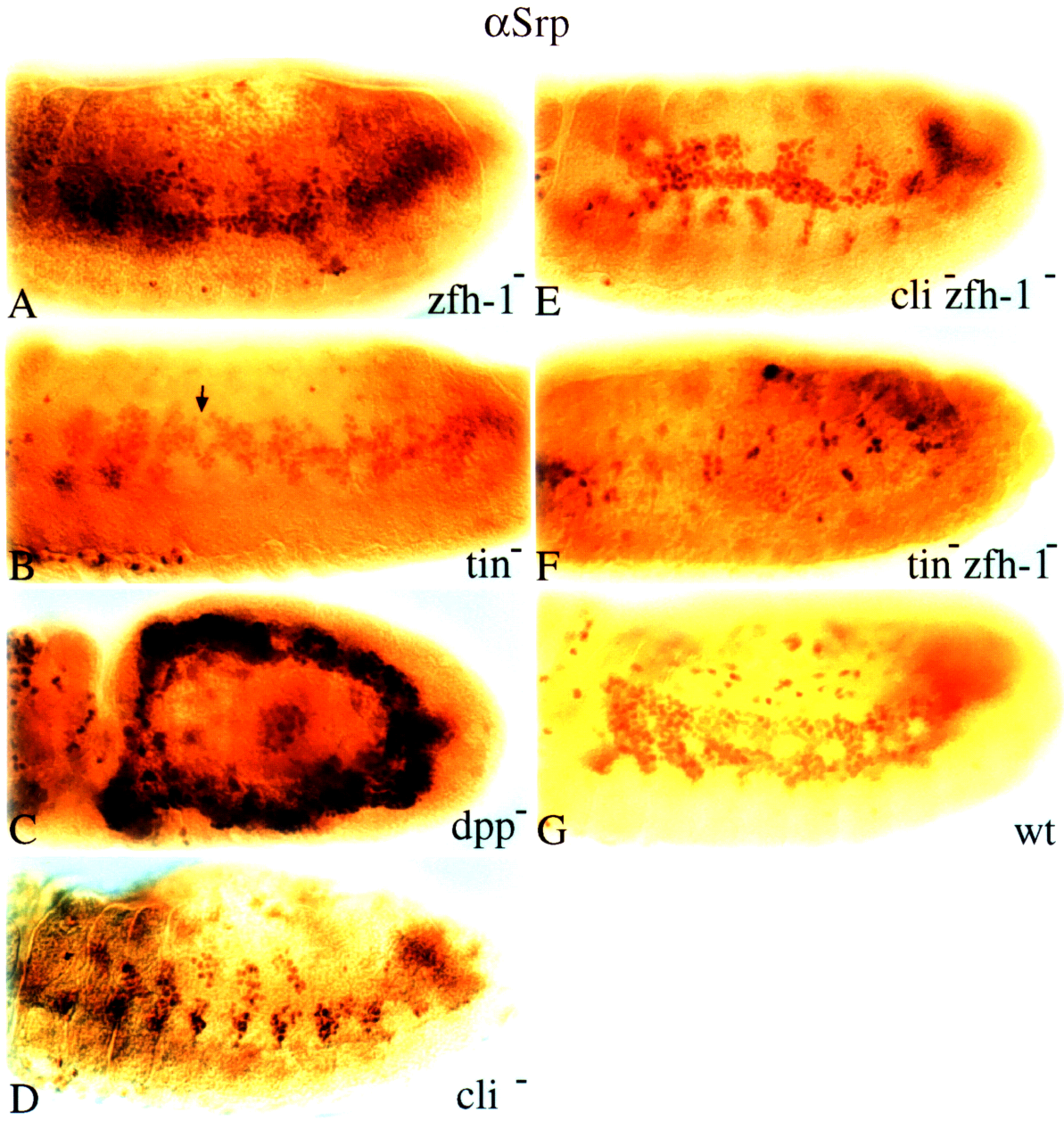
that the number of fat body precursors is virtually abolished, in embryos lacking both *zfh-1* and *tin* function (Broihier et al., 1998 and Fig. 4-4F). Because the phenotypes seen in the double mutant are more severe than those observed in either single mutant, it can be concluded that *tin* and *zfh-1* share overlapping functions in the development of both tissues. It has been recently shown that *cli* expression in gonadal mesoderm is markedly reduced in embryos lacking *zfh-1* activity. In *cli;zfh-1* double mutants, the gonadal mesoderm defect is identical to that seen in *zfh-1* single mutants. Taken together, these results indicate that *zfh-1* acts upstream of *cli* in gonadogenesis (Broihier at al., 1998). We have also examined the effect of removing both *cli* and *zfh-1* function on the development of the fat body. In *cli;zfh-1* double mutants, the defect observed in the fat body is identical to that seen in *zfh-1* mutants alone (compare Fig. 4-4E with Fig. 4-4A). Interestingly, a larger number of fat body cells are present in the double mutant than in *cli* single mutants (compare Fig. 4-4E with 4-4D; see Discussion). These results suggest that *zfh-1* and *cli* interact similarly in both gonadal mesoderm and fat body development.

Thus, we have demonstrated that many genes required for development of gonadal mesoderm act in a similar manner to direct fat body development. These combined results favor the hypothesis that steps leading to the specification of gonadal mesoderm and fat body initially occur via the same genetic pathway.

Figure 4-4. Genes required for gonadal mesoderm development have a similar requirement in the development of the fat body.

Anterior left in all panels; lateral views. All embryos are approximately at stage 13. (A-G) Fat body development visualized using an anti-Srp antibody. (A-F) Mutants; (G) Wildtype. (A) *zfh-1*$^{-}$. The number of fat body cells is reduced when compared to (G), often resulting in gaps within the tissue. (B) *tin*$^{-}$. The characteristic bridges of fat body cells between parasegments fail to form (arrow). This structure resembles that seen in wild-type stage 12 embryos. (C) *dpp*$^{-}$. Fat body precursors develop, although the tissue morphology cannot be assayed due to the severe developmental defects associated with these embryos. However, the number of fat body cells observed is greater than in *tin* mutants (compare with panel B). (D) *cli*$^{-}$. Fat body precursors form, but fail to differentiate into the proper structure. (E) *cli*$^{-}$; *zfh-1*$^{-}$. Fewer fat body cells develop, resulting in a phenotype indistinguishable from that seen in *zfh-1* mutants (compare with panel A). Interestingly, this phenotype is less severe than that observed in *cli* mutants (compare with panel D, see DISCUSSION). (F) *tin*$^{-}$, *zfh-1*$^{-}$. Fat body cells are virtually abolished.

Fig. 4-4. Genes required for gonadal mesoderm development act similarly in fat body development



DISCUSSION

Previous work has shown that both gonadal mesoderm and the fat body develop from lateral mesoderm derived from the *eve* domain of the mesoderm (Broihier et al., 1998; Moore et al., 1998; Riechmann et al., 1997). Our analysis suggests that prior to their specification as distinct cell types, gonadal mesoderm and fat body precursors exist as a common pool of cells that require a unique set of genes for their determination and development.

Positional relationship between gonadal mesoderm and fat body

We have shown that gonadal mesoderm and dorsal fat body precursors are found in identical positions in different parasegments from stages 11-13 of embryogenesis. Specifically, we found that in PS4-9, the dorsal component of fat body develops in the same D-V location where gonadal mesoderm forms in PS10-12. Moreover, in PS10-12, fat body precursors are found immediately ventral to where gonadal mesoderm develops. These observations provide the first evidence suggesting that gonadal mesoderm and fat body are developmentally closely-related tissues.

Genetic relationship between gonadal mesoderm and fat body

Our results demonstrate that mutations in genes disrupting gonadal mesoderm development have similar phenotypic consequences on the development of the fat body. Moreover, we found that the genetic hierarchy controlling gonadal mesoderm development is the same as that functioning in the development of the fat body. These results provide further evidence that both tissues follow a common developmental pathway.

We have shown that *tin* and *zfh-1* are required for the development of both gonadal mesoderm and fat body. Moreover, we have demonstrated that both tissues require the *dpp*-independent expression of *tin* throughout the mesoderm that occurs before stage 10 of embryogenesis. This is consistent with the observation that *tin* expression cannot be detected in lateral mesoderm from stage 10 onward (Azpiazu and Frasch, 1993). However, the effect of loss of *tin* function on both gonadal mesoderm and fat body cannot be detected until later embryonic stages. It is only when both *tin* and *zfh-1* are simultaneously removed that the formation of gonadal mesoderm and fat body is virtually abolished, revealing the early and overlapping functions of both genes in the developmental pathways of both tissues. These partially redundant functions for *tin* and *zfh-1* suggest that *zfh-1* acts at the same time as *tin* in the determination of lateral mesoderm. This is consistent with the fact that like *tin*, *zfh-1* is expressed throughout the mesoderm prior to

stage 10 (Lai et al., 1991). Therefore, we propose that prior to stage 10, gonadal mesoderm and fat body have not yet been specified, but exist as a population of precursor cells requiring the functions of both *tin* and *zfh-1*. This is further supported by the observation that at stage 10, Zfh-1 is expressed at uniform levels in lateral mesoderm within PS4-12, whereas high levels of Zfh-1 expression specifically in PS10-12 are not detected until stage 11 (Broihier et al., 1998). Furthermore, the expression of all known gonadal mesoderm and fat body cell-specific markers is only observed during or after stage 10 (Boyle et al., 1997; Broihier et al., 1998; Riechmann et al., 1997).

The *cli* gene is also required for both gonadal mesoderm and fat body development. However, in contrast to *tin* and *zfh-1*, *cli* does not affect the determination of lateral mesoderm. In *cli* mutants, precursors of both gonadal mesoderm and fat body form but do not differentiate (Boyle et al., 1997; this work). This demonstrates that like *zfh-1* and *tin*, *cli* affects both fat body and gonadal mesoderm development in a similar manner. It is not known at what point the *cli* gene is required in the development of either tissue. Cli protein is found throughout the mesoderm prior to stage 11, but becomes specifically expressed in SGPs at later timepoints. In contrast, Cli protein cannot be detected in fat body cells once they have been specified. Therefore, there are two ways by which *cli* could be involved in the development of gonadal mesoderm and fat body. One possibility is that *cli* is required before stage 11 for both tissues, but does not belie its function until later stages in development. A precedence for this type of gene behavior has been shown through our studies of *tin* (see above). In this model, *cli* could also play a role in gonadal mesoderm development at later embryonic stages, consistent with its expression pattern in SGPs throughout embryogenesis. Conversely, *cli* could function early in fat body development, but not play a role in gonadal mesoderm development until later in the differentiation of this tissue. Although both explanations are formally possible, our results favor the first model. The fact that *cli* mutants have similar effects on both fat body and gonadal mesoderm development suggests that *cli* functions at a stage before gonadal mesoderm and fat body have been specified as unique cell types. Moreover, the *cli* expression pattern indicates that it is unlikely to function in fat body development after stage 10. We cannot at this point discern whether or not *cli* continues to play a role in gonadal mesoderm development after this stage.

Our analysis of fat body development in *cli;zfh-1* double mutants demonstrates that these two genes interact in a similar manner for both gonadal mesoderm and fat body development, further indicating that the two tissues initially follow a common genetic pathway. However, it is surprising that more fat body cells develop in the *cli;zfh-1* double mutant than in *cli* single mutant embryos. Interestingly, a similar result has been observed

using the 412 retrotransposon as a marker for gonadal mesoderm development. In *cli* mutants, fewer 412-expressing cells are detected than in *zfh-1* mutants, whereas the double mutant is indistinguishable from embryos lacking *zfh-1* function alone (Broihier and Lehmann, unpublished observations). These results indicate that lack of *zfh-1* activity bypasses *cli*'s requirement in both gonadal mesoderm and fat body development. One possible explanation for this result is that without *zfh-1* function, both cell types are developmentally stalled at a stage before *cli* activity is required. Therefore, loss of *cli* function does not affect these precursor cells, and they behave as in *zfh-1* single mutants. However, the fact that residual gonadal mesoderm and fat body cells still express tissue-specific markers in *zfh-1;cli* double mutants suggests that some aspects of differentiation proceed in these cells. Further investigation into the functional relationship between *zfh-1* and *cli* will be necessary to address these observations.

Our results further suggest that precursors of gonadal mesoderm and fat body are determined independently of the visceral mesoderm, another *eve*-domain derivative. Although *zfh*-1 and *cli* are required at an early stage for both gonadal mesoderm and fat body development, neither is necessary for visceral mesoderm formation. In addition to its role in visceral mesoderm specification (Azpiazu and Frasch, 1993; Bodmer, 1993), we have shown that *tin* is also required for both gonadal mesoderm and fat body development. However, we have demonstrated that these latter functions for *tin* are dependent on its early, ubiquitous expression throughout the mesoderm. This is in contrast to previous work demonstrating that dorsally restricted *tin* expression is necessary for visceral mesoderm formation (Frasch, 1995; Staehling-Hampton et al., 1994). Therefore, *tin*'s role in visceral mesoderm specification is distinct from its requirement in the development of gonadal mesoderm and fat body. Given that *zfh-1, tin* and *cli* all appear to function in gonadal mesoderm and fat body development before stage 10, our results suggest that at this stage, the developmental pathways leading toward gonadal mesoderm and fat body versus visceral mesoderm specification have already diverged.

Control of decision between gonadal mesoderm and fat body cell fates

We have shown that the transcription factors *abdA*, *AbdB*, and *srp* are key players in the control of gonadal mesoderm versus fat body development along the A-P axis. In principle, two different mechanisms could account for the initial specification of each cell type within a parasegment. The first possibility is that *abdA* and *srp* could merely act to promote gonadal mesoderm and fat body development, respectively, with no effect on the alternate tissue. Therefore, loss of function of these genes would result in lack of cell differentiation, and possibly cell death. The second possibility is that *abdA* and *srp* also

function in repressing development of the alternate cell type, thereby creating a switch mechanism that chooses either gonadal mesoderm or fat body cell fates. Our results favor the latter hypothesis. In *abdA* mutants, fat body develops in place of gonadal mesoderm. In *srp* mutants, gonadal mesoderm markers are expressed where fat body normally develops. Moreover, ectopic *abdA* promotes gonadal mesoderm at the expense of fat body development in the dorsal component of lateral mesoderm. Therefore, the progeny of dorsolateral mesoderm cells either give rise to gonadal mesoderm or fat body along the A-P axis, depending on the presence or absence of *abdA* and *srp*.

Our results from the *abdA srp* double mutant demonstrate that the development of gonadal mesoderm from dorsolateral mesoderm in PS10-12 is executed through *abdA*-dependent negative regulation of *srp* function in this region. It is not known at what level this regulation occurs, although a likely possibility is that *abdA* directly affects *srp* transcription, given that *abdA* encodes a homeodomain protein (Karch et al., 1990). The phenotype observed in the *abdA srp* double mutant also shows that aspects of gonadal mesoderm development can occur in the absence of *abdA* activity, as long as fat body development is abolished. This suggests that the developmental "ground state" of dorsolateral mesoderm is gonadal mesoderm. In light of this theory, it is interesting that in other insect species as well as in many chilopods (centipedes), gonadal primordium develop from multiple abdominal segments. Most strikingly, in *Pyrrhocoris apterus* (fire beetle), gonadal tissue arises in abdominal segments one through eight (reviewed in Johannsen and Butt, 1941; Seidel, 1924).

It is unclear what mechanism controls the D-V decision between gonadal mesoderm and fat body within PS10-12. Whereas *abdA* is required to promote gonadal mesoderm versus fat body development in dorsolateral mesoderm in PS10-12, fat body develops from ventrolateral mesoderm within the same parasegments. It is possible that *abdA* expression does not extend into the region where the ventral fat body precursors are found. Alternatively, a ventrally-localized factor analogous to *tin* in the specification of dorsal mesoderm derivatives could inhibit *abdA* function in more ventral regions of lateral mesoderm. The results from the *hs-abdA* experiment argue that a combination of these theories could prove correct. We have demonstrated that ectopic *abdA* expression can inhibit *srp* expression, and therefore fat body development, in more ventral regions of the embryo. This suggests that in wild-type embryos, *abdA* is either not present, or is not active in the more ventral cells. However, gonadal mesoderm does not develop in place of fat body in this ventral region of ectopic *abdA* activity (Boyle and DiNardo, 1995), arguing for a ventrally-localized factor that inhibits some aspects of *abdA* function in these cells. These results suggest that prior to *abdA* function, D-V differences within lateral mesoderm

cells have already occurred. Further analysis of the spatial pattern of *abdA* expression in the mesoderm may help to address how these differences along the D-V axis are generated.

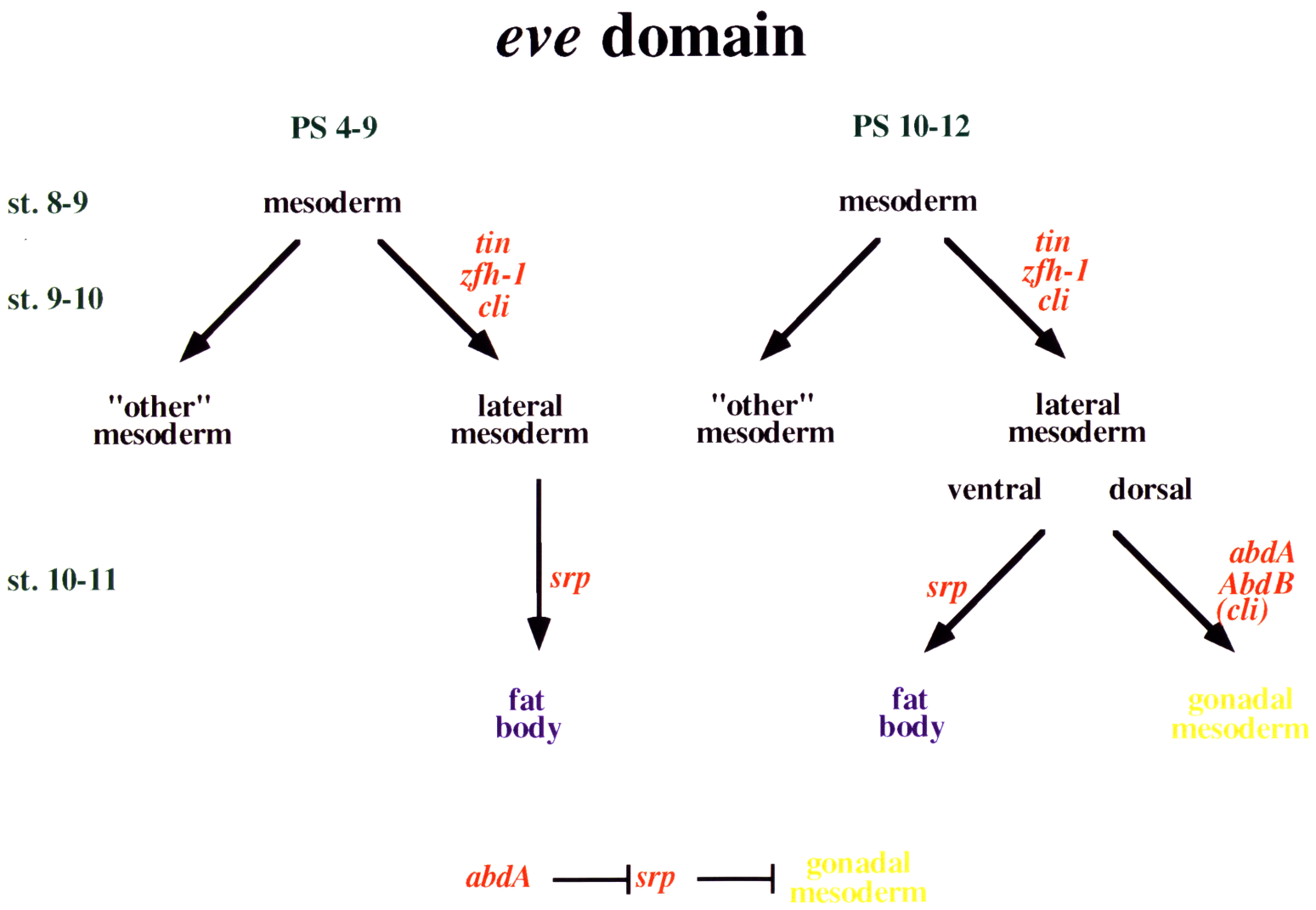
A model for gonadal mesoderm and fat body development

Our combined results lead to a developmental and genetic model of the pathway toward specification of two mesodermal tissues, the gonadal mesoderm and embryonic fat body (Fig. 4-5). Since both tissues are derived from within the *eve* domain of the mesoderm, we will only focus on this mesodermal component. During germ band extension (stage 8-9), the early functions of both *tin* and *zfh-1* determine lateral mesoderm while other mesodermal sub-types, including those that will become visceral mesoderm, are determined as distinct cell-populations. The *cli* gene then renders lateral mesoderm cells competent to fully differentiate into either fat body or gonadal mesoderm identity. During late stage 10, the combined functions of *abdA, AbdB*, and *srp* control the decision between gonadal mesoderm and fat body cell fates along the A-P axis. In PS10-12, *abdA* and *AbdB* function to repress *srp* expression in dorsolateral mesoderm, thereby allowing gonadal mesoderm development in this location. Ventrolateral mesoderm cells, expressing *srp*, develop into fat body. In PS4-9, *abdA* function is absent, resulting in all lateral mesoderm cells adopting a fat body cell fate. It is possible that *cli* has an additional role in gonadal mesoderm development at this stage. Presumably other factors, such as those determining D-V differences within lateral mesoderm cells and their derivatives, remain to be identified. Taken together, these studies provide a model for the events leading to the specification of gonadal mesoderm and fat body cell fates that includes gene functions, developmental steps, and regulatory interactions.

Figure 4-5. Model for lateral mesoderm development within the *eve* domain of the mesoderm.

In PS4-9, *tin*, *zfh-1*, and *cli* are required before stage 10 to determine lateral mesoderm. During stages 10-11, *srp* is involved in specifying fat body cell fates. In PS10-12, the same genes are required before stage 10 for lateral mesoderm determination. During stages 10-11, the combined functions of *abdA* and *AbdB* specify gonadal mesoderm identity by repressing fat body development in dorsolateral mesoderm. *abdA* executes this function through negative regulation of *srp* . It is possible that *cli* has a continued requirement in gonadal mesoderm development at this stage. *srp* is required for specifying fat body identity in ventrolateral mesoderm. Although *abdA, AbdB*, and *srp* are all required for specifying gonadal mesoderm and fat body cell fates, respectively, along the A-P axis, it is not known what controls the cell fate decision between these two tissues along the D-V axis.

Fig. 4-5: Model for lateral mesoderm development in Drosophila



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CHAPTER 5

Discussion

Drosophila germ cell migration: implications for a mouse model

The genetic screens presented in this thesis were successful in outlining the steps required for germ cell migration in Drosophila, as well as the identification of genes and developmental processes necessary for the specification and differentiation of the somatic gonadal mesoderm. However, it remains to be determined if any of the genes discovered through these screens play a role in actively guiding the germ cells to their proper target cells, the SGPs. For example, no putative ligand/receptor interactions analagous to the SCF/c-kit paradigm in the mouse have been identified in the Drosophila system. Efforts to identify the homologous factors in the fly via molecular techniques have so far proven unsuccessful (A. Forbes, personal communication). It may be that these putative signaling molecules in the fly are required for many other developmental processes and have a large maternal component, thereby rendering their detection through genetic screens for zygotically acting genes virtually impossible. It therefore may be necessary to integrate in vitro studies similar to those used in the mouse into the genetic system that has been used thus far in Drosophila. Preliminary studies have proven that germ cells can be induced to migrate in culture (Jaglarz and Howard, 1995). Efforts are underway in our lab to set up an in vitro migration system to identify chemotactic molecules acting on Drosophila germ cells. Perhaps by using known soluble factors proven to affect mouse germ cell development in culture (see Introduction), the identification of additional factors involved in Drosophila germ cell migration may be possible. Combining these results with the information gained through genetic screens may aid in determining the developmental framework within which these soluble factors are acting.

Although molecules analagous to those shown to act on mouse germ cell development were not identified through our screens in the fly, other developmental

processes discovered through this work may have direct relevance to mammalian systems. Chapter 4 describes how the gonadal mesoderm is a close developmental relative of the fat body, and that both tissues seem to develop from a common primordium. This situation is anatomically similar to that seen in the mouse developing urogenital ridge. In mouse embryos, the urogenital ridge gives rise to both the gonad as well as components of the nephric system. The gonadal component can first be seen as a thickening of the coelomic side of the urogenital ridge. The other side forms the mesonephros, which in the male develops to become an integral component of the developing testes (Buehr et al., 1992; Merchant-Larios et al., 1993). Although a complete analysis of the developmental origin of these two tissues has not been presented, one molecule, the product of the *Wilm's Tumor-1 (WT-1)* gene, is expressed and functions in both the gonad and nephric tissue (Armstrong et al., 1992; Kreidberg et al., 1993; Pelletier et al., 1991). Since germ cells are able to recognize and initially colonize the genital ridge in *WT-1* mutants, it appears that this gene is required for the differentiation, rather than specification, of somatic gonadal tissue (Kreidberg et al., 1993). Perhaps it plays an analagous role to the Drosophila *cli* gene in the differentiation of both gonadal mesoderm and fat body (see Chapter 4; Moore et al., 1998b). Interestingly, in both *WT-1* and *cli* mutants, germ cells initially associate with their somatic partner cells, but then lose this attachment at later developmental stages (Boyle et al., 1997; Kreidberg et al., 1993; Rongo et al., 1998). Further investigation of the developmental relationship bewteen the mouse somatic gonad and mesonephric tissue may reveal more similarities between gonad development in the mouse and fly.

Fat body as "second best tissue"

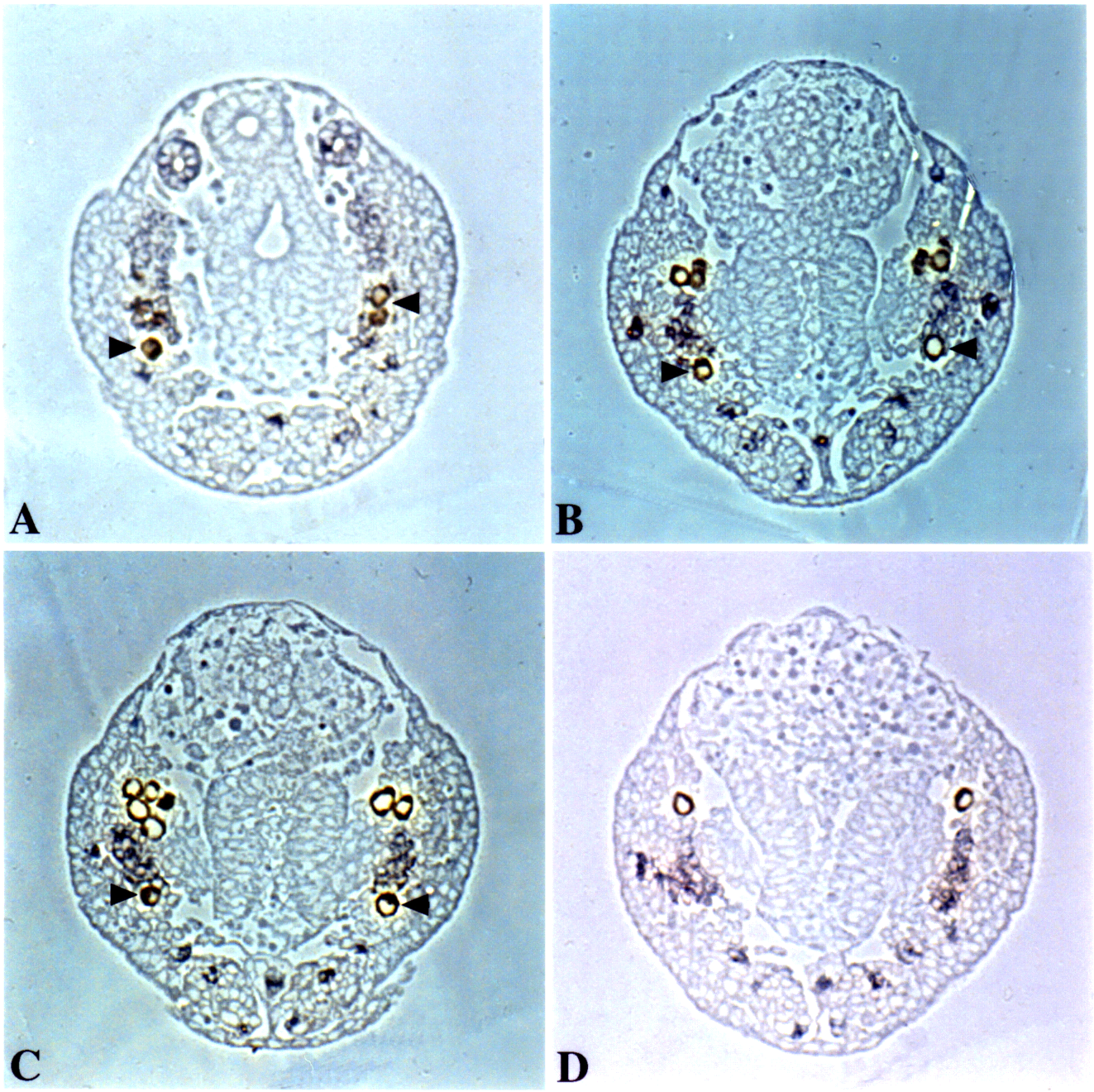
One of the first clues leading to the discovery that Drosophila gonadal mesoderm and fat body are closely-related tissues was that in certain mutant backgrounds, "lost" germ cells that do not coalesce into a gonad are often found within developing fat body tissue. An example of one mutant, *trx*$^{-}$, is shown in Figure 5-1. In this mutant, the posterior germ cells do not associate with SGPs, but instead are found associated with fat body cells (Fig. 5-1A-C, arrowheads). It is unclear if proper specification of fat body identity is required for this phenotype, since the extreme morphological defects associated with *srp* mutants, lacking fat body cells, preclude careful analysis of *srp trx* double mutants. However, it seems plausable that in the absence of properly specified gonadal mesoderm, germ cells preferentially adhere to fat body cells rather than other, more distantly-related mesodermal tissues. In light of this suggestion, it is interesting that lost germ cells in *trx* mutants are often found at the boundary of fat body-somatic musculature primordia (Fig. 5-1B,C, arrowheads). Therefore, it seems as if in *trx* mutants, lost germ cells continue to migrate

through the mesoderm past the area where SGPs normally develop, until they reach a tissue (somatic mesoderm) that is less attractive than the fat body.

Figure 5-1: Lost germ cells associate with fat body cells in *trx* mutant embryos.

(A-D) Serial, 2-micron sections of a stage 13 *trx*$^{-}$ embryo double-labeled with anti-Vas (brown) to detect germ cells, and *svp* antisense RNA (blue) to detect fat body cells. Dorsal is up in all sections. (A) Section taken posterior to where the gonad forms reveals "lost" germ cells associated with fat body cells (arrowheads). (B,C) Two sets of germ cells can be detected, the more ventral group (arrowheads) representing lost germ cells. Note that lost germ cells are found at the ventral border of fat body tissue (see text). (D) Section taken anterior to site of lost germ cells.

Fig. 5-1: Lost germ cells are associated with fat body precursors in *trx* mutant embryos



A similar situation is found in the developing mouse embryo lacking the function of the *Ftz-F1* gene. *Ftz-F1* is required for development of both the gonad and adrenal gland. Like *WT-1*, *Ftz-F1* acts in the differentiation of these tissues, rather than their specification. In *Ftz-F1* mutants, germ cells initially colonize the genital ridge; however, these somatic cells soon degenerate, and germ cells are subsequently found associated with mesonephric tissue (Luo et al., 1994). Given that the somatic gonad may be closely related to the mesonephros, mouse germ cells may preferentially adhere to nephric tissue in the absence of properly developed gonadal tissue.

Determining dorso-ventral boundaries of gonadal mesoderm and fat body development

As discussed in Chapter 4, the genetic mechanisms underlying the specification of dorsolateral versus ventrolateral mesoderm within the developing Drosophila embryo remain to be elucidated. However, preliminary evidence suggests that the *wg* signaling pathway may be involved. It has been shown that *wg* is necessary for the development of *slp* domain mesodermal derivatives (Reichmann et al., 1998; see Chapter 2 Discussion). In *wg* mutants, fat body tissue, an *eve* domain derivative, develops from the *slp* domain as well as the *eve* domain. However, it appears that fewer fat body cells are specified along the D-V axis (Reichmann et al., 1997; Moore and Lehmann, unpublished results). Moreover, gonadal mesoderm fails to develop in *wg* mutants (Boyle et al., 1997; Moore et al., 1998a), suggesting that it is the dorsal component of lateral mesoderm that is missing in this genetic background. Conversely, when *wg* is ectopically expressed via a heat shock promoter after a time when it functions in the *slp* pathway, more SGPs are specified than normally found in wild-type embryos (Boyle et al., 1997; see Chapter 3). Preliminary evidence suggests that this increase in SGP number is due to an expansion of SGP development into ventrolateral mesoderm of PS10-12 at the expense of fat body cell specification (Moore and Lehmann, unpublished results). It remains to be determined whether this effect is due to *wg* signaling directly within the mesoderm, or if this cell specification process occurs through a secondary signal relying on the *wg* signaling pathway.

Although the dorsal boundary of lateral mesoderm specification appears to rely on *dpp* signaling (Reichmann et al., 1998; Broihier and Lehmann, unpublished results), it is unclear what genetic pathway directs the ventral developmental boundary of this tissue. One candidate is the Epidermal Growth Factor (EGF) pathway. It has been recently shown that the development of a particular group of ventral mesodermal cells, the DM cells, is dependent on genes in the EGF pathway. Moreover, overexpression of the ligand acting in

this pathway is sufficient to specify additional DM cells (Lüer et al., 1997). It has been demonstrated that in *dpp* mutants, visceral mesoderm is diminished (Azpiazu and Frasch, 1993; Bodmer, 1993), whereas additional SGPs and fat body cells are specified (Reichmann et al., 1998; Broihier and Lehmann, unpublished results). Therefore, it may follow that in the absence of ventral mesodermal DM cells, lateral mesoderm derivatives may expand into their domain. Preliminary evidence suggests that this is the case. In *faint little ball* (*flb*) mutants, in which the EGF-receptor is inactive, additional gonadal mesoderm as well as fat body cells are specified (Moore and Lehmann, unpublished reults). Although it has yet to be determined if this expansion occurs ventrally, this result suggests that lateral mesoderm derivatives are specified at the expense of more ventral mesoderm cell-types. Additional experiments aimed at determining the direction of this lateral mesoderm expansion are needed, as well as to determine if the EGF pathway signals directly to the mesoderm, or relies on a secondary signal to direct the development of ventral mesodermal cell-types.

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