A GENETIC ANALYSIS OF GERM CELL MIGRATION AND GONADAL MESODERM DEVELOPMENT IN DROSOPHILA

by
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A genetic analysis of germ cell migration and gonadal mesoderm development in Drosophila

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Abstract

Cellular movements play an integral role in metazoan development. During Drosophila embryogenesis, germ cells migrate from their site of formation at the posterior pole toward the somatic gonadal precursors (SGPs), which are the somatic cells which will give rise to the support cells of the ovary or testis. Since there are numerous similarities between germ cell migration in Drosophila and in vertebrate species, genes required for Drosophila germ cell migration may also play a role in the guidance of vertebrate germ cells. In order to uncover genes required zygotically for proper germ cell migration, genetic screens for genes of the second and third chromosomes were undertaken.

Phenotypic analysis of the mutants identified in these screens reveals that germ cell migration can be subdivided into a limited number of discrete steps, each requiring a particular set of genes. Many of the genes isolated in these screens are involved in the specification or differentiation of SGPs. Furthermore, an analysis of the origin of SGPs indicates that these cells are specified from within the mesodermal eve domain.

Phenotypic characterization of two genes, waldo and schnurri, isolated in the second chromosome screen is presented here. waldo is an uncharacterized gene that is required for germ cell migration on the posterior midgut (PMG), and may be required for the adhesion of germ cells to the endoderm. schnurri is required for the specification of lateral mesoderm, including the SGPs and the fat body.

The role of zfh-1 in germ cell migration and SGP development is also analyzed. The transcription factor, zfh-1, is required for the migration of germ cells from the PMG to the SGPs. It is expressed in two distinct types of mesodermal cells that sequentially interact with migratory germ cells. The first of these cell types, the caudal visceral mesoderm, guides germ cells from the PMG to the lateral mesoderm. zfh-1 is also required for SGP development. Few SGPs are present in zfh-1 loss-of-function mutant embryos, whereas additional SGPs are specified when Zfh-1 is ectopically expressed. Analysis of a tinman zfh-1 double mutant demonstrates that these two proteins have overlapping functions in the development of lateral mesoderm.

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Title: Professor of Cell Biology
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CHAPTER 1

Introduction

Background and Objective

Metazoan development is characterized by extensive cell rearrangements. This can include the movement of groups of interconnected cells, as in epiboly or gastrulation, or in the migration of groups of individual cells. Cellular migrations may be subject to regulation at many levels, in order to precisely control the timing and trajectory of migrating cells. This regulation is likely to require a complex interplay between the migratory cells and their environment. For example, migrating cells must be cued, either by cell-intrinsic or extrinsic factors, to adopt a motile morphology and begin migrating. The direction of their movement must also be regulated. This is likely to depend on the local environment through which the cells are migrating, and may also require guidance cues emanating from the cells which are at the endpoint of the migratory path. While initial migratory steps may be independent of these target cells, the final migratory steps, and the cues which signal the migratory cells to cease their migration, probably rely on interactions between the migrating cells and their target cells or tissue. As a result of the complexity in the number of different cells and molecules which may impinge on a given cellular migratory process, the study of cell migration may lead to insights in diverse areas of developmental biology.

The goal of this thesis work has been to develop germ cell migration in Drosophila as a model system in which to analyze a developmentally-regulated cell migration. We have taken a genetic approach to uncover molecules required for this migration. To this end, we have carried out mutageneses of the second and third chromosomes of Drosophila and screened for mutants yielding aberrant patterns of germ cell migration. Why undertake the study of germ cell migration when other migratory cell types within the Drosophila embryo are already the focus of intense analysis? There are many unique aspects of germ cells and their migration that may allow for novel insights into cell migration. First, because germ cells migrate as individual cells, their migration may be regulated differently from the migration of tracheal cells, which migrate as interconnected cells within an epithelial sheet, or from the guidance of motor axons, in which the cell bodies are not migratory. Second, we might identify gene
products which contribute to the unique nature of germ cells. Third, because aspects of germ cell migration are similar between Drosophila and vertebrate species such as Xenopus and the mouse (see below), we might identify genes which have conserved roles in primordial germ cell migration. Finally, since germ cells interact with both the endoderm and the mesoderm during their migration (see below and Chapter 2), we may uncover gene products required for the development of both of these germ layers.

This introduction is organized in the following manner. I begin by describing what is known about germ cell formation and migration in Drosophila. In light of the fact that aspects of germ cell migration are conserved between Drosophila and vertebrate species, I then describe the migratory path of germ cells in these organisms and discuss the molecules which may be involved in guiding their migration. In the screens which we conducted, many genes were identified that are required for the development of the precursors of the somatic cells of the gonad, called the somatic gonadal precursors (SGPs). I have focused my work on the role of these genes in germ cell migration and SGP specification. Therefore, I describe the development of the mesoderm in Drosophila, within which the SGPs develop, and in particular what is known about the specification of distinct cell types within this germ layer. Since the genetic pathway leading to the formation of somatic muscle is increasingly well understood, I go into some detail about how these cells are specified, since it may serve as a model for the mechanisms leading to the specification of other cell types within the Drosophila mesoderm.

**Germ cell formation in Drosophila**

Germ cells differ from somatic cells in Drosophila in both the position and the timing of their formation. The initial nuclear divisions in Drosophila development occur in the absence of cytokinesis. After 10 rounds of division, the nuclei migrate from the center of the embryo to its cortex. The nuclei that reach the posterior pole will give rise to the germ cells, hence also referred to as pole cells. Approximately 10 germ cells cellularize shortly after the nuclei reach the posterior pole. After cellularization, germ cells undergo two more rounds of cell division, for a total of around 40. While germ cells cellularize at nuclear cycle 10, somatic cells undergo four more rounds of cell division during the syncytial blastoderm stage prior to cellularizing after nuclear cycle 13.

Pole cell formation is controlled by a specialized cytoplasm, called pole plasm, which is found exclusively at the posterior pole. Pole plasm has been shown to instruct germ cell formation by transplantation experiments. When cytoplasm from the posterior pole is transplanted to the anterior pole, ectopic pole cells form at this position (Illmensee
and Mahowald, 1974). These pole cells will develop into fertile gametes when they are returned to the posterior pole, demonstrating that they are functional germ cells. The ability of pole plasm to direct pole cell formation is believed to be the result of unique organelles contained within it known as polar granules. These organelles are composed of RNA and protein and are visible by electron microscopy as electron dense particles that are often associated with mitochondria.

Numerous genes have been identified which are required in the mother for assembly of the germ plasm (see Lehmann and Rongo, 1993, for review). Collectively, they are known as the grandchildless group of genes, as mutant mothers lay embryos lacking germ cells. The phenotypes of these mutants reveal the other role of pole plasm in Drosophila embryogenesis. As well as lacking germ cells, embryos laid from mutant mothers also lack abdomens, as gene products required for posterior patterning are also localized to the pole plasm. Pole plasm is thought to be assembled in a largely stepwise manner, as the functions of the grandchildless genes can be ordered in a pathway (Lehmann and Rongo, 1993). A central event in the formation of pole plasm is the localization of \textit{oskar (osk)} RNA to the posterior pole. If \textit{osk} RNA is mislocalized to the anterior pole by replacing its localization signal with that of the \textit{bicoid} RNA, functional germ cells form at the anterior pole (Ephrussi and Lehmann, 1992), as they did when pole plasm was transplanted to the anterior pole. While the study of the grandchildless genes has led to insights into the mechanisms by which RNAs and proteins are asymmetrically localized within the embryo, it is still unclear how these products instruct germ cell formation.

Some experiments have addressed the mechanisms underlying germ cell formation. In the absence of nuclei, centrosomes are able to organize germ cell formation (Raff and Glover, 1989). These authors demonstrated that in embryos injected with aphidicolin, DNA replication and nuclear migration are blocked, while centrosomes still migrate to the embryo cortex. Germ cells lacking nuclei formed around the centrosomes at the posterior pole. Since centrosomes are known to organize the cytoskeleton, these experiments led to the idea that germ cell formation requires centrosome-mediated cytoskeletal rearrangements.

There are few genes identified that are thought to function downstream of germ plasm assembly in germ cell formation. The product of the \textit{germ cell-less (gcl)} gene is one tantalizing candidate. \textit{gcl} was originally identified as a posteriorly-localized RNA and encodes a protein with no known homology domains (Jongens et al., 1992). In the absence of \textit{gcl} mutants, anti-sense \textit{gcl} RNA was expressed in order to inhibit wild-type \textit{gcl} function. Although pole buds did form in these embryos, in the most severe cases,
they regressed from cortical regions and germ cells were not formed. In other cases, although germ cells formed, they died during their migration to the embryonic gonad. These data are consistent with the model that gcl is required for germ cell specification, or perhaps survival. Polar granule component (Pgc) is another gene thought to be necessary for germline development (Nakamura et al., 1996). Pgc encodes an untranslated RNA which is localized to the posterior pole and incorporated into germ cells. Ultrastructural analysis indicates that Pgc RNA is present throughout polar granules and may therefore be an essential component. Nakamura et al. (1996) generated flies expressing antisense Pgc RNA in order to approximate the loss-of-function phenotype. They found that while germ cells form in embryos carrying antisense Pgc RNA, most do not successfully migrate to the embryonic gonad. The authors conclude that Pgc has a role in the differentiation of functional germ cells, perhaps through a requirement in polar granule formation or stability.

It is also likely that the mitochondrial 16S large rRNA (mt-lr RNA) plays a role in germ cell formation as injection of this RNA into UV-irradiated embryos rescues the ability of these embryos to form pole cells, demonstrating that mt\_lr RNA is a UV-sensitive component of germ plasm (Kobayashi and Okada, 1989). However, these pole cells do not give rise to functional germ cells. Moreover, injection of mt\_lr RNA is not sufficient for pole cell formation at ectopic locations within the embryo, demonstrating that there are additional factors required to bring about germ cell formation. The potential role of mt\_lr RNA in germ cell formation suggests that the close association of polar granules and mitochondria in pole plasm may have functional significance.

A final feature distinguishing germ cells from somatic cells is that in Drosophila, as well as in C.elegans, germ cells activate zygotic transcription later than somatic cells. This has been documented in several different ways. By treating permeabilized embryos with tritiated uridine, Zalokar (1976) observed that germ cells do not transcribe RNA at the blastoderm stage, as do somatic nuclei. Appreciable RNA synthesis was undetectable in germ cells until the onset of gastrulation. More recently, the transcriptional activator Gal4-VP16 was localized to the posterior pole, where at the blastoderm stage, it is sufficient to drive transcription of a lacZ reporter in posterior somatic nuclei, but not in germ cells (Van Doren et al., 1998). lacZ transcription in germ cells was not observed until stage 8-9 of embryogenesis, in good agreement with Zalokar's results. Lastly, during the period in which they are transcriptionally quiescent, germ cells have been shown to lack a specific phosphorylated form of RNA polymerase II (RNAP II), which has been tied to transcriptional elongation (Seydoux and Dunn, 1997). An antibody for a specific phosphoepitope on RNAPII was observed in blastoderm-stage somatic cells, but
not in germ cells until stage 7. This is just prior to the time at which Gal4-VP16 is competent to activate transcription. The transcriptional repression in germ cells may be specific for RNAP II transcripts, as nascent rRNAs are detectable in Drosophila germ cells as early as stage 5 (Seydoux and Dunn, 1997).

Germ cell migration in Drosophila

Consequent to their formation, germ cells undergo a stereotyped migration. During gastrulation, germ cells are passively carried into the embryo as the posterior pole invaginates as the posterior midgut (PMG) pocket. Germ cells begin actively migrating during stage 10 when they extend cytoplasmic processes and move through the blind end of the posterior midgut pocket. The onset of germ cell motility appears to be controlled by the somatic cells of the PMG, rather than by the germ cells themselves (Jaglarz and Howard, 1994). If germ cells are heterochronically transplanted into host embryos either three hours older or younger than the germ cell donor embryos, germ cells begin migrating at a time appropriate for the host into which they are transplanted. This result argues the germ cell-specific factors necessary for their motility may be maternally-provided, as these factors are capable of being activated prior to the onset of zygotic transcription in germ cells (see above).

Numerous morphological changes occur in the PMG at the time of germ cell migration through this tissue (Callaini et al., 1995; Jaglarz and Howard, 1995). Endodermal cells extend 2µm long cytoplasmic processes which contact the germ cells situated in the midgut lumen. There is also a decrease in the level of F-actin visible on the apical side of the epithelium during the period of germ cell migration. Additionally, the contacts between the cells of the PMG relax, as apical contacts become interrupted and large intercellular spaces appear between the cells. Interestingly, these morphological changes still occur in embryos lacking germ cells, indicating that they are not driven by germ cell-endoderm interactions. However, it is likely that these structural changes are required for proper germ cell migration into the embryo. In support of this idea, the PMG does not undergo these structural transformations in huckebein (hkb) mutant embryos (Jaglarz and Howard, 1995). Germ cells are not able to migrate through the PMG in hkb mutants, which argues that these structural changes are a prerequisite for the passage of germ cells through the PMG.

Once germ cells pass through the endodermal epithelium into the interior of the embryo, they migrate dorsally over its basal surface until they are sandwiched between the endoderm and the overlying mesodermal layer. This migratory step requires the products of the nanos (nos) and wunen (wun) loci. Maternal Nos protein is present in
germ cells from the time of their formation at the posterior pole. However, its role in
germ cell migration cannot be directly ascertained in these embryos, as they lack
abdomens. This problem was circumvented by transplanting germ cells from nos mutant
mothers into wild-type embryos (Kobayashi et al., 1996), or by making nos-hunchback-
germline clones, in which nos' role in pattern formation is relieved (Forbes and Lehmann,
1998). nos mutant germ cells are able to migrate through the PMG, but they then fail to
migrate over its basal surface. Instead, most adhere to one another in a large cluster on
the tip of the PMG (Forbes and Lehmann, 1998). This migration defect may be caused
by changes in gene expression in the germ cells, as nos mutant germ cells prematurely
express enhancer traps normally not expressed until after gonad coalescence (Kobayashi
et al., 1996). In wun mutant embryos, germ cell migration on the PMG is not oriented
toward the dorsal side (Zhang et al., 1996). wun encodes a transmembrane protein that is
probably involved in lipid metabolism as the intracellular domain has sequence similarity
to phosphatidic acid phosphatase. While it is not known how this enzymatic activity
functions in germ cell migration, both the expression pattern of wun in wild-type embryos
and overexpression experiments suggest that germ cells are repelled from Wun-
expressing cells (Zhang et al., 1997).

Germ cells enter the mesoderm from the dorsal side of the endoderm from late
stage 10 to stage 11. They subsequently associate with somatic gonadal precursors
(SGPS) which are the mesodermal cell type giving rise to the support cells of the ovary or
testis. SGPs are specified during stage 11 as three clusters of mesodermal cells in
parasegments (PS) 10-12 (Brookman et al., 1992; Boyle and DiNardo, 1995; Boyle et al.,
1997). During stage 12, the three clusters extend toward each other, until at the onset of
stage 13, they form a continuous line of cells reaching from PS10 to PS12. The SGPs
within PS11-12 then migrate anteriorly until stage 14, when germ cells and SGPs
coalesce in PS10 into the embryonic gonad. SGP specification and development does not
require the presence of germ cells, as it occurs properly in embryos lacking germ cells
(Brookman et al., 1992).

Almost all of the mutants identified thus far which disrupt germ cell migration
into and within the mesodermal layer are required for SGP development. The homeotic
genes abdA and AbdB have both been implicated in SGP specification. All SGPs require
abdA function (Brookman et al., 1992; Boyle and DiNardo, 1995), while only those SGPs
which originate within PS12 require the function of AbdB (Boyle and DiNardo, 1995).
Correspondingly, the germ cell migration phenotype of abdA mutant embryos is more
severe than that of AbdB. In abdA mutants, germ cells migrate into two bilaterally-
symmetric groups as in wild type, but in the absence of SGPs, they scatter throughout the
posterior mesoderm (Moore et al., 1998a). In AbdB mutant embryos, most germ cells associate with SGPs, while a few are found dispersed posterior to the gonads (Moore et al., 1998a). The eyes absent/clift (cli) locus is also required for SGP development (Boyle et al., 1997). SGPs are specified in cli mutant embryos, although they fail to maintain this differentiated state. Consistent with the idea that SGPs are initially present, germ cell migration in cli embryos is not disrupted until stage 13 (Boyle et al., 1997).

Given that we knew that genes required for SGP specification have a role in germ cell migration, we anticipated uncovering such loci in our screens. We also hoped to identify loci more specifically required for guidance of germ cells toward the SGPs. Although there were no genes in Drosophila with mutant phenotypes or expression patterns suggestive of such a role, there are candidates in vertebrate germ cell migration. It was possible that some of these genes might have conserved functions in germ cell guidance as there are striking similarities between germ cell migration in Drosophila and germ cell migration in vertebrate systems. With that in mind, I now discuss vertebrate germ cell migration, and some of the molecules postulated to function in this process.

**Vertebrate germ cell migration**

The overall pattern of germ cell migration is strikingly conserved between Drosophila and vertebrate species such as mouse and Xenopus. Since the majority of studies on vertebrate primordial germ cell (PGC) migration were conducted with mouse PGCs, I focus on what is known about the control of PGC migration in the mouse.

It is not known how the PGC lineage is determined during mouse embryogenesis. However, they are first detectable at 7-7.25 dpc by virtue of alkaline phosphatase (AP) activity (Ginsburg et al., 1990). At this early stage, they are found posterior to the primitive streak in the extra-embryonic mesoderm. This region of the embryo is subsequently incorporated into the hindgut. At 9.5 dpc, PGCs migrate out of the endoderm toward the precursors of the somatic cells of the gonad, called the genital ridge. While the first germ cells to migrate to the genital ridge appear to contact these cells directly from the surface of the hindgut (Gomperts et al., 1994), the majority of PGCs migrate to the genital ridge along the dorsal mesentery which comes to support the hindgut. PGC migration to the genital ridge is complete by 12.5 dpc (Tam and Snow, 1981; reviewed in Wylie and Heasman, 1993). While these migratory steps appear similar to germ cell migration in Drosophila, mouse PGCs proliferate during their migration, while Drosophila germ cells do not. In the mouse, PGC numbers increase from less than 100 at the gastrula stage, to approximately 2,500 at the end of their migration (Wylie and Heasman, 1993).
Many studies have characterized the migratory ability of mouse PGCs in vitro (Cooke et al., 1996; Dolci et al., 1991; Donovan et al., 1986; ffrench-Constant et al., 1991; Garcia-Castro et al., 1997; Godin et al., 1991; Godin et al., 1990; Godin and Wylie, 1991; Pesce et al., 1993). The combined results from these investigations suggest that PGC proliferation and migration may be regulated by interactions between PGCs and adhesion molecules, as well as by the response of PGCs to growth factors. PGCs will adhere to several extracellular matrix (ECM) proteins in vitro, and there is some evidence that these interactions have significance in vivo. It has been demonstrated that the ability of PGCs to adhere to Fibronectin (FN) is developmentally regulated. By removing PGCs from embryos at different developmental stages, ffrench-Constant et al. (1991) demonstrated that prior to their migratory phase, about 90% of PGCs will adhere to FN. The affinity of PGCs for FN decreases during the period when they are migrating out along the dorsal mesentery, and reaches 0% at 12.5 dpc, when the PGCs have colonized the genital ridges. These data are consistent with the model that intermediate affinities of migratory cells for ECM components may promote cellular migration. FN is present along the dorsal mesentery in mouse embryos, consistent with a role for this ECM component in mediating PGC adhesion or migration in vivo (ffrench-Constant et al., 1991; Fujimoto et al., 1985). Laminin (LM) is also expressed along the PGC migratory route. PGCs were found to be associated with a laminin "ribbon" connecting the base of the dorsal mesentery to the genital ridge (Garcia-Castro et al., 1997). In vitro, the affinity of PGCs for LM also decreases over time, although not as dramatically as does PGC affinity for FN (Garcia-Castro et al., 1997).

The finding that genital ridge explants were shown to exert long range effects on PGC number and migratory direction in vitro (Godin et al., 1990), motivated efforts to identify the molecule(s) responsible for this response. There is evidence that TGFβ can act as a chemoattractant in vitro (Godin and Wylie, 1991). This growth factor is expressed along the dorsal body wall, including the genital ridge, consistent with a role for TGFβ in directing PGCs along the dorsal mesentery toward the genital ridge. There is stronger evidence that Steel (Sl, also known as stem cell factor) is involved in PGC guidance. Mice homozygous for viable mutations in Sl or its receptor White-Spotting (W), which encodes the receptor tyrosine kinase c-kit, lack germ cells and are sterile (reviewed in Fleischman, 1993). Furthermore, the expression patterns of c-kit and Sl support a role for the ligand/receptor pair in PGC migration, as c-kit is expressed within migrating PGCs (Manova and Bachvarova, 1991; Orr-Urtreger et al., 1990), and Sl is present in surrounding tissues (Matsui et al., 1990). Buehr et al. (1993) confirmed the functional importance of c-kit in PGC migration and proliferation. In W homozygous
embryos, the correct number of PGCs are specified, but they do not proliferate as in wild type. Additionally, PGC migration in W embryos is delayed relative to wild type, and there are clumps of PGCs in ectopic locations. These data suggest that Sl may function both as a chemoattractant and to promote PGC proliferation. In vitro, Sl factor has been shown to promote PGC survival; however, a chemotropic effect was not observed (Dolci et al., 1991; Godin et al., 1991). In these assays, only the soluble form of Sl was tested, leaving open the possibility that the membrane-bound form could function as a chemoattractant. Support for a functional role of membrane-bound Sl comes from the sterility of mice harboring the Sl-dickie mutation, in which only a soluble form of Sl is produced (Dolci et al., 1991).

Mesoderm Development in Drosophila

Mesoderm Formation

Recently, the mechanisms and molecules underlying mesoderm development have become increasingly well understood. Much of this thesis work focuses on the roles of specific genes in the development of somatic gonadal preursors (SGPs). Since many of these genes were previously known to function in mesoderm development, I review what is known to date about mesoderm specification and its subsequent development in the Drosophila embryo.

The first gastrulation movement during Drosophila development is the invagination of mesodermal cells on the ventral side of the embryo. These ventrally-situated cells express high levels of Dorsal protein in their nuclei. This results in the expression of two transcription factors: twist (twi) and snail (sna) in the presumptive mesoderm (Bouley et al., 1987; Thisse et al., 1988; reviewed in Leptin et al., 1992). While the lateral boundary of the mesoderm is set by Sna expression (Leptin, 1991), the anterior and posterior boundaries of the mesoderm are defined by expression of the Huckebein (Hkb) transcription factor at the poles of the embryo. Anteriorly, hkb interferes with the expression of twi and sna target genes, while posteriorly, it represses sna expression (Reuter and Leptin, 1994).

It is thought that Twi functions primarily to activate the transcription of mesodermal factors, while Sna acts to repress the expression of ventral neuroectoderm determinants within mesodermal cells (Leptin, 1991). Consistent with the idea that these two genes have largely independent functions, twi and sna mutant embryos exhibit distinct phenotypes (Leptin and Grunewald, 1990). In twi embryos, numerous small, irregularly positioned ventral folds form; while sna embryos have one ventral furrow,
which is about half the width of wild type. Furthermore, in twi sna double mutants, the ventral epithelium is identical to neighboring ectodermal cells, supporting the model that twi and sna have at least partially separable functions. The buckling of the ventral epithelium in sna mutants could, in principle, be an indirect result of the misexpression of ectodermal genes within the ventral furrow. However, the mesoderm invaginates in a sna allele in which neuroectoderm-specific genes are expressed in the mesoderm, suggesting that these functions of sna are separable (Hemavathy et al., 1997; Ip et al., 1994).

After the mesodermal cells invaginate, they lose their epithelial character, flatten, and divide once. Following this round of division, the mesoderm spreads dorsolaterally to form a monolayer beneath the overlying ectoderm (see Bate, 1993, for review). This migratory step requires the activity of the heartless (htl) FGF receptor (Beiman et al., 1996; Gisselbrecht et al., 1996). Since the mesoderm does not migrate in htl mutant embryos, these embryos lack dorsal mesoderm derivatives, such as the heart and the visceral mesoderm, as these tissues are induced through contact with the dorsal ectoderm (see below). Although htl is expressed throughout the mesoderm at this early stage (Beiman et al., 1996), it may not be activated in all of these cells. Activation of the Htl receptor has been followed using an antibody specific for activated MAP kinase (Gabay et al., 1997a; Gabay et al., 1997b), which is a cytoplasmic protein kinase downstream of receptor tyrosine kinase (RTK) signaling (Seger and Krebs, 1995). Expression of activated MAP kinase suggests that Htl is activated only in the first few rows of cells that migrate dorsally, since the antibody is present specifically in these cells. Based on this expression pattern, the as-yet-unidentified Htl ligand might be expected to be expressed in a dynamic pattern in the ectoderm, shifting from ventral to dorsal ectoderm over time.

However, it is not entirely clear if local activation of htl plays an instructive role in guiding mesodermal cell migration, since pan-mesodermal expression of activated Ras, another component of RTK signaling pathways, partially rescues the migration defect (Gisselbrecht et al., 1996). It is also possible that htl has additional roles in mesoderm development, as a dominant-negative htl receptor present throughout the mesoderm does not interfere with dorsolateral mesodermal migration, but does bestow visceral mesodermal defects to embryos in which this construct is activated (Beiman et al., 1996). Consistent with this idea, htl is expressed in subsets of mesodermal cells later in development (Shishido et al., 1993). We found that htl is required for SGP development, as fewer SGPs are specified in htl embryos. This phentotype could be the result of the failure of dorsolateral mesoderm migration in htl mutants, since SGPs originate dorsal to where the ventral furrow invaginates (see below). Interestingly however, those SGPs that
do form appear to be morphologically abnormal and do not coalesce (Moore et al., 1998a).

Dorsoventral diversification of the mesoderm

After the dorsolateral migration is complete, mesodermal cells undergo a second round of division. At this time, distinct cell types are specified along the dorsoventral axis. It has been shown that Decapentaplegic (Dpp), a member of the TGFβ family of signaling molecules, is required for the formation of visceral mesoderm and heart, two dorsal mesoderm derivatives (Frasch, 1995; Staehling-Hampton et al., 1994). It is thought that Dpp is secreted from dorsal ectoderm cells and received by underlying mesoderm cells, where it induces the expression of dorsal mesoderm-specific genes, such as \textit{tinman} (\textit{tin}) and \textit{bagpipe} (\textit{bap}) (Azpiazu and Frasch, 1993). Accordingly, in Dpp mutant embryos, these genes are not expressed in the dorsal mesodermal domain, whereas if \textit{Dpp} expression within the ectoderm is expanded, the mesodermal expression domains of \textit{tin} and \textit{bap} expand similarly (Frasch, 1995; Staehling-Hampton et al., 1994).

The homeobox-containing protein Tin is an important regulator of dorsal mesoderm cell fates (Azpiazu and Frasch, 1993). As the mesodermal monolayer forms, \textit{tin} RNA is expressed panmesodermally, where it appears to be a direct transcriptional target of \textit{twi} (Yin et al., 1997). \textit{tin} expression is subsequently expressed throughout the dorsal mesoderm, and then specifically in heart progenitors. These three phases of expression are unlikely to reflect the maintenance of \textit{tin} RNA in subsets of cells, as each phase of expression has a unique enhancer element, demonstrating that they are independently transcriptionally controlled (Yin et al., 1997). In \textit{tin} mutant embryos, neither visceral mesoderm nor heart progenitors form, indicating the primary role of this gene in the specification of both of these dorsal cell types (Azpiazu and Frasch, 1993). \textit{tin} is also required for the expression of the homeobox-containing gene \textit{bagpipe} (\textit{bap}) within the visceral mesoderm primordium. \textit{bap} is also required for visceral mesoderm development, as the number of visceral mesodermal cells is greatly reduced in \textit{bap} mutants (Azpiazu and Frasch, 1993).

SGPs arise immediately ventral to the visceral mesoderm in PS 10-12 (Boyle et al., 1997). It has been shown that the number of SGPs increases in \textit{bap} mutant embryos, suggesting that in wild-type embryos, the dorsal border of SGPs is set by a repressive interaction between the visceral mesoderm and SGPs (Azpiazu and Frasch, 1993; Boyle et al., 1997). Since SGPs are only specified in three parasegments, the question arises as to what mesodermal tissue forms in the analogous dorsoventral location in other parasegments. Recent work has demonstrated that the fat body arises ventral to the
visceral mesoderm in PS4-9 and PS13 (Moore et al., 1998b; Riechmann et al., 1998). Similar genetic hierarchies function in the development of SGPs and the fat body, suggesting that the anlagen of these two tissues are specified equivalently (Moore et al., 1998b). Cell fate transformations between SGPs and fat body precursors in different genetic backgrounds has provided insight into how these two tissues are determined in wild-type development (Moore et al., 1998b; Riechmann et al., 1998). In serpent (srp) mutant embryos, the development of fat body precursors is blocked (Abel et al., 1993; Rehorn et al., 1996), and SGPs are present in all parasegments. Conversely, in abdA mutant embryos, SGP specification is inhibited, and additional fat body precursors are found in their place. Finally, in abdA srp double mutants, SGPs are present in all parasegments demonstrating that srp is epistatic to abdA. Therefore, the role of abdA is to negatively regulate srp in PS10-12, thus permitting SGP development (Moore et al., 1998b).

The Segmental Origin of Mesodermal Derivatives

The mesoderm is also subdivided into distinct cell types along the anteroposterior (A/P) axis. The primordia of the somatic musculature, the heart, the visceral mesoderm, the fat body, and the gonadal mesoderm all arise as clusters of cells positioned at regular intervals along the A/P axis. These primordia fall into two classes: those originating within the anterior portion of the parasegment (heart and somatic muscle) and those arising within the posterior portion of the parasegment (visceral mesoderm, fat body, and gonadal mesoderm). These primordia originate anterior and posterior to each other when the mesoderm is still a monolayer. During stage 11, the cells of the posterior domain migrate inside the cells of the anterior domain (Borkowski et al., 1995), so that the visceral mesoderm retains contact with the endoderm while the somatic musculature remains adjacent to the ectoderm. At stage 12, the primordia of the heart, the visceral mesoderm, the fat body, and the gonadal mesoderm lose their segmental appearance as they elongate and fuse along the A/P axis.

The two mesodermal domains are established around the time of gastrulation through the functions of the pair-rule genes. The mesodermal domain corresponding to the anterior parasegment has been named the Sloppy-paired (Slp) domain, as the function of this pair-rule gene is essential for heart and somatic muscle development (Riechmann et al., 1997). The mesodermal domain situated in the posterior of the parasegment has been termed the Even-skipped (Eve) domain, as the visceral mesoderm, fat body, and gonadal mesoderm do not form in eve mutant embryos (Azpiazu et al., 1996; Moore et al., 1998a; Riechmann et al., 1997). However, visceral mesoderm and fat body
precurors form in *eve slp* double mutant embryos, demonstrating that *eve* is not required to activate gene expression in these tissues; rather, it is required to repress *slp* function in the Eve domain (Riechmann et al., 1997). Consistent with this conclusion, *slp* expression is expressed uniformly throughout the trunk region of the embryo in *eve* mutants (Riechmann et al., 1997).

*eve* is thought to function in mesoderm segmentation around the time of ventral furrow formation, at which point it is expressed both in the ectoderm and in the mesoderm. Azpiazu et al. (1996) transplanted *eve*+ cells into *eve* mutant embryos in order to determine in which cells *eve* is required. They found that *bap* expression was restored to *eve*+ clones only when these clones were present within the mesoderm. This demonstrates that *eve* functions within mesodermal cells and argues that the initial subdivision of the mesoderm along the anteroposterior axis is unlikely to require an inductive interaction between the mesoderm and the ectoderm, as has been demonstrated for D/V patterning. The first gene to be expressed in a repeated pattern is *bap* in visceral mesoderm primordia at stage 9 (Azpiazu and Frasch, 1993). This is several hours after *eve* and *slp* are thought to function, suggesting that their effect on the transcription of tissue-specific gene expression may be indirect.

The segment polarity genes are likely to act downstream of the pair-rule genes in mesoderm development. *wingless (wg)* may function downstream of *slp* in the development of Slp domain mesodermal derivatives. *wg* is required both for formation of the muscle pioneer cells (Baylies et al., 1995) and for the heart (Park et al., 1996). It appears that Eve domain derivatives form in lieu of Slp domain derivatives in wg embryos as the visceral mesoderm and the fat body primordia become continuous (Azpiazu et al., 1996). Consistent with a role for *wg* in the specification of derivatives within the Slp domain, it is present at low levels in the mesodermal Slp domain, and at high levels in the overlying anterior compartment of the ectoderm (Azpiazu et al., 1996; Baylies et al., 1995). *engrailed (en)* and *hedgehog (hh)* are both required for the development of mesodermal derivatives arising within the Eve domain. There are slight reductions in *bap* expression in *en* or *hh* mutant embryos, and a more severe reduction in double mutant embryos. Furthermore, ectopically expressed En or Hh is able to partially rescue the mesodermal defects in *eve* mutant embryos (Azpiazu et al., 1996). Proper segmentation of the mesoderm may require antagonistic interactions between *wg* and *hh*, since ectopic expression of either protein has more dramatic effects on the mesoderm when the other gene's function is removed (Azpiazu et al., 1996).

It is not known whether these segment polarity genes act in within the mesoderm or the ectoderm. It has been suggested that they function solely within the ectoderm.
These conclusions are based on the strong ectodermal expression of the segment polarity genes at this stage, and the fact that overexpression either within the ectoderm or the mesoderm is able to rescue expression of mesodermal markers. This leads to a model whereby mesodermal pair-rule gene expression sets up a prepattern within the mesoderm, which is refined by inductive \textit{wg} and \textit{hh} signals from the ectoderm (Azpiazu et al., 1996).

The role of \textit{wg} in mesoderm development is not limited to an early role in formation of the Slp domain. It is also required for development of the SGPs, which arise within the Eve domain (Boyle et al., 1997). SGPs are not present in \textit{wg} mutant embryos (Moore et al., 1998a), whereas additional SGPs are present in \textit{HSwg} embryos, or when an activated form of Armadillo is expressed throughout the mesoderm (Boyle et al., 1997). These experiments suggest that \textit{wg} is likely to function at multiple times in mesodermal development. While it functions between gastrulation and stage 10 in the development of Slp derivatives, it is also required for SGP specification, which is evident at stage 11.

**Somatic Muscle Development**

As discussed previously, the somatic musculature of the larva arises within the Slp mesodermal domain of the embryo. The expression of Twi protein distinguishes these cells from those arising within the Eve domain. While Twi is expressed in all mesodermal cells at early embryonic stages, at stage 10 it acquires a segmental expression pattern, with high protein levels present in the somatic muscle precursors. Subsequently, Twi is lost from cells that will differentiate into the larval musculature, and maintained in the adult muscle precursors.

\textit{twi} has been shown to have a pivotal function in somatic muscle development, through the analysis of both loss and gain of function situations. Baylies and Bate (1996) demonstrated that in embryos in which high Twi levels are maintained throughout the mesoderm, the number of visceral mesoderm and heart precursors is reduced, with somatic-like muscle precursors forming in their place. Strikingly, ectopic Twi expression in the ectoderm is sufficient to induce aspects of muscle differentiation there. In these embryos, ectodermal cells will express myosin and fuse to form multinucleate cells, while the development of the epidermis and the nervous system is impaired. Finally, by using temperature-sensitive \textit{twi} alleles in order to bypass the early requirement of \textit{twi} in muscle specification, they demonstrated that \textit{twi} is necessary for somatic muscle development.

The transcription factor D-Mef2 has also been shown to be required for development of the somatic muscle. D-Mef2 is a member of the MADS family of
transcription factors, which is an evolutionarily conserved family of genes regulating muscle-specific transcription. D-Mef2 is expressed in somatic, cardiac, and visceral muscle precursors (Lilly et al., 1994). This expression pattern suggests that unlike twi, D-Mef2 is not required for the specification of the somatic musculature, but rather for the expression of downstream factors, such as Myosin, required for the differentiation of multiple muscle types. Consistent with this idea, the segregation of different muscle types occurs properly in D-Mef2 mutant embryos, although their subsequent differentiation is aberrant (Bour et al., 1995; Lilly et al., 1995).

While genes such as twi and D-Mef2 have been shown to function broadly in somatic muscle development, the mechanisms leading to the specification of unique muscle fibers are also increasingly well understood. There are 30 different muscles within each hemisegment which may be distinguished by their size, location, epidermal attachment site, and pattern of innervation (Bate, 1993). All of the information necessary to pattern each muscle is believed to be present in a single precursor cell, called the founder cell (Bate, 1990). Founder cells seed the muscle pattern by fusing with nearby naive myoblasts to generate multinucleate muscle fibers. This idea is modeled after the process of myogenesis in grasshopper embryos, where muscle pioneers are a morphologically distinct class of cells responsible for generating the muscle pattern (Ho et al., 1983). In Drosophila, evidence for muscle founders comes primarily from gene expression in wild-type and mutant embryos. The homeobox gene S59 is expressed in a small number of myoblasts prior to fusion. During myoblast fusion, these putative founders fuse with surrounding cells, at which time S59 expression is initiated in the nuclei of fusing myoblasts (Dohrmann et al., 1990). In embryos in which myoblast fusion is blocked, the initial pattern of S59 expression is wild-type, indicating that the formation of founder cells is independent of myoblast fusion. These mononucleate S59 cells often elongate and attach correctly to the epidermis, indicating that their unique identities have already been established. However, in the absence of myoblast fusion, additional cells are not recruited to express S59 (Rushton et al., 1995).

The selection of the founder myoblasts from the pool of cells competent to differentiate into the somatic mesoderm has striking similarities to neuroblast specification in the neuroectoderm. In the neuroectoderm, clusters of cells competent to acquire a neuroblast fate are generated through the action of the proneural genes, including the genes of the Acheate-Scute complex (As-C) (see Goodman and Doe, 1993 for review). However, all but one of these cells are prevented from developing as neuroblasts through a process of lateral inhibition mediated by the neurogenic genes, such as Notch (Goodman and Doe, 1993). It has been shown that one of the genes of the
As-C, lethal of scute (l' sc), is required for the specification of muscle founders. Carmena et al. (1995) demonstrated that during stage 11, 19 clusters of L'sc-expressing cells appear in each hemisegment. Gradually, one cell in each cluster accumulates higher levels of L'sc and initiates S59 expression. This cell, called a muscle progenitor, divides asymmetrically, giving rise to two founder cells, which seed the formation of two different muscles. In neurogenic mutant embryos, a single cell from each promuscle cluster is not singled out, and the entire cluster can coexpress S59 and L'sc. This is consistent with the observation that muscle-specific gene expression is expanded in neurogenic mutants (Corbin et al., 1991). Surprisingly, the muscle phenotype of l'sc mutant embryos is not as strong as might be predicted from the expression pattern, suggesting that other genes may play similar, redundant functions in the specification of muscle progenitors (Carmena et al., 1995).

The asymmetric division of the muscle progenitor cell to give rise to two distinct muscle founders has recently been shown to depend on numb (nb) and inscuteable (insc) (Carmena et al., 1998; Ruiz-Gomez and Bate, 1997). These genes were first described for their roles in the asymmetric divisions of neuroblasts, where it has been shown that insc is required for nb localization, even though the proteins are localized to opposite sides of dividing neuroblasts (Doe and Spana, 1995). As in neuroblasts, Nb and Insc are localized to opposite sides of dividing muscle progenitors. Furthermore, in insc mutants, Nb is delocalized in muscle progenitors, leading to specific muscle defects. These defects result from aberrant asymmetric cell division of progenitor cells, with both of the resulting founder cells adopting the same cell fate. As expected, this phenotype is similar to that observed in embryos with ectopic Numb, whereas the reciprocal cell fate transformations occur in nb loss-of-function mutant embryos. Since nb is thought to function in cell fate decisions by antagonizing Notch (N) signaling, this suggests that N functions at two distinct steps in muscle development. First, N is required for the selection of a single progenitor cell from the promuscle cluster, and second, it is required for the asymmetric division of this cell to give two distinct muscle pioneers.

While all muscle pioneers are likely to be selected through the combined action of the proneural and neurogenic gene families, muscles must establish individual patterns of gene expression in order to assume their unique identities. The generation of this diversity within each parasegment is likely to depend on the expression of unique sets of transcription factors within each pioneer cell. Much remains to be learned about the genetic pathways responsible for this process of individuation, although there are a number of genes whose expression patterns in subsets of pioneer cells suggest that they may act to differentiate one pioneer from another. As one example, there is strong
evidence that the gap gene Krüppel (Kr) is necessary for the development of a subset of precursors. It is expressed in this subset and required for gene expression within these cells. Consistent with a cell-autonomous function for Kr in establishing unique muscle identities, only those muscles which express Kr are disrupted in Kr mutant embryos (Ruiz-Gomez et al., 1997). The identification of additional genes required for the specification of discrete sets of founder cells may provide insight into the mechanisms allowing for the generation of 30 distinct muscle types.

After the muscle pioneers form, the founders fuse with surrounding myoblasts and attach to the ectoderm. During the process of fusion, myotubes extend filopodia toward their epidermal attachment sites. While it is not yet clear what role the ectoderm plays in the specification of the muscle progenitors, ectodermal cells along the segment border play a key role in myotube migration. Segment border cells are attractive to myoblasts in culture, and myotube migration is disrupted in embryos mutant for segment polarity genes in which these cells are absent or mispositioned (Volk and VijayRaghavan, 1994). The interaction of the muscle progenitors and ectodermal cells in myotube migration is a good example of the interactions that occur between these two germ layers during mesoderm development. As more is learned about the specification and differentiation of mesodermal cell types, additional interactions between the mesoderm and the ectoderm will certainly be uncovered.
Specific Aims
The aim of this thesis has been to advance germ cell migration in Drosophila as a model system for the study of a developmentally-regulated cell migration. Since germ cells adhere to gonadal mesodermal cells during much of embryogenesis, the analysis of germ cell migration was bound to lead to insights into the specification and differentiation of this mesodermal cell type. Chapter 2 describes a comprehensive screen of the third chromosome for genes required zygotically for proper germ cell migration. In this chapter, I characterize the germ cell migration phenotypes that were uncovered in this screen, and discuss what insights these phenotypes provide about the migratory steps germ cells follow. Chapter 3 describes a smaller-scale, yet similar, screen of the second chromosome that laid the groundwork for the third chromosome screen. The preliminary characterization of two mutants that were identified in this screen is also presented. In Chapter 4, I present my analysis of one of the mutants that was identified in the screen of the third chromosome. I show that this gene, zfhl, is essential for the development of two distinct mesodermal cell types that sequentially interact with migratory germ cells. Furthermore, I demonstrate that zfhl and tinman have overlapping functions in specification of lateral mesoderm derivatives. In the Appendix, data that the dorsal boundary of lateral mesoderm is set by Dpp signaling from the ectoderm is presented. Finally, in the Afterword, I discuss a few of the implications of this work, and suggest possible future directions that work on germ cell migration might follow.


PREFACE

The work presented in Chapter 2 was a collaboration between a number of people in the lab. The third chromosome screen was carried out by Lisa Moore, Mark Van Doren, and myself. Lisa Moore and I collaborated on the analysis of the mesodermal origin of the gonadal mesoderm. Lynn Lunsford provided technical assistance in the secondary screen.

This chapter has been published as:

*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. 1). The PGCs, often referred to as pole cells in Drosophila, are the first to cellularize at the posterior pole of the embryo (Fig. 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. 1B). The PGCs then migrate through the PMG wall, moving along its basal surface to the dorsal side of the embryo (Fig. 1C). From this position they move toward lateral mesodermal cells in parasegments 11-13 (PS 11-13, Fig. 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. 1F). Finally, the PGCs and gonadal mesoderm coalesce in PS 10 to form the embryonic gonad (Fig. 1H). Germ cell migration in Drosophila therefore provides a model system for the study of cellular movements and cell-cell interactions.

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 *abdominalA (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 *AbdominalB (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.

Fig. 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-Nos 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) anlagen, 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.
Figure 2-1: Germ cell migration in wild-type embryos
MATERIALS AND METHODS

EMS mutagenesis and establishment of balanced lines
See Fig. 2 for an outline of the screen. A ru st e' 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' 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} pP/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*/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' 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' 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 MgCl₂, 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-Nos 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 devitellinization. Non-devitellinized embryos were removed from the blocks, and the remaining embryos were rehydrated and subjected to antibody staining as described in Eldon and Pirotta (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 (Whatmann), 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 I), 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: \textit{abdA} (Cumberledge et al., 1992), \textit{AbdB} (Brookman et al., 1992), and \textit{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 \textit{ru st e’ ca} markers. Once mapped to an interval, mutants were crossed to deficiencies (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 from Clontech according to the method of Lehmann and Tautz (1994). Antisense RNA probes were prepared for the detection of $lacZ$ using the pC4 $\beta$-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: $abda^{max}$, $AbdB^{D1013}$ (both gifts from Welcome Bender), $cno^2$, $Df(3R)crbS87-5$, $Df^{9D}$, $fkh^{E200}$, $ftz^{7B}$, $htl^{AB42}$ (a gift from James Skeath), $hh^U$, $hkb^2$, $kni^{cC}$, $opa^{hp}$, $srp^{al}$, $srw^I$, $tl^{L10}$, $tin^{AGC14}$ (a gift from Manfred Frasch), $td^{sQ}$, $trx^{B11}$ (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. The crossing scheme used to generate the single balanced mutant lines is shown in Fig. 2. We screened embryos directly by using the fat facets-lacZ transgene (faf-lacZ; Fischer-Vize et al., 1992) to visualize germ cells, and also a “blue balancer” (Ubx-lacZ TM3) to distinguish homozygous mutant embryos from their siblings (an example is shown in Fig. 2). The protein product of the faf-lacZ transgene is localized to the posterior pole of embryos and incorporated into germ cells, where β-galactosidase activity is maintained throughout embryogenesis. 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.

The results of our screen of the third chromosome are summarized in Table 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.
Fig. 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). 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.
**Figure 2:** Crossing scheme to establish lines isogenic for a mutagenized third chromosome

35mMMS

P: \[\frac{\text{ru st } P[\text{faf-lacZ}] e ca}{\text{ru st } P[\text{faf-lacZ}] e ca}\]  

F1: \[\frac{\text{ru st } P[\text{faf-lacZ}] e ca}{\text{ru st } P[\text{faf-lacZ}] e ca}\]  

F2: \[\frac{\text{ru st } P[\text{faf-lacZ}] e ca}{\text{ru st } P[\text{faf-lacZ}] e ca}\]  

F3: \[\frac{\text{ru st } P[\text{faf-lacZ}] e ca}{\text{ru st } P[\text{faf-lacZ}] e ca}\]  

<table>
<thead>
<tr>
<th>\text{P[hs-hid], p}</th>
<th>\text{TM3 Sb, P[Ubx-lacZ]}</th>
<th>\text{F[hs-hid], p}</th>
<th>\text{TM3 Sb, P[Ubx-lacZ]}</th>
<th>\text{TM3 Sb, P[Ubx-lacZ]}</th>
<th>\text{TM3 Sb, P[Ubx-lacZ]}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\text{P[hs-hid], p}</td>
<td>\text{TM3 Sb, P[Ubx-lacZ]}</td>
<td>\text{ru st } P[\text{faf-lacZ}] e ca</td>
<td>\text{ru st } P[\text{faf-lacZ}] e ca</td>
<td>\text{ru st } P[\text{faf-lacZ}] e ca</td>
<td>\text{ru st } P[\text{faf-lacZ}] e ca</td>
</tr>
</tbody>
</table>

let lay eggs for 5 days, remove parents  
heat shock at 37 °C for 2hrs on days 5 and 6

collect embryos, stain with X-gal; test for lines with aberrant pole cell migration under stereo microscope
Classification of mutant phenotypes
The results of the secondary screen enabled us to categorize the mutants into classes based on phenotypic similarity (Table 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 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 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. (\textsuperscript{b}) 28 lines were not included in the secondary screen either due to death of the stock, or loss of the balancer chromosome. (\textsuperscript{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

<table>
<thead>
<tr>
<th>Phenotypic Classes</th>
<th>n</th>
<th>% of selected lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>specific effect on germ cells</td>
<td>70</td>
<td>21</td>
</tr>
<tr>
<td>pattern formation</td>
<td>110</td>
<td>34</td>
</tr>
<tr>
<td>dominant/multiple a</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>lost b</td>
<td>28</td>
<td>9</td>
</tr>
<tr>
<td>false positive c</td>
<td>113</td>
<td>34</td>
</tr>
</tbody>
</table>
Complementation analysis suggests a high degree of saturation

Seventy Class I mutant lines that displayed a strong, highly penetrant germ cell migration defect fall into 9 complementation groups (Table 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 II alleles and many of the mutants identified in previous screens for defects in pattern formation (Table 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). When combining the results for the allele frequencies of genes in both the Class I and Class II 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 I and Class II 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. Complementation analysis of Class I and Class II mutants. (*) 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

**Class 1: specific germ cell migration defect**

<table>
<thead>
<tr>
<th>Allele</th>
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<tr>
<td>abdominal A (abdA)</td>
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</tr>
<tr>
<td>Abdominal B (AbdB)</td>
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</tr>
<tr>
<td>columbus (clb)</td>
<td>15</td>
</tr>
<tr>
<td>heartless (htl)</td>
<td>4</td>
</tr>
<tr>
<td>fear-of-intimacy (foi)</td>
<td>3</td>
</tr>
<tr>
<td>tinman (tin)</td>
<td>1</td>
</tr>
<tr>
<td>trithorax (trx)</td>
<td>17</td>
</tr>
<tr>
<td>trithoraxgleich (trg)</td>
<td>4</td>
</tr>
<tr>
<td>zinc finger homeodomain-1 (zfhl-1)</td>
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</table>

**Class 2: pattern formation mutants**

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<th>Category</th>
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<td>gut development</td>
<td>huckebein (hkb)</td>
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</tr>
<tr>
<td></td>
<td>serpent (srp)</td>
<td>11</td>
</tr>
<tr>
<td>dorsal/ventral polarity</td>
<td>shrew (srw)</td>
<td>8</td>
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<tr>
<td></td>
<td>toloid (tld)</td>
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<tr>
<td>gap</td>
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<td></td>
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<td>knirps (kni)</td>
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<td></td>
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<tr>
<td>pair-rule</td>
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<td></td>
<td>odd-paired (opa)</td>
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<td></td>
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</tr>
<tr>
<td>cellular differentiation</td>
<td>crumbs (crb)</td>
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</tr>
<tr>
<td>cellularization</td>
<td>thread (th)\textsuperscript{a}</td>
<td>6</td>
</tr>
</tbody>
</table>
Placing genes on the chromosomal map

Rough mapping of 2 representative alleles from each of the 6 remaining complementation groups in Class I 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 deletions 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. 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. 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. 3C). Those germ cells that do leave the PMG often appear disorganized within the mesoderm, and do not correctly navigate toward SGPs (data not shown). 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. 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. 4C). In contrast, 412 expression appears normal in clb mutant embryos, implying that this gene is not required for the specification of SGPs (data not shown). Results consistent with those described above are seen using a variety of markers, including anti-Cli, Dwnt-2, and anti-Zfh-1, which recognize gonadal mesoderm at various points in development (data not shown).

-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. 3E). For the present analysis, we have used the abdA<sup>nei</sup> 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 (data not shown), in a manner similar to that seen in embryos lacking the Bithorax-Complex (Brookman et al., 1992).
Whereas \textit{abdA} is required for the development of all gonadal mesoderm cells, mutations in \textit{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, \textit{trx}, that has a germ cell migration phenotype very similar to that seen in \textit{AbdB} mutants (Fig. 3G). The "lost" germ cells in \textit{trx} and \textit{AbdB} mutants remain in an area ventral and posterior to the gonad until after coalescence. The \textit{trx} gene is known to be required for maintaining the expression of homeotic genes including \textit{abdA} and \textit{AbdB} (Breen and Harte, 1993; Mazo et al., 1990). Several lines of evidence suggest that the defect seen in \textit{trx} mutants is due to reduced function of \textit{AbdB}, including the result that a \textit{hs-AbdB} construct can partially rescue the \textit{trx} germ cell migration defect (Moore and Lehmann, unpublished results). Surprisingly, initial results from our analysis of gonadal mesoderm development appeared to be inconsistent with this theory. In embryos lacking zygotic \textit{trx}, 412 appears to be expressed at normal levels (data not shown), whereas in \textit{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 \textit{trx} show 412 expression levels identical to those seen in \textit{AbdB} mutants (data not shown). These results suggest that \textit{trx}, like \textit{AbdB}, is required for a subset of SGPs to maintain their identity and as a result, to maintain their association with germ cells (Boyle and DiNardo, 1995).

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

Mutations in the \textit{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. 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 \textit{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 \textit{tin} function (Fig. 4D). This result is consistent with previous studies demonstrating expression of another SGP marker, \textit{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 their 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. 3K). Once again, the fault appears to lie with gonadal mesoderm as highlighted by 412 expression (Fig. 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. 4C with Fig. 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.

Fig. 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
Fig. 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-l*. 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-l* 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). 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. 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 visceral mesoderm development (Azpiazu et al., 1996). In fact, we find that the gonadal mesoderm appears to develop correctly in h mutants (data not shown). 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.

Fig. 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-G) Mutations in ftz, opa, and hh all result in the failure of germ cells to associate with mesodermal cells; (D-H) The number of gonadal mesoderm cells is severely reduced in all mutants shown.
Fig. 2-5: Segmental origin of gonadal mesoderm lies within the *eve* domain of the mesoderm
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).

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. 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 (data not shown), 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).

Fig. 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

Migration through Midgut

- srp, hkb

Attachment to Mesoderm

- zfh-1, clb
- htl

Alignment with Gonadal Mesoderm

- abdA, AbdB
- trx, trg

Gonad Coalescence

- tin
- foi
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 \textit{abdA}, \textit{AbdB}, \textit{cli}, and \textit{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. \textit{zfh-1} and \textit{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 \textit{abdA}, \textit{AbdB}, \textit{cli}, and \textit{tin}.

We and others have found the \textit{tin} gene to be required for development of gonadal mesoderm, as exemplified by the lack of expression of gonadal mesoderm specific markers in \textit{tin} mutants (Boyle et al., 1997; Fig. 4D). Although the drastically reduced expression of \textit{cli} in \textit{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. 3I). More recent work has shown that most SGPs are at least partially specified in \textit{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 \textit{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 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 \textit{htl} mutant embryos (Beiman et al., 1996; Gisselbrecht et al., 1996). Our phenotypic analysis of both germ cell migration and gonadal mesoderm defects in \textit{htl} embryos demonstrates that this gene is required at an early stage in the development of yet another mesodermal cell type, the gonadal mesoderm. \textit{htl} is also necessary for the Dpp-dependent maintenance of \textit{tin} expression in dorsal regions of the mesoderm (Gisselbrecht et al., 1996). Therefore, \textit{htl} could be acting through \textit{tin} to specify SGPs, since \textit{tin} is required for gonadal mesoderm development (see above). Conversely, \textit{htl} could be required for a signaling process that is independent of its role in maintaining \textit{tin}'s dorsal expression pattern. Further experiments are necessary to distinguish between these two possibilities, but the finding that \textit{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 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. 5 and data not shown). 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 I 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.
REFERENCES


PREFACE
The screen presented in this chapter was a collaboration between Lisa Moore and myself. I conducted the subsequent analysis of the *waldo* and *schnurri* phenotypes.
CHAPTER 3

Second chromosome screen and characterization of waldo and schnurri germ cell migration phenotypes

SUMMARY
This chapter outlines a relatively small-scale screen for mutations located on the second chromosome required zygotically for germ cell migration. This screen was the predecessor to the saturation mutagenesis carried out on the third chromosome. While we did not saturate the second chromosome, we did establish that such a mutagenesis and screen were capable of identifying genes involved in guiding germ cells on their migratory route through the embryo. Additionally, many of the mutant phenotypes characterized in this screen are similar to those described in the third chromosome screen, suggesting that the same genetic steps are affected.

Several interesting mutants were uncovered in this screen; two of which, waldo and schnurri, are described here. waldo (wdo) is an uncharacterized gene with a unique germ cell migration phenotype among the mutants that we have identified. It appears to be required for germ cell migration on the basal surface of the endoderm. Phenotypic analysis demonstrates that the endoderm fails to develop properly in wdo mutants. I discuss a model in which wdo encodes an adhesion molecule required for germ cell/endoderm adhesion. schnurri (shn) codes for a transcription factor believed to be in the decapentaplegic (dpp) signal transduction cascade. I describe the germ cell migration phenotype in shn mutant embryos, and suggest that it is likely to result from a failure of somatic gonadal precursor (SGP) development. Since dpp is not required for SGP development, and in fact represses lateral mesoderm development, shn and dpp have opposite effects on the development of this tissue. I suggest several potential roles for shn in lateral mesoderm development.
INTRODUCTION

Genes controlling the migration of several different cell types in the Drosophila embryo have recently been identified through genetic screens (Samakovlis et al., 1996; Van Vactor et al., 1993). Similarly, we expected that a genetic approach would also be successful in the identification of genes involved in guiding germ cell migration. To test this hypothesis, we carried out an EMS mutagenesis of the second chromosome and screened about 3200 lines for aberrant germ cell migration. While we clearly did not saturate this chromosome for mutations, we did isolate a number of lines in which germ cell migration is disrupted. Many of these mutant phenotypes resemble those identified in the saturation mutagenesis of the third chromosome (Chapter 2), suggesting that they may be involved in the same genetic steps. Furthermore, both screens were successful in identifying genes required for gonadal mesoderm development, here called somatic gonadal precursors (SGPs).

In this chapter, I outline the second chromosome screen and my characterization of two mutants that were identified in it. The first of these mutants, waldo (wdo), is required for a very early step of germ cell migration. In wdo mutant embryos, germ cells do not successfully navigate to mesoderm, but appear to prematurely detach from the basal surface of the posterior midgut (PMG). None of the third chromosome mutants that we identified are required for this step, although one previously-identified gene, wunen (wun), has been shown to be necessary to guide the endodermal migration of germ cells (Zhang et al., 1996; Zhang et al., 1997). In wun mutant embryos, germ cells do not migrate dorsally on the PMG as they do in wild-type embryos. Instead, their migration on the PMG is unoriented (Zhang et al., 1996). wun encodes a transmembrane protein with an intracellular domain with sequence similarity to type 2 phosphatidic acid phosphatase (Zhang et al., 1997). It is believed to act as a repulsive signal in germ cell migration, since it is expressed on the regions of the PMG on which germ cells do not typically travel (Zhang et al., 1997). There are several possible mechanisms by which Wun might be involved in germ cell guidance. On the one hand, Wun might be constitutively active and required for the production of a cell surface factor that destabilizes germ cell filopodia. In this way, germ cells would be steered away from Wun-expressing cells (Van Doren and Lehmann, 1997). Alternatively, Wun might produce a signal inside the Wun-expressing cells only in response to a germ cell-specific ligand. This signal would then repel germ cells away from Wun-expressing cells (Zhang et al., 1997). Further characterization of the function of the Wun extracellular domain may help to distinguish between these possibilities.
While germ cells in wdo mutant embryos are lost at a similar time as germ cells in wun mutant embryos, phenotypic analysis suggests that the adhesion of the PMG and the germ cells may be affected in wdo mutants. I show that the PMG develops abnormally in wdo embryos, suggesting that the germ cell migration phenotype may result from a defect in endodermal development. Furthermore, I have genetically mapped wdo to a poorly characterized region of the left arm of chromosome 2.

I have also analyzed the role of the schnurri (shn) gene in germ cell migration. shn was initially identified by Nüsslein-Volhard et al. (1984) in their screen for genes on the second chromosome required for embryonic patterning. Several lines of evidence suggest that shn may be in the dpp pathway. First, mutations in shn and dpp or its receptors yield similar phenotypes. For instance, there are similar midgut defects in dpp and shn mutants (Arora et al., 1995; Grieder et al., 1995), and both are required for dorsal mesoderm development (Arora et al., 1995). Second, the cuticle phenotype of a weak dpp allele is enhanced in a shn background (Arora et al., 1995). Finally, shn is thought to be downstream of dpp since dpp overexpression phenotypes in the mesoderm and in the endoderm depend on wild-type shn function (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). shn encodes a zinc finger containing transcription factor and has an expression pattern overlapping the dpp expression pattern, consistent with its proposed role in dpp signaling (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995).

I describe the shn germ cell migration phenotype and demonstrate that it results from a failure in the development of somatic gonadal precursors (SGPs). shn is likely involved in the patterning of the lateral domain of the mesoderm, as the fat body, which is the sister tissue of gonadal mesoderm (Moore et al., 1998), also fails to develop properly in shn mutants. This is a phenotype that shn does not share with dpp, as we have shown that dpp signaling is not required for SGP development (Chapter 4, Appendix). In light of these observations, I discuss several possible models for shn function in the development of the lateral mesoderm.
MATERIALS AND METHODS

EMS Mutagenesis and establishment of balanced lines
See Fig. 3-1 for an outline of the screen. 100 cn P[fa-f-lacZ] males were mutagenized with 35 mM EMS from Sigma, in a method identical to that described in Chapter 2. These males were mated to 300 virgins of the genotype l(2)91DTS b pr cn sca/P[ftz-lacZ] CyO (ftz-CyO). A total of 3700 F1 males of either genotype were each mated to 2-3 l(2) 91DTS b pr cn sca/ftz-CyO virgins. These crosses were placed at 29°C for 4 days in order to kill larvae containing the dominant temperature sensitive (DTS) mutation. After this period, the vials were placed at room temperature. Many of the F2 "lines" were contaminated with escaper DTS-carrying flies. We found that, on average, these flies eclosed a few days later than the flies of the desired genotype. Therefore, in order to generate lines, we verified that the first few flies to eclose were of the genotype cn P[fa-f-lacZ]/ftz-CyO, transferred these flies into fresh vials, and allowed them to lay eggs.

Screening of Embryos
The timing of egg collections and method of X-gal staining was identical to that described in Chapter 2 for the third chromosome. However, the germ cell migration phenotype of the embryos was not scored directly in the staining dishes, as in the third chromosome screen. Instead, glycerol slides were prepared for all the lines, and the stained embryos were viewed using a Zeiss Axiophot microscope. By this method, 202 lines did not appear to display wild-type germ cell migration.

In order to look more carefully at the phenotype of embryos from these lines, antibody staining was performed using polyclonal anti-Vasa and anti-βgal antibodies. After dechorionation, embryos were transferred into scintillation vials containing 1.75 ml PBS, 0.25 ml 37% glutaraldehyde, and 8 ml heptane and were fixed for 20 minutes with gentle shaking. Antibody staining was carried out as described in Eldon and Pirrotta (1991). The protocol used for cuticle preparations is described in Chapter 2.

Complementation Analysis
The lines with phenotypes somewhat specific for germ cell migration were divided into two groups based on the strength of the migration phenotype. 13 lines were judged to have strong phenotypes, with many "lost" germ cells; while 19 had weak phenotypes, in which a minority of germ cells migrated incorrectly. Complementation tests were scored both for lethality and for the germ cell migration phenotype of the transheterozygote.
Initially, complementation tests were conducted between mutants within each class. While some of the lines within the "strong" class were found to be allelic to each other (see Table 3-2), all of the mutants within the "weak" class complemented each other. Subsequently, all of the 19 mutants in the "weak" class were crossed to representative alleles from the "strong" complementation groups. In these tests, all of the "weak" mutants were also found to complement all of the "strong" mutants.

The pattern formation class of mutants includes those whose cuticles resemble that of genes known to be required in embryonic patterning. For this class, complementation tests were performed between individual lines and representative alleles of candidate genes. The results of this analysis are indicated in Table 3-2.

The class of mutants with gross developmental defects was subdivided into three subclasses prior to complementation analysis (see Table 3-2). The first group, consisting of 22 lines, is comprised of lines in which the initial gastrulation movements, such as germ cell extension, do not occur correctly. In many of these lines, germ cells are not internalized into the embryo. These lines were not analyzed further. In the other two subclasses, embryos appear to gastrulate properly. These classes are distinguished by the strength of the germ cell migration phenotype. First, all of the lines with "strong" germ cell migration phenotypes were crossed together, as were all of the "weak" lines. Second, alleles from "strong" and "weak" complementation groups were crossed with each other. Finally, an allele from each complementation group was crossed to an allele from complementation groups on the second chromosome with poorly differentiated cuticles.

**Fly Stocks**

The following fly stocks were obtained from the Bloomington stock center and were used in complementation analyses: bib1, bsk1, clt1, en1, eve1D, faI1, flb1, faS1, flz1, fzy1, ghOl, Df(2R)gsb, Kr1, mmY1, odd7L, pim1L, pral6L, raw1, rib1, shg2, snal, tup1, twi1, ush2, and wg1L.

The P element stocks titled P(lacW)kxxxxx were obtained from the Berkeley Drosophila Genome Project through T. Laverty. The recombination frequencies between these P elements and the wdo locus were determined by multiplying the number of recombinants by two, as half of the recombinant progeny were lethal, and dividing by the total number of progeny scored.

The RD2 line (Hursh et al., 1993) which contains a dpp reporter gene construct was obtained from R. Padgett and W. Gelbart.
**wald**o **alleles**
We identified two **wald**o alleles: \textit{wald}^{20.89} and \textit{wald}^{35.16}. The germ cell migration phenotype of \textit{wald}^{35.16} is similar to, but stronger than, the phenotype of \textit{wald}^{20.89}, suggesting that 35.16 is a stronger allele than 20.89. Since both mutagenized chromosomes are likely to have more than one lesion, phenotypic analysis was performed on transheterozygous embryos.

**schnurri** **alleles**
Four \textit{schnurri} alleles were identified in our screen, but because the molecular nature of a strong \textit{schnurri} allele is known, I used this allele in the phenotypic analysis. \textit{schnurri}^{1B} was identified by Nüsslein-Volhard et al. (1984) and has since been shown to contain a nonsense mutation producing a truncated protein in which the two C terminal Zn fingers are absent (Chen and Hoffmann, personal communication). Since there is still some question as to whether this allele is a null (see Discussion), I repeated the phenotypic analysis with \textit{l(2)04738}, here called \textit{schnurri}^{P}, a \textit{P} element allele thought to be a strong loss-of-function allele (Arora et al., 1995; Grieder et al., 1995). The germ cell migration and mesodermal phenotypes of this allele were, in general, slightly weaker than the phenotypes observed in \textit{schnurri}^{1B} embryos.

**Whole-mount Antibody staining**
Antibody staining was performed using horseradish peroxidase with biotinylated secondary antibodies and the Elite kit (Vector laboratories). Embryos were fixed and stained as described in Chapter 4. The following primary antibodies were used in this work: anti-FasIII (N. Patel) at 1:10, anti-Vasa (A. Williamson) at 1: 5,000, anti-βgal (Cappel) at 1:10,000, anti-Srp (M. Leptin) at 1:1,000, and anti-Cli (M. Boyle and N. Bonini) at 1:1,000.

**Whole-mount in situ hybridization**
Whole-mount in situ hybridizations were performed as in Lehmann and Tautz (1994). Antisense digoxigenin-coupled RNA probes were synthesized using the Boehringer-Mannheim 'Genius' kit as described in Gavis and Lehmann (1992). The lacZ riboprobe was made with the pC48-gal plasmid and T7 polymerase (Thummel et al., 1988). The 412 retrotransposon riboprobe was made with pSK2.4#3 plasmid (Brookman et al., 1992) and T7 polymerase.
RESULTS

Second chromosome zygotic screen
Prior to the third chromosome screen (see Chapter 2), we conducted a similar, but smaller scale, screen of the second chromosome, which is outlined in Figure 3-1. The genetics in the two screens are virtually identical. The primary difference is that in the third chromosome screen, a chromosome containing a HS-hid construct was utilized in order to facilitate the generation of isogenic lines, while in the second chromosome screen, we used a chromosome bearing a dominant temperature sensitive (DTS) mutation.

Table 3-1 summarizes the results of the second chromosome screen. We conducted a primary screen of 3194 lines by X-gal staining. 83% of these lines were homozygous lethal, which, using the Poisson distribution, translates into an average of 1.8 lethal hits/chromosome. In the primary screen, 202 lines did not appear to have wild-type germ cell migration. These lines were subjected to a secondary screen in which the larval cuticle preparations were prepared in order to analyze overall embryonic development, and anti-Vasa antibody was used in order to more carefully analyze the germ cell migration defect. This analysis allowed us to place the lines into phenotypic classes, shown in Figure 3-1.

Table 3-2 summarizes the results of the complementation analysis (see Materials and Methods for details). These data were used to calculate the average number of alleles/locus generated in the screen. For known genes, we obtained an average of 2.4 alleles/locus; however, if all single mutants are included, the average number of alleles/locus falls to 1.5. The difference between these two values probably reflects the fact that the phenotypes of some of the many single mutants result from synthetic effects of multiple mutations. Regardless, the low allele frequency that we obtained indicates that we did not saturate this chromosome for mutations disrupting germ cell migration. However, this work did demonstrate the feasibility of identifying genes required for germ cell migration with this type of a screen, which was an important conclusion before we proceeded with a screen of the scale of the third chromosome screen. We focused our subsequent analysis on the second chromosome mutants on the lines with "specific" germ cell migration phenotypes. My analysis of two of these genes: waldo (wdo) and schnurri (shn) is presented here.
Figure 3-1. Outline of screen for mutants affecting germ cell migration on chromosome 2

$\frac{l(2)\text{ DTS(91) b pr cn sca}}{P\{ftzlacZ\} CyO}$ $\times$ $\frac{cn\ P(faf-lacZ)}{cn\ P(faf-lacZ)}$

$\frac{l(2)\text{ DTS(91) b pr cn sca}}{P\{ftzlacZ\} CyO}$ $\times$

$\frac{cn\ P(faf-lacZ)}{cn\ P(faf-lacZ)}$ $^*$

with

$\frac{l(2)\text{ DTS(91) b pr cn sca}}{P\{ftzlacZ\} CyO}$ $\times$

$\frac{cn\ P(faf-lacZ)}{cn\ P(faf-lacZ)}$ $^*$

or

$\frac{cn\ P(faf-lacZ)}{cn\ P(faf-lacZ)}$ $^*$

$\frac{P\{ftzlacZ\} CyO}{P\{ftzlacZ\} CyO}$

29 C

only $\frac{cn\ P(faf-lacZ)}{P\{ftzlacZ\} CyO}$ $^*$ should survive

remove escapers

Generate stocks:

$\frac{cn\ P(faf-lacZ)}{cn\ P(faf-lacZ)}$ $^*$

(if homozygous viable)
Table 1: Screen for genes required for germ cell migration and gonad formation on chromosome 2

<table>
<thead>
<tr>
<th>lines scored</th>
<th>3194</th>
</tr>
</thead>
<tbody>
<tr>
<td>lines selected</td>
<td>202</td>
</tr>
<tr>
<td>lethal hits/chromosome</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenotypic Classes</th>
<th>n</th>
<th>% of selected lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;specific&quot; effect on germ cells</td>
<td>29</td>
<td>14%</td>
</tr>
<tr>
<td>pattern formation</td>
<td>21</td>
<td>10%</td>
</tr>
<tr>
<td>gross developmental defects</td>
<td>38</td>
<td>19%</td>
</tr>
<tr>
<td>non-2nd chromosome mutations</td>
<td>17</td>
<td>8%</td>
</tr>
<tr>
<td>false positives</td>
<td>97</td>
<td>48%</td>
</tr>
</tbody>
</table>
Table 3-2. Complementation Analysis

If one or more lines failed to complement a previously-identified mutation, or if more than one mutant defined a new complementation group, the number of alleles identified is in the "# alleles" column. When lines complemented each other, and all known loci, they are listed in the "singe mutants". This distinction was made because it is likely that some of the phenotypes result from synthetic effects of more than one lesion (see Results and Discussion). (a) complementation tests were not performed between the lines which did not gastrulate.
Table 2: Complementation Analysis

**Class I: specific/strong germ cell migration defect**

<table>
<thead>
<tr>
<th>Allele</th>
<th># alleles</th>
<th>Single mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>clift/eyes absent (cli)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>schnurri (shn)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>shotgun (shg)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>waldo (wdo)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>9.35</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>22.38</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

**Class II: pattern formation mutants**

<table>
<thead>
<tr>
<th>Class</th>
<th># alleles</th>
<th>Single mutants</th>
</tr>
</thead>
</table>
| a. Mutants in known genes  
Krüppel (Kr) | 1         |               |
| odd skipped (odd) | 2         |               |
| paired (prd)      | 2         |               |
| snail (sna)       | 2         |               |
| twist (twi)       | 4         |               |
| wingless (wg)     | 2         |               |
| b. Mutants in unidentified genes/synthetic effects  
tail-up/ushaped-like | 6         |               |
| terminal defects  | 1         |               |
| gap gene-like     | 1         |               |

**Class III: gross developmental defects**

<table>
<thead>
<tr>
<th>Class</th>
<th># alleles</th>
<th>Single mutants</th>
</tr>
</thead>
</table>
| a. “strong” germ cell migration phenotype  
faint sausage (fas) | 2         | 8             |
| unidentified/synthetic      |           |               |
| b. “weak” germ cell migration phenotype  
faint little ball (flb) | 2         | 4             |
| unidentified/synthetic      |           |               |
| c. gastrulation problems    | 22^3      |               |
**waldo (wdo) is required for an early step of germ cell migration**

We isolated two alleles of *waldo (wdo)*. In *wdo* mutant embryos, germ cells migrate through the posterior midgut (PMG) as in wild type. However, it appears that germ cells do not properly adhere to the basal surface of the PMG epithelium, as they disperse prior to their dorsally-oriented migration on the PMG (compare Fig. 2A,B). During germband retraction, most germ cells are found within the embryonic yolk (Fig. 2C), supporting the conclusion that they are lost prior to association with mesoderm. Eventually, germ cells end up scattered throughout the posterior half of the embryo (Fig. 2E). Although the vast majority of germ cells migrate abnormally in *wdo* mutants, there are usually a few germ cells which are able to reach approximately the correct location (arrow in Fig. 2C).

I analyzed the development of the endoderm in *wdo* mutants in order to determine if the germ cell migration phenotype might be the result of a defect in the development of the endoderm. In wild type, the Drosophila homolog of the angiotensin converting enzyme (RACE) is expressed strongly in the anterior and posterior midgut invaginations (Tatei et al., 1995 and Fig. 3A). Although RACE is initially expressed properly in *wdo* mutants (Fig. 3B,D), by stage 13, RACE expression indicates that there is a defect in endoderm morphogenesis. At the onset of germband retraction in wild-type embryos, the epithelia of the anterior and posterior midgut become mesenchymal and migrate toward each other as paired lobes before joining at stage 13 to form bands on either side of the yolk (Skaer, 1993 and Fig. 3C). In *wdo* mutant embryos, the migration of the anterior and posterior midgut does not occur and these cells remain clustered at the ends of the embryo (Fig. 3D).

Since endodermal cells grow out along underlying visceral mesoderm cells (Skaer, 1993), it is possible that the defect in endodermal migration observed in *wdo* mutants stems from a problem in the development of the visceral mesoderm. In order to test this possibility, visceral mesoderm development was measured with several molecular markers. Expression of Fasciclin III (FasIII) is initiated in the visceral mesoderm beginning at stage 11, when these cells are aligned in rows on either side of the endoderm (Patel et al., 1987 and Fig. 4A). In *wdo* mutant embryos, FasIII is expressed normally and the early morphogenesis of the visceral mesoderm appears to be wild-type (Fig. 4B). *decapentaplegic (dpp)*, a member of the TGFβ family of signaling proteins, is expressed in two domains within the visceral mesoderm (St. Johnston and Gelbart, 1987 and Fig. 4C). These domains of *dpp* expression are largely wild-type in *wdo* mutants, although the midgut constrictions do not form (Fig. 4D). Since the formation of the midgut constrictions depends on inductive interactions between the
endoderm and the mesoderm (Skaer, 1993), it is not surprising that the constrictions do not form in wdo mutants as the endoderm is not closely apposed to the visceral mesoderm. The analysis of FasIII and dpp expression in the visceral mesoderm is consistent with the idea that the development of the visceral mesoderm is normal in wdo mutant embryos. Therefore, the failure of the endoderm to migrate over the visceral mesoderm is likely to result from a failure in endodermal development.

SGP development was also analyzed in wdo mutant embryos. Initially, I looked at SGP development to determine whether or not the few germ cells which do migrate into the mesodermal layer are able to locate SGPs. Using 412 RNA as an SGP marker and anti-Vasa to label germ cells, it appears that germ cells do attach to SGPs (arrows in Fig. 5B,D). However, there appears to be a reduced number of SGPs in wdo mutants. Using 412 RNA, this reduction was apparent as early as stage 11 (compare Fig. 5A,B). In order to look more closely at SGP development, anti-Zfh-1 was used to label SGPs in wdo mutant embryos. A reduction in the number of SGPs was also evident using the anti-Zfh-1 antibody. At stage 13, the number of SGPs is reduced by approximately 50% (compare Fig. 6A,B). While many of the germ cell migration phenotypes that were characterized in the third chromosome screen (see Chapter 2) are likely caused by a failure of SGP development, this is unlikely to be the case in wdo mutant embryos, since the migration of germ cells on the endoderm occurs properly in embryos in which the entire mesodermal layer is absent (see Discussion and Jaglarz and Howard, 1995).

The defects in the development of the endoderm and the mesoderm in wdo mutant embryos raise the possibility that wdo plays a role in the development of multiple tissue types. The idea that wdo has pleiotropic effects is supported by the wdo cuticle phenotype. 14/79 (18%) of cuticles analyzed were wild-type, 25/79 (32%) had variable segmentation defects, and 40/79 (50%) were poorly differentiated. The high degree of variability in the cuticle pattern may indicate that the alleles used in this analysis are not nulls. While this is likely to be the case, it has been difficult to directly test, since I was unable to identify deficiencies uncovering the wdo locus (see below).
Figure 3-2. Migratory germ cells prematurely detach from the endoderm in *waldo* mutant embryos.

(A,C,E) *wdo* mutant embryos. (B,D,F) wild-type embryos. All embryos are labeled with anti-Vasa antibody and are situated anterior left. In (B), germ cells in a wild-type st11 embryo have successfully transferred to the mesodermal layer. In the st11 *wdo* embryo in (A), most germ cells have dissociated from the endoderm and are situated in the yolk. This phenotype is apparent at st12, when in wild type, germ cells are associating with SGPs (D). In a *wdo* mutant embryo (C), a few germ cells may have correctly navigated to SGPs (arrow), but the majority of germ cells are still within the yolk. At st14, germ cells in *wdo* mutants appear more widely dispersed, with many now present in ectodermal regions (E). In a wild-type embryo at this stage, germ cells and SGPs are coalescing in PS10 (F).
Figure 3-2: Migratory germ cells prematurely detach from the endoderm in wdo mutant embryos

\( wdo \)  \hspace{1cm}  \text{wild type}

\begin{tabular}{ll}
A & B \\
\includegraphics[width=0.4\textwidth]{wdo_st11} & \includegraphics[width=0.4\textwidth]{wild_type_st11} \\
C & D \\
\includegraphics[width=0.4\textwidth]{wdo_st12} & \includegraphics[width=0.4\textwidth]{wild_type_st12} \\
E & F \\
\includegraphics[width=0.4\textwidth]{wdo_st1314} & \includegraphics[width=0.4\textwidth]{wild_type_st1314}
\end{tabular}
Figure 3-3. The anterior and posterior midgut primordia do not migrate along the visceral mesoderm in \textit{wdo} mutants.

(A,C) are wild-type embryos, (B,D) are \textit{wdo} mutant embryos. Embryos are double-labeled with anti-Vasa antibody which recognizes the germ cells, and a riboprobe for \textit{RACE} RNA, which recognizes the endodermal primordia. Anterior is to the left in all panels. A comparison of panels (A,B) demonstrates that \textit{RACE} RNA is initially properly expressed at st10 in \textit{wdo} mutants. In (C), the bilaterally symmetric arms of the anterior and posterior midgut extend toward each other in wild type. In the \textit{wdo} embryo in (D), the endodermal primordia are not migrating toward each other, but remain as groups of cells near the anterior and posterior poles of the embryo.
Figure 3-3: The anterior and posterior midgut primordia do not migrate along the visceral mesoderm in *wdo* mutants.
Figure 3-4. Aspects of visceral mesoderm development occur properly in wdo mutant embryos.

(A,C) wild type, (B,D) wdo mutants. Panels (A,B) were labeled with anti-FasII antibody which labels the visceral mesoderm. (The embryo in (A) is a wdo/ftz-CyO heterozygote that has also been labeled with βgal antibody to recognize the balancer chromosome.) Embryos in (C,D) carry the RD2 dpp reporter gene construct, which expresses Dpp specifically in the visceral mesoderm. All embryos are anterior left. A comparison of (A,B) indicates that the visceral mesoderm as seen with FasIII is correctly specified in wdo mutant embryos. In (C), dpp expression in two distinct domains within the visceral mesoderm is apparent. In the wdo embryo in (D), dpp is still expressed in approximately the proper visceral mesoderm domains, although the midgut constrictions are not present in these embryos.
Figure 3-4: Aspects of visceral mesoderm development occur properly in *wdo* mutant embryos.
Figure 3-5. *wdo* mutant embryos have reduced 412 RNA expression

(A,C) wild type (B,D) *wdo* mutant embryos. Embryos were double labeled with anti-Vasa (brown) which recognizes germ cells and a riboprobe for 412 RNA (purple), which labels gonadal mesoderm. All embryos are anterior left. In (A), germ cells are found associating with gonadal mesoderm. Note that at this stage, 412 expression is not restricted to the gonadal mesoderm, but is found in lateral mesoderm in all parasegments. In the *wdo* mutant embryo in (B), a few germ cells adhere to gonadal mesoderm (arrow). However, the expression of 412 appears reduced relative to wild type. This reduction of 412 RNA expression is also evident at st14 (compare C,D).
Figure 3-5: *wdo* mutant embryos have reduced 412 expression

**A** wild type

**B** waldo

**C**

**D**

412 RNA, anti-Vasa
Figure 3-6. Fewer SGPs are present in wdo mutant embryos.

(A) wild type (B) wdo mutant embryo. Embryos in both panels were labeled with anti-Zfh-1 antibody to recognize the gonadal mesoderm. Anterior is to the left. A comparison of (A,B) indicates that the number of SGPs is reduced by approximately 50% in wdo embryos relative to wild type at this stage.
Figure 3-6: Fewer SGPs are present in \textit{wdo} mutant embryos

\textbf{A} \hspace{2em} \textbf{B}

\textbf{anti-Zfh-1}

\textbf{wt}

\textbf{wdo}
*wdo* may map between 28C and 29C (Where's *waldo*?)

The *wdo* locus was roughly mapped using an *al dp b pr c px sp* chromosome. The recombination frequencies between the *wdo* locus and the *dp* and *b* genes suggest that *wdo* is located 15 cM proximal to *dp* which corresponds to a genetic position of 26.1 and a cytological position of between 28C and 29C. All of the deficiencies available from Bloomington in the region between *dp* and *b* were crossed to both *wdo* alleles (Table 3). These crosses were scored both for transheterozygous lethality and for a germ cell migration phenotype. All of these deficiencies complement *wdo* mutations. Subsequently, additional deficiencies in the region of 28C-28D were ordered and complementation tests performed (Table 3). All of these deficiencies also failed to uncover *wdo*.

In order to obtain additional evidence that *wdo* was located in this area, I mapped *wdo* relative to a number of *w*+ P elements in the region (Table 4). *wdo* maps 2.8 cM from *P{lacW}k10210*. In general, recombination values between *wdo* and the other P elements tested increase as one moves either proximally or distally from *P{lacW}k10210* (Table 4). The region extending 2.8 cM distal to 2.8 cM proximal of this P element extends from approximately 28C-29D. These mapping data support the idea that *wdo* is located within this chromosomal region.
Table 3-3: Deficiencies between *dp* and *b* that complement *waldo*

<table>
<thead>
<tr>
<th>Bloomington #</th>
<th>Name</th>
<th>Breakpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>1712</td>
<td>In(1) w&lt;sup&gt;m4&lt;/sup&gt;; Df(2L)2802/CyO</td>
<td>25F2-3; 25F4-26A1</td>
</tr>
<tr>
<td>1357</td>
<td>Df(2L)J136-H52</td>
<td>27C2-9; 28B3-4</td>
</tr>
<tr>
<td>3077</td>
<td>Df(2L)spd, al&lt;sup&gt;l&lt;/sup&gt; dp&lt;sup&gt;ov1&lt;/sup&gt;/CyO</td>
<td>27D-E; 28C</td>
</tr>
<tr>
<td>n.a.</td>
<td>TE80x1</td>
<td>28D4; 28E1</td>
</tr>
<tr>
<td>n.a.</td>
<td>TE128x11</td>
<td>28E4-7; 29B2-C1</td>
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<tr>
<td>n.a.</td>
<td>TE128x14</td>
<td>28F1-2; 29A2-B1</td>
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<tr>
<td>n.a.</td>
<td>TE128x36</td>
<td>29B</td>
</tr>
<tr>
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<td>29A1-2; 29E1-2</td>
</tr>
<tr>
<td>n.a.</td>
<td>TE128x16</td>
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<tr>
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<td>29C1-2; 30C8-9</td>
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<td>29C3-5; 30C8-9</td>
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<tr>
<td>3702</td>
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<td>29F7-30A1; 30C2-5</td>
</tr>
<tr>
<td>1045</td>
<td>Df(2L)Mdh, cn&lt;sup&gt;l&lt;/sup&gt;/Dp(2;2)Mdh3, cn&lt;sup&gt;l&lt;/sup&gt;</td>
<td>30D-30F; 31F</td>
</tr>
<tr>
<td>1469</td>
<td>Df(2L)J39/In(2L)Cy; Dp(2;Y)cb50, Dp(1;Y)BSYy&lt;sup&gt;+&lt;/sup&gt;/C(1)RM</td>
<td>31C-D; 32D-E</td>
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<tr>
<td>3079</td>
<td>Df(2L)Pr1/CyO</td>
<td>32F1-3; 33F1-2</td>
</tr>
<tr>
<td>3129</td>
<td>Df(2L)esc10, b&lt;sup&gt;l&lt;/sup&gt; pr&lt;sup&gt;l&lt;/sup&gt;/CyO</td>
<td>33A8-B1; 33B2-3</td>
</tr>
<tr>
<td>3344</td>
<td>Df(2L)prd1.7, b&lt;sup&gt;l&lt;/sup&gt; Adh&lt;sup&gt;n2&lt;/sup&gt; pr&lt;sup&gt;l&lt;/sup&gt; cn&lt;sup&gt;l&lt;/sup&gt;/CyO</td>
<td>33B2-3; 34A1-2</td>
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<td>35B1-3; 35E6</td>
</tr>
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<td>1491</td>
<td>Df(2L)r10, cn&lt;sup&gt;l&lt;/sup&gt;/In(2LR)</td>
<td>35E1-2; 36A6-7</td>
</tr>
<tr>
<td>3180</td>
<td>Df(2L)H20, b&lt;sup&gt;l&lt;/sup&gt; pr&lt;sup&gt;l&lt;/sup&gt; cn&lt;sup&gt;l&lt;/sup&gt; sca&lt;sup&gt;l&lt;/sup&gt;/CyO</td>
<td>36A8-9; 36E1-2</td>
</tr>
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<td>420</td>
<td>Df(2L)TW137, cn&lt;sup&gt;l&lt;/sup&gt; bw&lt;sup&gt;l&lt;/sup&gt;/CyO, Dp(2;2) M(2)m&lt;sup&gt;+&lt;/sup&gt;</td>
<td>36C2-4; 37B9-C1</td>
</tr>
<tr>
<td>3189</td>
<td>Df(2L)TW50, cn&lt;sup&gt;l&lt;/sup&gt;/CyO, Dp(2;2) M(2)m&lt;sup&gt;+&lt;/sup&gt;</td>
<td>36E4-F1; 38A6-7</td>
</tr>
<tr>
<td>3632</td>
<td>Df(2L)pr-A14, cn&lt;sup&gt;l&lt;/sup&gt; bw&lt;sup&gt;l&lt;/sup&gt;/SM5</td>
<td>37D2-7; 39A4-7</td>
</tr>
<tr>
<td>3076</td>
<td>Df(2L)E55, rdo&lt;sup&gt;l&lt;/sup&gt; hk&lt;sup&gt;l&lt;/sup&gt; pr&lt;sup&gt;l&lt;/sup&gt;/CyO</td>
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<td>Df(2L)TW84/CyO</td>
<td>37F5-38A1; 39D3-E1</td>
</tr>
<tr>
<td>167</td>
<td>Df(2L)TW161, cn&lt;sup&gt;l&lt;/sup&gt; bw&lt;sup&gt;l&lt;/sup&gt;/CyO</td>
<td>38A6-B1; 40A4-B1</td>
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Table 3-4: Recombination frequencies between *waldo* and lethal P lines

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<th>P Line</th>
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<th># Recombinants/Total</th>
<th>cM</th>
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<tr>
<td>K10316</td>
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<td>8/200</td>
<td>4.0</td>
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<tr>
<td>K06009</td>
<td>28D1-2</td>
<td>9/238</td>
<td>3.8</td>
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<td>K10210</td>
<td>28D7-9</td>
<td>14/502</td>
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<td>K07602</td>
<td>28E3-4</td>
<td>13/258</td>
<td>5.0</td>
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<td>K09030</td>
<td>28E4-5</td>
<td>13/240</td>
<td>5.4</td>
</tr>
<tr>
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<td>7/152</td>
<td>4.6</td>
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<td>6.9</td>
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<tr>
<td>K07118</td>
<td>29C1-3</td>
<td>17/275</td>
<td>6.2</td>
</tr>
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<td>K107704</td>
<td>29D1-2</td>
<td>10/196</td>
<td>5.1</td>
</tr>
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<td>K16002</td>
<td>29F1-2</td>
<td>13/156</td>
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schnurri (shn) is necessary for germ cell migration and lateral mesoderm development

shn codes for a Zn finger-containing transcription factor believed to act downstream of dpp signaling (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). Four shn alleles were identified in our screen; however, I analyzed the phenotype of two previously identified shn alleles, shn\textsuperscript{IB} and shn\textsuperscript{P}, since they are reported to be strong loss-of-function alleles (Arora et al., 1995; Grieder et al., 1995). In general, the phenotypes of shn\textsuperscript{IB} were more severe than shn\textsuperscript{P}, although I primarily describe the shn\textsuperscript{P} phenotypes here, since recent evidence indicates that shn\textsuperscript{IB} is not a well-behaved loss-of-function allele (see Discussion).

Germ cells in shn mutant embryos migrate properly while they are attached to the PMG and transfer into the mesodermal layer in two bilaterally-symmetric groups as in wild type (compare Fig. 7A,B). However, the majority of germ cells do not migrate toward SGPs during stages 11 and 12. Instead, some "lost" germ cells disperse, while others clump in inappropriate locations within the mesoderm (Fig. 7D). This phenotype resembles the phenotype of abdA mutants, in which SGPs are not specified (Chapter 2 and Boyle and DiNardo, 1995), suggesting that SGPs may fail to develop in shn mutants. Furthermore, shn is expressed in the mesoderm, and not in the germ cells (Arora et al., 1995; Grieder et al., 1995), suggesting that shn function is required in the mesoderm for proper germ cell migration.

In order to test if shn is required for SGP development, SGPs were labeled with several molecular markers. As seen with a probe for the 412 retrotransposon, SGP number is reduced in both shn\textsuperscript{P} (Fig. 8B) and shn\textsuperscript{IB} (Fig. 8C) embryos by stage 14, although there were generally slightly more SGPs in shn\textsuperscript{P} embryos. In shn\textsuperscript{P} embryos, SGP development was further analyzed with the anti-Cli antibody. At stage 14, a comparable number of SGPs were observed with anti-Cli (Fig. 9D) as were seen with 412 (Fig. 8B). I also found that there were fewer SGPs present at stage 11 of development (compare Fig. 9A,B), suggesting that shn activity is required early in gonadal mesoderm development. The germ cell migration phenotype is, therefore, likely to be the result of a reduction in SGP number in shn mutant embryos. The SGPs which are present appear to attract and adhere to germ cells, but presumably, there are too few SGPs to adhere to all germ cells, and many scatter in the mesoderm.

Both the gonadal mesoderm and the fat body precursors arise within the lateral mesoderm of the eve domain (Moore et al., 1998b Riechmann et al., 1998). To determine whether shn is required only for SGP development, or for the development of the lateral
mesoderm in general, fat body development in shn mutant embryos was analyzed using an anti-Serpent antibody (Rehorn et al., 1996). I found that the number of fat body precursors is reduced in shn mutant embryos and that the bridges of fat body cells which normally span from parasegment to parasegment at stage 13 are not present (compare Fig.10A,B).

These data suggest that shn may act early in the development of the lateral mesoderm, as it is required for the development of both the gonadal mesoderm and the fat body. Since we have shown that dpp signaling is not required for lateral mesoderm development (Broihier et al., 1998; Moore et al., 1998b), and in fact antagonizes the development of both SGPs and fat body (Appendix A), a role for shn in lateral mesoderm specification is somewhat surprising (see Discussion).

Figure 3-7. Germ cell/SGP interactions are disrupted in shn mutant embryos.

(A,C) wild type (B,D) shnP mutant embryos. All embryos are anterior left and were labeled with anti-Vasa antibody which recognizes germ cells. In (A,B) germ cells migrate into the mesoderm. This step of germ cell migration occurs properly in shn embryos. In (C), germ cells in wild type have coalesced in PS10 at st14. In (D), germ cells in a shn mutant embryo are scattered bilaterally in the posterior mesoderm.
Figure 3-7: Germ cell/SGP interactions are disrupted in *shn* mutant embryos

**wild type**

A

B

**shnP**

C

D
Figure 3-8. 412 expression indicates that the number of SGPs is reduced in *shn* mutant embryos.

(A) wild type, (B) *shn*\(^P\) embryo, (C) *shn*\(^1B\) embryo. All embryos are anterior left and were labeled with a biotinylated riboprobe for 412 RNA. All embryos are st14. (A) SGPs in a wild-type coalesced gonad are shown in (A). The number of SGPs is reduced somewhat in *shn*\(^P\) embryos (B), and more dramatically in *shn*\(^1B\) embryos (C). While the reduction in *shn* embryos is slightly variable, in general, the phenotype of *shn*\(^1B\) embryos was more severe than that of *shn*\(^P\) embryos.
Figure 3-8: 412 expression indicates that the number of SGPs is reduced in shn mutant embryos.
Figure 3-9. SGPs are not properly specified in shn mutant embryos.

(A,C) wild type (B,D) shnP embryos. All embryos are anterior left and are labeled with anti-Cli antibody which recognizes SGPs. (A) wild-type embryo at st11, with SGPs visible as three clusters of cells in PS10-12 (arrows). In a shnP embryo at the same stage (B), only a couple of SGPs are present in PS12 (arrow). In (C), SGPs in a wild-type embryo at st13/14 are coalescing. In a similarly staged shn embryo (D), a reduced number of SGPs are visible. These cells are present in two clusters (arrows) and are not coalescing.
Figure 3-9: SGPs are not properly specified in \textit{shn} mutant embryos
Figure 3-10. The number of fat body precursors is reduced in shnP embryos.

(A) shnP embryo (B) wild-type embryo. Both embryos are anterior left and are labeled with anti-Srp antibody which recognizes fat body precursors. In (B), the characteristic "ladder" structure of a stage 13 wild-type embryo is observed. In the shnP embryo in (A), the number of fat body precursor cells is diminished and the fat body does not adopt its characteristic structure.
Figure 3-10: The number of fat body precursors is reduced in in $shnP$ embryos.
DISCUSSION

A second chromosome screen identified genes required for migration of germ cells
The second chromosome screen of 3200 lines identified a number of genes with roles in
germ cell migration. This small-scale screen allowed us to master the techniques
necessary for carrying out the larger third chromosome screen. It also confirmed the fact
that we were able to identify mutant phenotypes by screening embryos carrying the
P{faf-lacZ} transgene. Additionally, as discussed in Chapter 2, many of the genes that
were identified in the third chromosome screen are required for the development of the
gonadal mesoderm. We were likewise successful in isolating genes on the second
chromosome required for gonadal mesoderm development. For example, we isolated
alleles of eyes absent/clift (cli), which has been shown to be involved in SGP
differentiation (Boyle et al., 1997). Furthermore, as I have shown here, the shn locus, of
which 4 alleles were identified, is required for SGP development. Overall, the
phenotypes of mutants on the second chromosome resemble those from the third
chromosome screen, suggesting that many of the same genetic steps are disrupted in the
mutants.

While many of the mutants from the second and third chromosome screens have
similar phenotypes, the overall distribution of phenotypic classes is quite different
between the two screens (compare Table 1: Chapter 2 and Table 1: Chapter 3). There are
likely to be several reasons for these differences. The false positive class from the second
chromosome was 48% of the lines, compared to 34% of the third chromosome lines.
This difference is likely a result of the fact that we were not as experienced choosing
mutant phenotypes during the second chromosome screen, and by the fact that the lines
were screened differently in the two screens. In the second chromosome screen, glycerol
slides were prepared from all the lines, while in the third chromosome screen, we
screened the lines in the staining dishes underneath the dissecting scope. This made it
easier to select only those lines with reasonably penetrant phenotypes.

19% of the lines from the second chromosome screen displayed gross
developmental defects. There is not an analogous class from the third chromosome,
although we did obtain alleles of several genes which produce poorly differentiated
cuticles. Complementation tests with the second chromosome lines indicated that few of
them fall into complementation groups or were allelic to known second chromosome
genes. This suggests that many of these phenotypes may be the result of synthetic effects
of more than one mutation. It is not clear why we would see more synthetic effects on
the second chromosome than on the third, given that the number of lethal
hits/chromosome was similar in the two screens.

**wald**o may be required for adhesion of the germ cells to the endoderm

Germ cells in *wdo* mutant embryos prematurely detach from the PMG and scatter through
the yolk. Many germ cells appear to dissociate from the ventral side of the PMG,
suggesting that they were lost prior to their normal dorsally-oriented migration. One
model for *wdo* function is that it might be required for orienting the dorsal migration of
germin cells on the PMG, and that in the absence of this directional cue, germ cells detach
from the endoderm. However, phenotypic characterization of *wunen (wun)* mutant
embryos makes this an unlikely possibility. In *wun* mutants, germ cells migrate
randomly over the endodermal surface, but most remain attached to it (Zhang et al.,
1996).

A more likely possibility is that *wdo* is required for the adhesion between the
PMG and the germ cells. While *wdo* activity could be required either in germ cells or in
the PMG to mediate this adhesion, the PMG phenotype suggests that the defect in *wdo*
mutants may lie in the development of the endoderm. In these embryos, the anterior and
posterior midgut primordia do not migrate toward each other as in wild type. Instead,
both tissues remain as spherical groups of cells at the ends of the embryo. This
phenotype could be caused by a problem in the development of the endoderm or the
visceral mesoderm, which is the substrate over which the endodermal cells migrate.
However, FasIII expression in *wdo* embryos indicates that the overall development of the
visceral mesoderm occurs properly in these embryos, suggesting that the defect may lie in
the endodermal cells themselves.

Interestingly, the PMG phenotype of *wdo* mutant embryos is similar to the
phenotype of embryos lacking PS integrin subunits. *l(1) myospheroid (mys)* encodes a
PS integrin β subunit. In embryos in which the maternal and zygotic contribution of *mys*
have been removed, the midgut rudiments do not extend toward each other (Roote and
Zusman, 1995). This phenotype is similar to, although stronger than, the phenotypes of
multiple edematous wings (mew) and inflated (if) mutant embryos. These genes encode
αPS1 and αPS2 subunits, respectively. Roote and Zusman (1995) propose that the
integrin subunits are required for the initial attachment of the endoderm and the visceral
mesoderm and suggest that integrin function is required in both of these tissues in order
to mediate adhesion. Since germ cell migration is unaffected in embryos in which the
maternal and zygotic contributions of *mys, mew*, or *if* are removed (S. Zusman, personal
communication), it is unlikely that *wdo* encodes a gene whose sole function is the
regulation of integrin expression. However, \textit{wdo} might code for another adhesion molecule required on the PMG both for its attachment to the visceral mesoderm and adhesion to germ cells. One interesting possibility is that \textit{wdo} encodes Drosophila Laminin B1 (LanB1), which maps to 28D1-28D12 (Montell and Goodman, 1988). Laminins are localized to basement membranes, consistent with a role for \textit{wdo} in the adhesion of germ cells to the basal surface of the PMG epithelium. Since there are no known LanB1 alleles, the easiest way to determine whether or not \textit{wdo} encodes LanB1 might be to look for aberrations in the LanB1 sequence in our \textit{wdo} alleles.

While \textit{wdo} is required for SGP development, this phenotype is likely to be unrelated to the germ cell migration defect, since germ cells disperse in \textit{wdo} mutant embryos before there is a requirement for mesodermal cells in attracting or guiding migratory germ cells. Rather, the mesodermal phenotype and cuticle phenotype of \textit{wdo} mutants suggests that \textit{wdo} has many roles during development. This is consistent with the model that \textit{wdo} could encode for an adhesion molecule. There are many ways that an adhesion molecule could block proper cell specification in the mesoderm. For example, PS integrins are known to be concentrated between germ layers (Bogaert et al., 1987; Leptin et al., 1989; Zusman et al., 1990) and in \textit{mew}, \textit{if}, and \textit{mys} mutant embryos, the mesodermal layer is detached from the ectoderm at the extended germband stage (Roote and Zusman, 1995). Since this is the time at which a Dpp signal emanates from the dorsal ectoderm and induces dorsal mesoderm derivatives (Staehling-Hampton et al., 1994; Frasch, 1995), it is possible that such a detachment could disrupt proper mesodermal patterning. This hypothesis is consistent with the observation that there are fewer visceral mesoderm cells in \textit{mys} and \textit{if} mutants (Roote and Zusman, 1995).

\textit{schnurri} is necessary for SGP development

\textit{schnurri} (\textit{shn}) is a transcription factor thought to be activated in response to \textit{dpp}. Genetic and phenotypic characterization of \textit{shn} and other \textit{dpp} pathway mutants suggests that \textit{shn} acts positively in the \textit{dpp} pathway (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). Here I show that germ cells in \textit{shn} mutants do not migrate toward SGPs. This germ cell migration phenotype results from a dramatic reduction in the number of SGPs. Fat body precursors, which also arise within the lateral mesoderm, are likewise reduced in \textit{shn} embryos. This suggests that in these embryos the specification of lateral mesoderm does not occur correctly.

These results contrast with the role of \textit{dpp} in lateral mesoderm development (See Chapter 4 and the Appendix). \textit{dpp} signals from the dorsal ectoderm to the mesoderm where it induces dorsal mesoderm derivatives. In contrast, SGPs are repressed by \textit{dpp} as
additional SGPs form in \textit{tkv} mutant embryos and ectopic activation of the \textit{dpp} pathway inhibits lateral mesoderm development (see Appendix). These results suggest that \textit{dpp} signaling is responsible for defining the dorsal boundary of lateral mesoderm.

There are several ways in which \textit{shn} might function in lateral mesoderm development. First, it is possible that \textit{shn} functions independently of \textit{dpp} in mesoderm development. In addition to having reduced lateral mesoderm derivatives, \textit{shn}1B embryos also have reduced dorsal mesoderm derivatives (Arora et al., 1995; Staehling-Hampton et al., 1995). While this phenotype has been interpreted as being the result of a disruption of \textit{dpp} signaling, \textit{shn} could instead act prior to the separation of dorsal and lateral mesoderm. Another possible role for \textit{shn} in mesoderm development would be to inhibit \textit{dpp} signaling in the lateral mesoderm. If so, then the reduction of lateral mesoderm in \textit{shn} mutants would be expected to be associated with an increase in the number of visceral mesodermal cells. In \textit{shn}1B embryos, this is not the case, as these embryos display reduced visceral mesoderm (Arora et al., 1995; Staehling-Hampton et al., 1995). However, \textit{shn}1B may be an unusual \textit{shn} allele. It codes for a truncated protein (Chen and Hoffmann, personal communication) and is the only \textit{shn} allele to exhibit genetic interactions with \textit{dpp} pathway members (Chen et al., 1998). Therefore, this allele may not represent the null phenotype.

Another way to test for a genetic interaction between \textit{shn} and \textit{dpp} would be through analysis of a \textit{shn tkv} double mutant. If \textit{shn} activity is required to inhibit \textit{dpp} signaling, then the requirement for \textit{shn} may be relieved in the double mutant, and additional lateral mesodermal cells would form. On the other hand, if \textit{shn} functions independently of \textit{dpp}, then gonadal mesoderm would not form properly in these embryos. I have made \textit{shn tkv} double mutants with several different \textit{shn} alleles and expect that the analysis of mesodermal development in these embryos will elucidate the relationship between \textit{shn} and \textit{dpp}. 


This chapter has been published as:

CHAPTER 4

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

SUMMARY

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

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

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

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

Some genes involved in SGP specification have recently been identified. The homeobox-containing gene tinman (tin) is expressed throughout the mesoderm as it invaginates into the embryo. tin expression is subsequently maintained only in dorsal
mesoderm, where it is required for the specification of the heart and the visceral mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993). Expression of tin in dorsal mesoderm depends on dpp, which is expressed in the overlying dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Although SGPs arise ventral to visceral mesoderm, and therefore outside of the dorsally-restricted tin expression domain, SGP-specific gene expression is not initiated properly in tin mutant embryos (Boyle et al., 1997). The homeotic gene abdominal A (abdA) has also been shown to be required for the specification of SGPs (Cumberledge et al., 1992; Boyle and DiNardo, 1995; Greig and Akam, 1995; Boyle et al., 1997). In the absence of proper SGP specification in tin or abdA mutant embryos, germ cells still migrate toward and associate with laterally-positioned mesodermal cells (Boyle et al., 1997; Moore et al., 1998).

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

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

We show here that *zfh-1* is required for the initial interactions between germ cells and mesoderm, as germ cells do not correctly migrate to the lateral mesoderm in *zfh-1* mutant embryos. Mutant analysis demonstrates that *zfh-1* is necessary for the development of two distinct mesodermal lineages which both function in germ cell migration: the caudal visceral mesoderm and the somatic gonadal precursors. Analysis of embryos in which Zfh-1 is ectopically expressed demonstrates that *zfh-1* is sufficient for the development of additional SGPs. Furthermore, we demonstrate that *tin* and *zfh-1* have overlapping functions in the development of lateral mesoderm derivatives, including gonadal mesoderm and the fat body. Finally, by studying the spatial and temporal association of germ cells with mesodermal cells, we find that germ cells follow multiple migratory paths to reach the SGPs.
MATERIALS AND METHODS
Fly stocks
We used a transheterozygous combination of two EMS-induced zfh-1 alleles, zfh-165.34/zfh-175.26 for all analyses (Moore et al., 1998). The alleles show the same phenotype in trans to each other, in trans to a deficiency uncovering zfh-1 (Df(3R)20 e der177), or when transheterozygous to another strong zfh-1 allele, zfh-12. (Both of these stocks were provided by Z.C. Lai.) An anti-Zfh-1 antibody does not recognize protein in zfh-165.34 or zfh-175.25 embryos, but both alleles produce zfh-1 RNA. These same zfh-1 alleles were used to make germ line clones.

Two overlapping deficiencies, Df(3L)vin4 and Df(3L)vin6, were used to uncover the byn locus (Bloomington). In addition the following stocks were used: Df(3R)GC14 (tin-) (M. Frasch), abdAMX1 (W. Bender), dppH46 (B. Gelbart), srp9L (Bloomington), ctiI (Bloomington), cti14.40 (generated in the Lehmann lab), cliD1 (M. Boyle), and HSzfh-1/CyO (Z.C. Lai), described in Lai et al. (1991).

The tin zfh-1 double mutant was made by standard meiotic recombination. Recombinants were generated between Df(3R)GC14 and both zfh-165.34 and zfh-175.26. The cli zfh-1 double mutant was generated using the cliD1 allele and both zfh-165.34 and zfh-175.26. The phenotypes of both double mutants were identical with either zfh-165.34 or zfh-175.26. In all experiments, we used balancers carrying P-lacZ transgenes: P{UbxlacZ-TM3} or P{ftzlacZ-CyO} to distinguish homozygous mutant embryos from balancer-bearing siblings.

Whole-mount antibody staining
Antibody staining was performed using either horseradish peroxidase with biotinylated secondary antibodies and the Elite kit (Vector Laboratories), or with alkaline phosphatase (AP) with directly-conjugated secondary antibodies. Embryos were fixed by gentle shaking for 25 minutes in 8 mls heptane, 0.25 mls 37% formaldehyde, and 1.75 mls PBS, 50 mM EDTA and devitellinized as for in situ hybridization (Ephrussi et al., 1991). Following rehydration, antibody staining using biotinylated secondaries was conducted as described in Eldon and Pirrotta (1991). Antibody staining with multiple primary antibodies was carried out as described in Patel (1994). Whole-mount embryos were embedded in PolyBed812 (Polysciences) following the protocol of Ephrussi et al. (1991).
or in 85% glycerol, then analyzed with a Zeiss Axiophot using Nomarski optics. Slides were taken using either 64T or 160T Kodak film. Figures were composed using Photoshop 3.0 and Freehand 7.

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

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

**Whole-mount in situ hybridizations**

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

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

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

The number of SGPs in HSzhf-1 embryos at late stage 12 or stage 13 was compared to OR embryos subjected to an identical heat shock. There are an average of 48 SGPs (s.d.=13.6, n=18) in HSzhf-1 embryos at this stage. In OR controls, there are 28.3 SGPs (s.d.=3.8, n=16). A two-sided t test assuming equal variance gives p=9.0 X 10^{-7}. Thus, more SGPs are present in HSzhf-1 embryos than in wild type.

Heat-shock protocol

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

Zfh-1 is necessary for germ cell migration

In a large scale mutagenesis screen aimed at identifying genes required for germ cell migration and gonad formation (Moore et al., 1998), we isolated six alleles of zfh-1 (Fortini et al., 1991; Lai et al., 1993). Germ cell formation and the initial steps of germ cell migration are not affected in zfh-1 mutant embryos. The first deviation from wild-type development occurs in late stage 10 when germ cells are positioned on the endoderm in close juxtaposition with mesoderm. In wild-type embryos, germ cells leave the endoderm and navigate into lateral mesoderm where they contact somatic gonadal precursors (SGPs) (Fig.1B) (Boyle et al., 1997). Germ cells and SGPs remain in contact through the remainder of embryogenesis until the two cell types coalesce at stage 14 to form the embryonic gonad (Fig.1D). In zfh-1 mutant embryos, the majority of germ cells do not attach to the SGPs at stage 11 (Fig.1A). Instead, some germ cells remain attached to the gut, leading to a cluster of germ cells in the middle of the embryo during later stages of development (Fig.1C). Germ cells that enter the mesoderm in zfh-1 mutant embryos disperse throughout the posterior mesoderm with some cells migrating across the mesodermal layer and into the overlying ectoderm (Fig.1E, arrowheads). Occasionally, small gonads form in mutant embryos (data not shown).

The germ cell migration defect we observe in zfh-1 mutant embryos demonstrates that zfh-1 function is required for the transition of germ cells from the endoderm to the mesoderm and suggests that zfh-1 is required for interactions between germ cells and SGPs. Zfh-1 protein is found in the germ cells as well as in many mesodermal derivatives (Lai et al., 1991; see below). Thus, zfh-1 could be required either in the germ cells or in the mesoderm to promote the interaction between these two cell types. In order to distinguish between these two possible modes of zfh-1 function, we analyzed the requirement for zfh-1 activity in germ cells and in mesodermal lineages.
Fig. 4-1. Germ cells in zfh-1 mutant embryos do not successfully navigate to the SGPs.

Whole-mount embryos in (A-D) are anterior left and dorsal up; transverse sections (E-F) are dorsal up. zfh-165.34/zfh-175.26 embryos (A,C,E) or wild-type embryos (B,D,F). Germ cells labeled with anti-Vasa in brown (all panels) and gonadal mesoderm with the 412 retrotransposon RNA in blue (E,F). (B) wild-type embryo at stage 11, germ cells attach to lateral mesoderm (arrow). (A) zfh-1 mutant embryo at stage 11, germ cells migrate past their target mesodermal cells, into posterior or ectodermal regions (arrow). Open arrow in (B) denotes A/P level of sections in (E-F). (E) section of zfh-1 mutant embryo at stage 11 with ectodermally-located germ cells. (F) wild-type embryo at the same stage with germ cells adhering to gonadal mesoderm cells. (D) wild-type embryo at stage 13 with germ cells within lateral mesoderm. In stage 13 zfh-1 mutant embryo (C) lost germ cells are found near the PMG (arrow), and at the posterior end of the embryo, reflecting those germ cells which migrated too far posteriorly at stage 11.
Figure 4-1: Germ cells in $zf{h-1}$ mutant embryos do not successfully navigate to SGPs.
Maternal Zfh-1 is not required for germ cell migration

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

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

Proper germ cell migration requires caudal visceral mesoderm migration

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

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

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

We cannot directly assess the role of the caudal visceral mesoderm in guiding germ cells in zfhl mutant embryos because zfhl is required for both the development of the caudal visceral mesoderm and the somatic gonadal precursors (see below). In order to further investigate the role of the caudal visceral mesoderm in germ cell migration, we analyzed germ cell migration in brachyenteron (byn) mutant embryos (Singer et al., 1996). Like zfhl, byn is required for the migration of the caudal visceral mesoderm, but unlike zfhl, it is not required for gonadal mesoderm development (R. Reuter, pers. communication, our observations). In byn embryos, many germ cells remain attached to the PMG surface (arrowhead in Fig. 2F), while some navigate correctly to the SGPs. The germ cell migration defect in byn embryos suggests that the germ cell migration phenotype of zfhl embryos may be at least in part attributable to a defect in caudal visceral mesoderm migration. Since byn and zfhl both disrupt caudal visceral mesoderm migration and show similar defects in germ cell migration, we propose that in wild-type embryos, the caudal visceral mesoderm facilitates the transition of many germ cells from the endoderm to the lateral mesoderm (Fig. 8A).
Fig. 4-2. Caudal visceral mesodermal cells express Zfh-1 and interact with migratory germ cells.

Embryos in (A-D) and (G) wild type, (E) zfh-1^{65.34}/zfh-1^{75.26}, (F) byn\textsuperscript{vin4}/byn\textsuperscript{vin6}. (A-C) labeled with anti-Zfh-1. (D-E) labeled with anti-Vasa (brown) marking germ cells and a zfh-1 riboprobe (blue) marking the caudal visceral mesoderm. Open arrowheads point to the caudal visceral mesoderm which is in a slightly deeper plane of focus than the germ cells. We were able to use zfh-1 RNA as a caudal visceral mesoderm marker, as the zfh-1 alleles used in this analysis transcribe zfh-1 RNA. (F) is stained with anti-Vasa, and the section in (G) is labeled with anti-Zfh-1 (brown) and anti-Vasa (blue). (A) wild-type Zfh-1 expression at stage 9. The anterior-most cells are hemocyte precursors, whereas the posterior-most cells are caudal visceral mesoderm. Maternal Zfh-1 is still faintly visible in germ cells (arrowhead) at this stage. In (B-E), open arrowheads indicate caudal visceral mesoderm, and black arrows mark the posterior end of the embryo for reference. Panels (B) early stage 10 and (C) late stage 10 demonstrate normal caudal visceral mesoderm migration. Embryos in (D-E) compare the positions of caudal visceral mesoderm and germ cells at late stage 10 in wild-type (D) and zfh-1 mutants (E). In wild type, caudal visceral mesodermal cells have migrated anteriorly to the position of the germ cells on the PMG. In zfh-1 mutants, caudal visceral mesoderm remains posterior to the germ cells. Transverse section in (G) illustrates the association of germ cells (blue) and caudal visceral mesoderm (brown, open arrowheads), in wild type. (F) byn mutant embryo at stage 13. A cluster of germ cells is situated on the PMG surface. There are small gonads in this embryo, but they are out of the plane of this photograph.
**Figure 4-2:** Caudal visceral mesodermal cells express Zfh-1 and interact with migratory germ cells
SGPs express high levels of Zfh-1

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

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

Although *abdA* is required for SGP specification, the initial stages of germ cell migration are unaffected in *abdA* mutant embryos. In *abdA* mutants, germ cells migrate properly from the endoderm into the lateral mesoderm during stage 11; lost germ cells are not apparent until mid stage 12 (Moore et al., 1998). Given that Zfh-1 is expressed at low levels in lateral mesodermal clusters in PS10-12 in an *abdA*-independent manner, we asked whether germ cells adhere to these cells in *abdA* mutant embryos. Indeed, germ cells specifically associate with mesodermal cells expressing low levels of Zfh-1 in *abdA* mutants (Fig. 3D), suggesting that germ cells can migrate toward lateral mesodermal cells which are not specified as gonadal mesoderm, but do express Zfh-1. Germ cells maintain this association until mid stage 12, when they disperse, presumably because SGP-specific gene products are required for the continued association of germ cells and gonadal mesoderm.
Figure 4-3. Zfh-1 is expressed in somatic gonadal precursors from stage 10 through stage 14.

Whole-mount embryos in (A-E) are anterior left and dorsal up; transverse sections in (F-H) are dorsal up. All panels except (D) wild type; (D) abdAMX1. All embryos are stained with anti-Zfh-1 in brown. (C) and (F-H) are also labeled with the germ cell marker anti-Vasa in blue. (A) Stage 10 embryo with Zfh-1 expression in lateral mesoderm clusters (circled). (B) The expression of Zfh-1 in lateral mesoderm clusters increases at stage 11 when these cells are specified as SGPs (circled) and remains at high levels in these cells until they coalesce with germ cells at stage 14 (E). Unlabeled germ cells associate with the PS12 cluster at stage 11 (arrowheads in inset in B). (C) Embryo of the same stage as in (B) with both germ cells and the SGPs labeled. Some germ cells associate with Zfh-1-expressing SGPs (circled) while others remain posterior (bracket). (F-H) These spatial relationships are highlighted in transverse sections. (F) Germ cells migrate from the PMG toward SGPs during early stage 11. Two germ cells (arrowheads) migrating around the visceral mesoderm toward Zfh-1-expressing SGPs (arrowhead in inset). In (G), germ cells are surrounded by Zfh-1-expressing SGPs at stage 11 (magnified in inset). (H) Section of a stage 11 embryo posterior to the SGP clusters. Germ cells (arrowheads) are in the mesoderm, but they are not in the vicinity of SGPs. (D) abdA mutant at stage 11, illustrating Zfh-1 expression in lateral mesodermal clusters (circled). Zfh-1 levels are comparable to those of a stage 10 wild-type embryo (compare to (A)). Unlabeled germ cells associate with Zfh-1-expressing cells in abdA mutants (arrowheads in inset in D).
Figure 4.3: Zfh-1 is expressed in SGPs
Germ cells follow different migratory paths to SGPs

To study the temporal and spatial relationship between migratory germ cells and SGPs in wild type, we prepared sections of embryos labeled with anti-Vasa to detect germ cells and anti-Zfh-1 to mark SGPs. In early stage 11 embryos, a few germ cells are still attached to the endoderm. These germ cells migrate into the mesodermal layer during stage 11 in a caudal visceral mesoderm-independent manner, as the caudal visceral mesoderm has migrated anteriorly past the end of the PMG by this stage (Fig. 8B; see Discussion). By mid stage 11, almost all germ cells are in lateral mesoderm. However, most of them have not yet reached the SGPs and are frequently situated next to Bagpipe-expressing visceral mesodermal cells which have migrated between the SGPs and the PMG (Azpiazu and Frasch, 1993; Fig.3F, arrowheads; Fig. 8B). Shortly thereafter, germ cells associate with Zfh-1-expressing SGP clusters in PS 11 and 12 (Fig.3G). Germ cells are not observed with the PS 10 cluster at stage 11, presumably because the PMG does not underlie the mesoderm in this parasegment. About one-third of germ cells migrate from the PMG into PS 13 and are therefore posterior to all SGP clusters at stage 11 (Fig.3C, brackets; Fig.3H; Fig. 8C, D). Because all germ cells are associated with SGPs by the beginning of stage 13, the more posteriorly-situated germ cells are likely to migrate anteriorly during toward SGPs during stages 11 and 12. Thus, while some germ cells reach the SGPs by stage 11, other germ cells take a more indirect route and do not contact SGPs until stage 13. We conclude that germ cells do not follow a single invariant path to reach SGPs (see Discussion).
Zfh-1 is necessary for gonadal mesoderm development

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

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

The observation that SGPs are not completely absent in zfh-1 mutants demonstrates that a small number of SGPs are specified in a zfh-1-independent manner. The specification of these SGPs does not require cli function as they are still present in cli; zfh-1 double mutants (data not shown). We present evidence below demonstrating that the tin gene is required for the specification of these SGPs.
Fig. 4-4. *zfh-1* is necessary for SGP-specific gene expression.

(A, C, E) *zfh-1*^{165.34}/*zfh-1*^{75.26} mutant embryos; (B, D, F) wild-type embryos. (A-D) stained with anti-Eya (brown) to label the SGPs. (A) The germ cells are faintly brown in the *zfh-1* mutant embryos in (A, C) as *zfh-1* mutant lines carry a lacZ-containing transgene expressed in their germ cells and anti-βgal was added in order to distinguish the balancer-containing embryos. Germ cells are distinguishable from SGPs in (A,C) due to their large unstained nuclei.) The number of Eya-expressing SGPs is reduced at stage 11 in *zfh-1* mutants (A) compared to wild type (B). Residual SGPs remain attached to germ cells at stage 13 in *zfh-1* mutants (C, arrowheads). *croc* RNA is expressed strongly in SGPs at stage 14 in wild-type embryos (F), while *croc* RNA is absent in *zfh-1* mutants; even when small gonads are visible, (E, arrowhead).

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Figure 4-4: *zfh-1* is necessary for SGP-specific gene expression.
Ectopic Zfh-1 is sufficient to induce additional SGPs

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

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

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

Fig. 4-5. Ectopic Zfh-1 alters the timing of SGP-specific gene expression and the size of the gonadal mesoderm primordium.

(A-F) anterior left and dorsal up. (A,C,E) HSzfj-1 embryos; (B,D,F) wild-type embryos after an identical heat shock regimen. (A,B) stage 13 embryos stained with anti-Eya antibody. Ectopic Zfh-1 interferes with germ band retraction, explaining the difference in appearance of the two embryos. (C,D) stage 10 embryos labeled with bagpipe riboprobe. (E,F) stage 11 embryos labeled with crocodile riboprobe. In HSzfj-1 embryos (A), the number of Eya-expressing cells is almost double that in wild type (B). The A/P extent of the gonadal mesoderm is not expanded in the HSzfj-1 embryo relative to wild type; rather, the gonadal mesoderm appears broader along the D/V axis. The number of bagpipe-expressing cells is reduced in HSzfj-1 embryos (C) relative to wild type (D). bagpipe is expressed in visceral mesodermal cells, which originate immediately dorsal to SGPs. In HSzfj-1 embryos (E), croc is expressed in the SGPs at stage 11 (bracket). In wild type, croc expression in the mesoderm is undetectable at stage 11 (F).
Figure 4-5: Ectopic Zfh-1 alters the timing of SGP-specific gene expression and the size of the gonadal mesoderm primordium.
Only the pan-mesodermal expression of tinman is required for SGP formation

tinman (tin) encodes a homeobox protein which is expressed in all mesodermal cells until stage 9 (Azpiazu and Frasch, 1993; Bodmer, 1993), but subsequently maintained only in dorsal mesoderm. The dorsally-restricted expression of tin requires Dpp, which is present in the overlying dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Although tin is not expressed in SGPs, it is required for the expression of SGP-specific markers, such as cli and Dwnt2 (Boyle et al., 1997). However, the analysis of germ cell migration in tin mutants suggests that aspects of SGP specification are independent of tin. In tin mutants, germ cells align with mesodermal cells at stage 13, although they disperse shortly thereafter (Moore et al., 1998). Because the onset of this phenotype is later than the onset of the germ cell migration phenotype in abdA, in which SGPs are not specified, SGPs may initially be present in tin embryos.

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

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

\textbf{Fig. 4-6. Tin, but not Dpp, is required for SGP development}

(A-D) anterior left, (A-B) dorsal up, (C-D) "frontal" views. (A,B) \textit{tin}^{Dj(3R)GC14} mutant embryos labeled with anti-Zfh-1. (C,D) \textit{dpp}^{H46} embryos stained with anti-Zfh-1 (C) or 412 riboprobe (D). The number of Zfh-1-expressing SGPs is reduced by 41\% at stage 11 in \textit{tin} mutants (A, circled) compared to wild type (see Fig. 3B). By stage 12, the number of Zfh-1 expressing cells is reduced by 76\% in \textit{tin} mutants (B, circled) compared to wild type. In this analysis, mesodermal cells were counted as SGPs if they express high levels of Zfh-1 and reside within PS10-12. SGP specification or coalescence does not require the presence of Dpp, and therefore dorsally-restricted \textit{tin} expression, as judged by either Zfh-1 protein expression (C) or 412 RNA expression (D).
Figure 4-6: Tin, but not Dpp, is required for SGP development
Tinman and Zfh-1 cooperate in gonadal mesoderm development

Both *tin* and *zfh-1* are important regulators of gonadal mesoderm cell fate. It is unlikely, however, that they fit neatly into a linear hierarchy controlling gonadal mesoderm determination. We have demonstrated that the early broad expression of *tin* is required for SGP development. However, *zfh-1* is not required for this expression (data not shown), suggesting that *zfh-1* is not upstream of *tin*. Furthermore, since germ cell association with SGPs is blocked in *zfh-1* mutants but not in *tin* mutants, it seems unlikely that *tin* acts upstream of *zfh-1* in SGP development. These observations suggest that *tin* and *zfh-1* function in parallel in gonadal mesoderm development.

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

Gene expression in the mesoderm was analyzed in double mutant embryos in order to elucidate the cause of the germ cell migration phenotype. We assayed for the presence of gonadal mesoderm with multiple markers, such as the anti-Eya antibody (data not shown) and the 412 retrotransposon (Compare Fig. 7A,B). While 412-expressing cells are detectable in either *tin* or *zfh-1* single mutants, they are abolished in double mutant embryos. This demonstrates that *tin* and *zfh-1* have parallel functions in gonadal mesoderm formation. We next tested whether *tin* and *zfh-1* act together in fat body development, as the fat body is another lateral mesoderm derivative. While both *tin* and *zfh-1* are involved in the development of the fat body, neither is absolutely required (Moore et al., submitted). Anti-Srp was utilized to label fat body precursors (Rehorn et al., 1996). Only a few residual Srp-expressing cells remain in *tin zfh-1* embryos (Fig. 7F), demonstrating that *tin* and *zfh-1* cooperate in the specification of two tissues derived from lateral mesoderm: the gonadal mesoderm and the fat body.
Fig. 4-7. *tin* and *zfh-1* cooperate in SGP specification. (A-F) anterior left, (A,B,E,F,G) dorsal up, (C,D) dorsal views. (A,C,E,G) *tin zfh-1* double mutant embryos. (B,D,F) wild-type embryos. (A-D) are labeled with anti-Vasa to mark germ cells (brown) and with 412 RNA to mark gonadal mesoderm (blue). (E,F) The fat body precursors are labeled with anti-Srp. The transverse section in (G) is labeled with anti-Vasa. (A) While germ cells congregate on the dorsal side of the PMG in stage 11 *tin zfh-1* mutants, they do not migrate into the mesoderm (compare to wild-type embryo in Fig. 1B). Section in (G) demonstrates that mesodermal cells contact germ cells on the PMG in *tin zfh-1* double mutants. (C) Germ cells remain in the vicinity of the endoderm at stage 13. No gonadal mesoderm differentiates in *tin zfh-1* mutants as demonstrated by the absence of 412 RNA expression in (A) and (C), compare to 412 expression in wild-type embryos in (B) and (D). The number of fat body cells is drastically reduced in *tin zfh-1* mutants (G), compare to (H).
Figure 4-7: *tin* and *zfh-1* cooperate in SGP specification

**tin zfh-1**

**A**

**B**

**C**

**D**

**E**

**F**

**G**
DISCUSSION

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

The caudal visceral mesoderm contacts migratory germ cells

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

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

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

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

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

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

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

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

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

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

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

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

**Germ cells navigate along different paths to reach the gonadal mesoderm**

Cell migration within developing tissues such as the tracheal system or the nervous system occurs along highly stereotyped paths (Van Vactor et al., 1993; Samakovlis et al., 1996). Our analysis of germ cell migration indicates that there is a surprising degree of plasticity in the routes germ cells take toward the gonadal mesoderm. The first germ cells to migrate from the PMG to the mesoderm do so along the caudal visceral mesoderm (Fig. 8A). These germ cells take the most direct route from the PMG to the SGPs, as caudal visceral mesodermal cells touch SGPs at late stage 10. At mid stage 11, germ cells transfer to the mesoderm without directly contacting caudal visceral mesoderm, as these mesodermal cells have migrated past the germ cells. Germ cells leaving the PMG at this time migrate around other mesodermal cells toward SGPs (Fig. 8B). In particular, they navigate around \textit{bagpipe}-expressing visceral mesoderm which has moved inside the SGPs (Azpiazu and Frasch, 1993; Boyle et al., 1997).

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

No matter which path they choose, we do not know how germ cells are attracted to SGPs. Our observations imply that the signal (whether it is a diffusible molecule or a gradient of adhesion molecules) is capable of acting over several cell diameters. In vitro studies suggest that this is the case in mouse, as genital ridge explants attract germ cells from long range (Godin et al., 1990). It will be interesting to determine whether germ cell-germ cell interactions are important in guiding the late arrivals to the developing gonads. Such interactions have been observed in mouse embryos where germ cells send out long processes to form extensive networks with one another (Gomperts et al., 1994). Subsequent studies of germ cell migration in Drosophila embryos will further elucidate how germ cell migration to the gonadal mesoderm is mediated.
Fig. 4-8. Germ cells follow different paths to reach SGPs. (A-C) transverse sections, (D) lateral view. (A) Germ cells which migrate from the PMG to SGPs during late stage 10 follow a direct path along the caudal visceral mesoderm. (B) During stage 11, germ cells migrate more extensively through mesoderm to reach SGPs. (C) Some germ cells migrate into PS13, where SGPs are not specified, and therefore must migrate anteriorly through lateral mesoderm. (D) Lateral view of a stage 11 embryo, indicating the plane of the sections in (B) and (C). The curved arrows in (A,B,D) indicate the direction of germ cell migration. Yellow (germ cells), red (gut), turquoise (caudal visceral mesoderm), green (mesoderm), light purple (low Zfh-1-expressing lateral mesoderm), dark purple (high Zfh-1-expressing SGPs), orange (visceral mesoderm).
Figure 4-8: Germ cells follow different paths to reach SGPs


APPENDIX

Dpp signaling delimits the dorsal boundary of lateral mesoderm

INTRODUCTION

decapentaplegic (dpp) is a Drosophila member of the TGFβ family of secreted signaling molecules. TGFβs have been shown to interact with two different types of serine threonine kinase transmembrane receptors (see Kingsley, 1994 for review). Since neither of these receptor types appears able to signal alone, they are though to form a heterodimeric signaling complex. In Drosophila, there are two type I receptors: thickveins (tkv) and saxophone (sax) (Nellen et al., 1994; Xie et al., 1994), and one type II receptor: punt. (Letsou et al., 1995; Ruberte et al., 1995).

dpp has been implicated in numerous processes, including the induction of dorsal mesoderm (Frasch, 1995; Staehling-Hampton et al., 1994). In this event, it is thought to be secreted from dorsal ectoderm and received by dorsal mesodermal cells, which are then induced to develop as heart or visceral mesoderm. This model is based on both expression and functional studies. The dorsoventral expression limit of ectodermal dpp exactly correspond to the mesodermal limit of dorsal mesoderm. Furthermore, in dpp mutants, visceral mesoderm does not develop; whereas when dpp is overexpressed, the visceral mesoderm anlage expands correspondingly (Frasch, 1995; Staehling-Hampton et al., 1994).

I was interested in analyzing the role of dpp in gonadal mesoderm development. I have previously shown that SGPs form in dpp mutants (Chapter 4) demonstrating that SGPs are not a dorsal mesodermal derivative. However, since the primordia of the gonadal mesoderm and visceral mesderm are directly apposed to one another (Boyle et al., 1997), it is possible that Dpp is involved in setting the boundary between these two mesodermal primordia. In this appendix, I demonstrate that this is indeed the case. In dpp pathway mutants additional SGPs are specified, while SGP formation is blocked when Dpp is overexpressed. These results demonstrate that the border between visceral mesoderm and gonadal mesoderm is defined by dpp signaling from the ectoderm.
MATERIALS AND METHODS

Fly stocks
The following fly stocks were used in this study: dpp\textsuperscript{H46} (V. Twombly and W. Gelbart), punt\textsuperscript{P} and tkv\textsuperscript{7} (J. Tresiman). The embryo collections from the dpp\textsuperscript{H46} and tkv\textsuperscript{7} flies were done at RT, while the punt\textsuperscript{P} embryos were collected at 29° C, as all punt alleles are temperature-sensitive. Flies carrying either the UAS-dpp or UAS-tkv\textsuperscript{QD} constructs were obtained from J. Treisman. M. Bate provided the twi-gal4; gal4-24B flies.

Antibody staining
Anti-Cli antibody was obtained from M. Boyle and N. Bonini and was used at 1:1000. Antibody staining was performed as described in Chapter 4. SGP counts were done on a Leica DMRXA microscope with the aid of the Metamorph imaging program.
RESULTS AND DISCUSSION

Additional SGPs are specified in embryos mutant for members of the dpp signaling pathway

The analysis of somatic gonadal precursor (SGP) development in dpp mutant embryos demonstrated that dpp is not required for the specification of these cells (Chapter 4). In fact, there appears to be an increased number of SGPs in dpp mutants. However, dpp mutant embryos are strongly ventralized, making the quantitation of SGP number difficult. In order to circumvent this problem, I analyzed SGP development in thickveins (tkv) and punt mutant embryos. tkv codes for a type I receptor, whereas punt encodes a type II receptor (Letsou et al., 1995; Nellen et al., 1994; Ruberte et al., 1995). These embryos are not ventralized, as maternal contributions of these products are responsible for dorsoventral patterning (Affolter et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). I found that additional SGPs were specified in tkv mutant embryos. SGP number was quantitated in tkv homozygous and heterozygous embryos. In tkv heterozygotes, there were an average of 38.2±5.8 (n=7) SGPs (Fig. 1A), while in tkv homozygotes, there were an average of 111.4±10.7 (n=8) SGPs (Fig. 1B). In other words, there are 2.9 times more SGPs in tkv homozygotes. There also appears to be an increased number of SGPs in punt mutant embryos (Fig. 1C), although the increase is not as dramatic as that observed in tkv mutants. This may be because the strongest punt allele is unlikely to be a null (Letsou et al., 1995; Nellen et al., 1994; Ruberte et al., 1995), or it may reflect the fact that another type II receptor is involved in this signaling event.

The additional SGPs in tkv embryos are most likely to arise dorsal to the lateral mesodermal domain and suggest that in wild-type embryos, dpp signaling represses gonadal mesoderm development and thereby sets the dorsal limit of gonadal mesoderm formation. Consistent with a role for tkv and punt in dorsal mesoderm formation, both are expressed in the mesoderm at this stage, and mutant embryos have visceral mesoderm defects (Brummel et al., 1994; Childs et al., 1993; Letsou et al., 1995; Penton et al., 1994; Ruberte et al., 1995). SGP-specific markers are not the only mesodermal genes repressed by dpp signaling. The expression of pox meso, a putative transcription factor expressed in ventral mesoderm, expands around the circumference of dpp mutant embryos (Staehling-Hampton et al., 1994).
Figure A-1: Additional SGPs are present in tkv mutant embryos
All embryos are approximately stage 14 and are stained with anti-Cli antibody. SGPs in all panels are circled with a dotted line. (A) tkv heterozygous embryo, which contain, on average, 38.2 SGPs. (B) tkv homozygous embryo, which contain an average of 111.4 SGPs. (C) punt mutant embryo.
Figure A-1: Additional SGPs are present in \textit{tkv} mutant embryos

\begin{align*}
\text{anti-Cli} & \\
\text{tkv/+} & \\
\text{tkv} & \\
punt & \\
\end{align*}
Ectopic expression of Dpp or activated Tkv blocks lateral mesoderm development

After determining that in the absence of dpp signaling, SGPs are specified from mesoderm arising within the dorsal domain, I next wanted to determine if ectopic expression of dpp pathway members is able to repress SGP development within lateral mesoderm. To ask this question, I made use of the gal4-UAS system (Brand and Perrimon, 1993) to overexpress Dpp (Frasch, 1995) or an activated form of the Tkv type I receptor, TkvQD (Nellen et al., 1996), throughout the mesoderm. Flies bearing either of these constructs were crossed to flies bearing two different mesodermal gal4 drivers: twi-gal4 (Baylies and Bate, 1996) or gal4-24B (Brand and Perrimon, 1993).

SGP formation was completely blocked when Dpp was expressed throughout the mesoderm (Fig. 2B), and was largely blocked when TkvQD was expressed throughout the mesoderm (Fig. 2C). These data indicate that when either Dpp or an activated form of its receptor, Tkv, are expressed in lateral mesoderm, it is sufficient to repress gonadal mesoderm development. To determine whether this effect is specific to gonadal mesoderm, or whether fat body precursors also respond to dpp signaling in this way, fat body development was also analyzed in these embryos. I found that fat body precursors respond in exactly the same way to either ectopic Dpp or ectopic Tkv. In embryos with ectopic Dpp, no fat body precursors develop (Fig. 3B), whereas in embryos with ectopic TkvQD, only a few of these cells are present (Fig. 3C).

Taken together, these data show that dpp signaling, via the tkv receptor, sets the boundary of lateral mesoderm formation. This conclusion is in agreement with the analysis of the expression patterns of ectodermal dpp and mesodermal markers. Frasch (1995) showed that the ventral boundary of ectodermal dpp almost exactly overlays the ventral boundary of mesodermal bap expression. Furthermore, there is very little, if any, overlap between the ventral boundary of dpp expression and the dorsal boundary of gonadal mesoderm (Boyle et al., 1997). Therefore, in wild type, lateral mesodermal cells develop as such because they do not receive the dpp signal.

The findings presented here are consistent with the results of Reichmann et al. (1998) who demonstrated that expressing Dpp ectopically in the ectoderm or in the mesoderm blocks fat body development. Our data extend these findings by demonstrating the role of tkv, and probably punt, in this signaling event. Furthermore, we show that in the absence of dpp signaling, additional SGPs are specified. This demonstrates that dorsal mesoderm is capable of developing into more lateral mesodermal derivatives when dpp signaling is absent. This result fits nicely with the observation that additional SGPs are specified in bap mutant embryos, which are similar
to \textit{dpp} mutant embryos in that visceral mesoderm cells do not develop (Boyle et al., 1997). In \textit{bap} mutant embryos, however, dorsal mesoderm cells receive the Dpp signal, but are unable to initiate the developmental program toward visceral mesoderm development (Azpiazu and Frasch, 1993; Frasch, 1995). This demonstrates that mesodermal cells are not committed to develop as dorsal mesoderm when they receive the Dpp signal. It also suggests that \textit{dpp} does not directly repress lateral mesoderm formation, but rather its effect may be mediated by downstream factors activated by \textit{dpp}, such as \textit{bap}.

\textbf{Figure A-2: SGP specification is inhibited when Dpp or Tkv$^{QD}$ is overexpressed}  
Embryos are stage 13/14 and are stained with anti-Cli antibody to recognize SGPs. (A) wild-type embryo. (B) twi-gal4; gal424B X UASDpp. Dpp is misexpressed throughout the mesoderm in these embryos, and SGPs are not present. (C) twi-gal4; gal424B X UASTkv$^{QD}$. An activated form of the Tkv receptor is ectopically expressed throughout the mesoderm, and only a few SGPs are present (arrow).
Figure A-2: SGP specification is inhibited when Dpp or TkvQD is overexpressed.
Figure A-3: Fat body development is inhibited when Dpp or TkvQD is overexpressed.

Embryos are stage 13 and have been double-labeled with anti-Cli (brown) which recognizes SGPs, and anti-Srp (purple) which labels fat body precursors. (A) wild-type embryo. (B) twi-gal4; gal424B X UAS-Dpp. The formation of the fat body is blocked when Dpp is overexpressed throughout the mesoderm. (C) twi-gal4; gal424B X UAS-TkvQD. Only a few fat body precursors are present in this embryo (arrows).
Figure A-3: Fat body development is inhibited when Dpp or Tkv$^{QD}$ is overexpressed.


The screens that we conducted for genes required zygotically for proper germ cell migration have identified numerous genes involved in this process. While many of the genes that were identified are required for gonadal mesoderm development, others have a more direct role in germ cell migration. In particular, two new genes identified in the third chromosome screen, *columbus* and *fear-of-intimacy*, are not required for the expression of gonadal mesoderm-specific markers. The fact that two such genes were identified in the third chromosome screen argues that other genes playing specific roles in germ cell/SGP interactions would likely be uncovered in additional screens.

At least two more zygotic screens could be undertaken; either the second chromosome could be rescreened and saturated for mutations, or the X chromosome could be screened. While there are certainly genes on the second chromosome (such as *wunen*) which we did not identify, another second chromosome screen might also facilitate the further analysis of some of the genes that we did uncover. We isolated a single allele of two genes with relatively specific effects of germ cell migration, 9.35 and 22.38 (Moore and Lehmann, unpublished data), and only two alleles of *waldo*. Additional alleles would make it easier to determine if the mutant phenotypes were interesting enough to warrant molecular analysis, and furthermore, they might facilitate a future cloning effort since we were unsuccessful in locating deficiencies uncovering either 9.35 or *wdo*.

Many gene products required for germ cell migration are likely to be maternally-provided. This is particularly true for the genes acting within the germ cells themselves, as germ cells are capable of initiating migration prior to the onset of germ cell-specific zygotic transcription (Jaglarz and Howard, 1994; Van Doren et al., 1998; and Introduction). Furthermore, many gene products provided by the mother are known to be localized to the posterior pole and taken up into germ cells. While germ cell-specific transcription may be required for later steps of migration, none of the genes that we identified appear to function in germ cells. Maternally-required genes could be identified by generating homozygous mutant germline clones in heterozygous adults using the FLP recombinase and the ovoD/FRT system (Chou and Perrimon, 1992). Such a screen might also identify somatically-required genes. For example, we did not isolate any genes specifically required for germ cell migration through the posterior midgut (PMG) epithelium. The only mutants that we identified that interfered with this transepithelial migration change the identity of the PMG. Since the cellular changes that take place in
the PMG occur early (stage 10) in embryogenesis, it is probable that some of the required gene products are maternally provided.

Other experimental approaches may also help identify genes specifically involved in germ cell migration. Recently, a technique has been developed to allow for the mis-expression of random genes in particular tissues (Rørth, 1996). The mis-expression is generated by a P element carrying a multimerized UAS element that has randomly inserted into the 5' end of random genes. These genes can be misexpressed in germ cells by using the germ cell-specific gal4VP16 driver (Van Doren et al., 1998). Preliminary results from our lab (Cho, Forbes, Starz-Gaiano, and Lehmann, unpublished data) indicate that strong, penetrant germ cell migration phenotypes are produced from a limited number of the UAS lines. If the genes responsible for the mutant phenotypes can be readily identified, this technique may be another way to identify elusive germ cell-specific genes. Finally, wild-type Drosophila germ cells have been shown to migrate on various substrates in vitro (Jaglarz and Howard, 1995). Once candidate germ cell-specific genes have been identified, the migratory ability of mutant germ cells can be tested in vitro. Additionally, if the in vitro assay is developed to allow for the testing potential chemotactic effects, as is possible for mouse germ cells, the readiness of germ cells to navigate toward particular sources could be measured.

It is not yet clear if any of the genes that we have identified play a role in germ cell migration in the mouse. Directed efforts to identify Drosophila homologs of c-kit or Steel factor have been unsuccessful thus far (Forbes, personal communication). Perhaps as additional molecules functioning in germ cell migration are uncovered, additional similarities will surface. One unexpected similarity between germ cell migration in Drosophila and the mouse is suggested by the discovery of the role of the caudal visceral mesoderm in Drosophila germ cell migration. The caudal visceral mesoderm seems to play an analogous role to the dorsal mesentery in the mouse. Germ cells in both systems use this cell type to navigate from the gut epithelium to the gonadal mesoderm. Because after germ cells migrate along the dorsal mesentery or the caudal visceral mesoderm they are situated in close proximity to the gonadal mesoderm, these tissues may play similar roles in enabling germ cells to respond to a spatially-restricted signal.


