

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

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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 that 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 that associates with germ cells to form the embryonic gonad. Moreover, mutations that we isolated affecting embryonic patterning as well as germ cell migration suggest that the origin of gonadal mesoderm lies within the *eve* domain of the developing mesoderm.

Key words: *Drosophila*, Germ cell migration, Gonadal mesoderm, abdominal A, Abdominal B, columbus, even-skipped, fear-of-intimacy, fushi-tarazu, heartless, hedgehog, huckebein, odd-paired, serpent, trithorax, trithoraxgleich, zinc finger homeodomain protein-1, Mesoderm, Cell migration

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 constitute 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 (French-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 (PS11-13, Fig. 1D,E). As the germ band retracts, PGCs associate and align with mesodermal cells in PS10-12 that will give rise to the somatic component of the gonad (Fig. 1F). Finally, the PGCs and gonadal mesoderm coalesce in PS10 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.

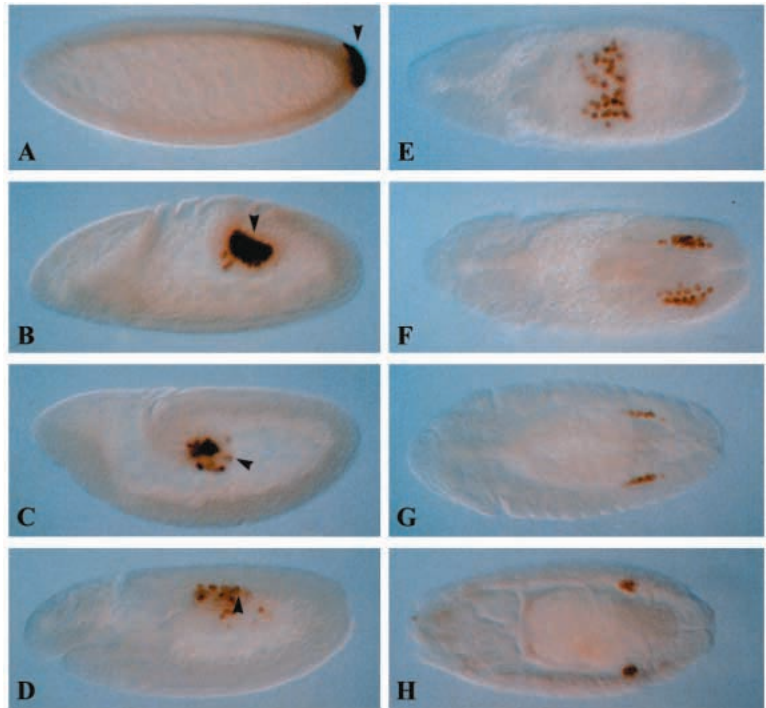


Fig. 1. Germ cell migration in wild-type embryos. Anterior is to left in all panels. Staging according to Campos-Ortega and Hartenstein (1985). (A-H) Germ cells visualized using an anti-Nanos antibody (arrowheads); (A-D) lateral views; (E-H) dorsal views. (A) Stage 5. Germ cells form at the posterior pole of the embryo. (B) Stage 8. During gastrulation, germ cells adhere to the posterior midgut (PMG) anlage, and are carried into the PMG lumen. (C) Stage 9-10. Germ cells begin their migration through the PMG. (D) Stage 11. Germ cells have migrated to the dorsal side of the PMG, and begin to associate with lateral mesoderm in PS11-13. (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 PS10-12. (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.

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, 1997).

The development of the somatic component of the gonad has also been the focus of numerous studies. It has been known for some time that mutations in the homeotic gene *abdominal A* (*abdA*) abolish gonad function (Karch et al., 1985; Lewis, 1978) and that *abdA* is required in the soma for gonad formation (Cumberledge et al., 1992). Moreover, a regulatory mutation in the *abdA* locus, *iab4*, causes specific defects in gonad coalescence (Boyle and DiNardo, 1995; Cumberledge et al., 1992; Warrior, 1994). More recent work has shown that both *abdA* and *Abdominal B* (*AbdB*) are required for the specification of somatic gonadal precursors (SGPs), those cells that give rise to gonadal mesoderm, in PS10-12 (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 PS11-12 migrate anteriorly toward PS10 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 PS10-12. 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 that have the most specific effects on gonad formation, and describe how these mutants provide further insight into the mechanisms governing the proper migration of germ cells in *Drosophila*.

MATERIALS AND METHODS

EMS mutagenesis and establishment of balanced lines

See Fig. 2 for an outline of the screen. A *ru st e^s ca* chromosome carrying the *fat facets-lacZ* (*faf-lacZ*) transgene (Fischer-Vize et al.,

1992), that had recently been isogenized was used for the target mutagenesis strain. This line had been selected for its low frequency of germ cells found outside the gonad at stage 15. A total of 2100 *ru st P[faf-lacZ] e^S ca* males were mutagenized with EMS (Sigma and ICN: 1500 with a 25 mM solution, and 600 with a 35 mM solution) in 1% sucrose for 24 hours according to standard procedures (Ashburner, 1989), with the modification that they were starved for 6 hours on a Kimwipe saturated with water prior to EMS treatment. These males were then mated to 4200 virgin females of the genotype *Df(3R)H99 P[hs-hid] p^P/ Ubx-lacZ TM3, Sb* [The *Df(3R) H99P[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 two *Df(3R)H99 P[hs-hid] p^P/Ubx-lacZ TM3, Sb* virgin females. These crosses were allowed to lay eggs for 5 days, after which the parents were discarded. The progeny were then subjected to 2 hours of heat shock on days 5 and 6 by placing vials directly in a 37°C water bath, with a 24 hour interval between heat shocks. Induction of ectopic *hid* expression in this manner presumably causes massive cell death and results in embryonic/larval lethality. We found that about 10% of our isolates contained flies that were not of the mutagenized *ru st P[faf-lacZ] e^S ca/Ubx-lacZ TM3, Sb* genotype. However, these 'lines' usually contained only 1-2 'escaper' progeny and did not pose a serious problem to the screening procedure. Lines that were kept for further analysis (see below) were inspected for 'escaper' flies and, if necessary, virgin females and males of the desired genotype were collected and used to establish balanced stocks. Lethal lines were determined by the absence of *ru st e^S ca* homozygotes.

Screening procedures and detection of β -galactosidase activity

Eggs of approximately 6-16 hours of age were collected from balanced lines using the block method as described in Nüsslein-Volhard et al. (1984). Eggs were collected from apple juice-agar plates and placed into 18-well staining blocks (design by Phillip Zamore), and processed for X-gal staining using the following procedure: Eggs were washed twice in PBT, and then dechorionated by placing in a 50% bleach solution for 5 minutes. After washing twice in PBT, they were fixed in heptane saturated with 2.5% glutaraldehyde for 7 minutes. The embryos were allowed to dry for 4 minutes in a fume hood and then washed in PBT for 30 minutes. Embryos were then stained for β -galactosidase activity using 10% X-gal in DMSO (Diagnostic Chemicals Limited), 1:50 in a staining buffer containing 10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM 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

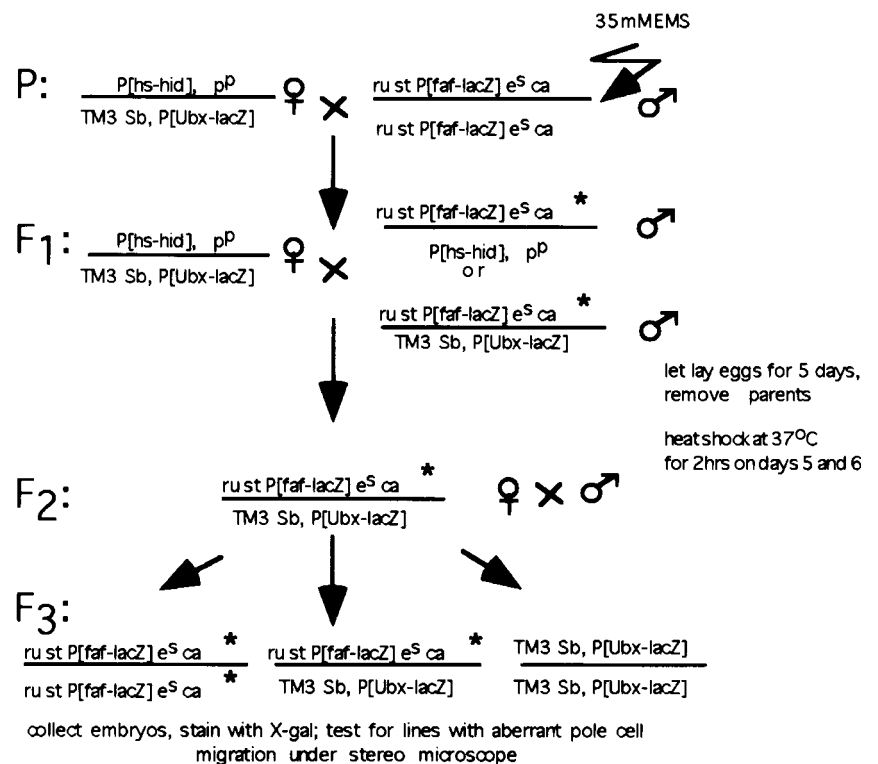


Fig. 2. Crossing scheme to establish lines isogenic for a mutagenized third chromosome (for an explanation of stocks used, see Materials and methods). Markers as described in Lindsley and Zimm (1992) *, designates mutagenized chromosome. Below the crosses is shown an example of embryos from a wild-type line containing the *faf-lacZ* chromosome over a 'blue balancer,' stained for β -galactosidase activity. Homozygous embryos are at stage 14, showing germ cells in coalesced gonads (arrow). 'Blue balancer' embryo is at stage 11.

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 Pirrotta (1991). Embryos were mounted onto slides in LX112 embedding medium (Ladd Research Industries, Inc.) according to Ephrussi et al. (1991), then analyzed with a Zeiss Axiophot microscope using Nomarski optics.

Cuticle preparations

Cuticle preparations were made of all potential mutant lines in a manner similar to that described by Nüsslein-Volhard et al. (1984), with the following modifications: Embryos were collected on apple juice-agar plates for 12 hours and allowed to age for 24 hours at 25°C. Unhatched eggs were collected into specialized 18-well staining blocks (design by Philip Zamore), dechorionated and fixed for 10 minutes in a 3:1 acetic acid:glycerol solution at 65°C. Embryos were washed twice in PBT and placed onto a slide. Excess PBT was removed with a filter paper (Whatman), replaced with a small drop of Hoyer's medium and covered by a 22×22 mm coverslip. Embryos were cleared by a 36 hour incubation at 65°C, and analyzed with a Zeiss Axiophot using dark field with a 20× 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 that showed obvious defects in pattern formation were crossed to mutants obtained from the Bloomington Stock Collection having similar phenotypes. In addition, once complementation groups were established from the 'specific' class of mutants, a representative allele from each group was crossed to three mutants previously known to show defects in gonad formation: *abdA* (Cumberledge et al., 1992), *AbdB* (Brookman et al., 1992) and *tin* (our observations; Boyle et al., 1997).

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

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

Whole-mount in situ hybridization

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

Antisense riboprobes were prepared for detection of the 412

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

Fly stocks

The following alleles were used for the complementation analyses described above, and all further phenotypic analyses: *abdA^{mx1}*, *AbdB^{D101.3}* (both gifts from Welcome Bender), *cno²*, *Df(3R)crbS87-5*, *Df^{9D}*, *fkh^{E200}*, *ftz^{7B}*, *htl^{AB42}* (a gift from James Skeath), *hh^{IJ}*, *hkb²*, *kni^{FC}*, *opa^{HP}*, *srp^{9L}*, *srw¹*, *tll^{LI0}*, *tin^{ΔGC14}* (a gift from Manfred Frasch), *tld^{9Q}* and *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 that failed to show a germ cell migration defect in this analysis were discarded.

Classification of mutant phenotypes

The results of the secondary screen enabled us to categorize the mutants into classes based on phenotypic similarity (Table 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

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

Lines scored	8842	
Lines selected	327	
Lethal hits/chromosome	1.9	
Phenotypic classes	<i>n</i>	% of selected lines
Specific effect on germ cells	70	21
Pattern formation	110	34
Dominant/multiple*	6	2
Lost†	28	9
False positive‡	113	34

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.
 †28 lines were not included in the secondary screen either due to death of the stock, or loss of the balancer chromosome.
 ‡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.

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 dorsoventral 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 long enough 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

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

	No. of alleles
Class 1: specific germ cell migration defect	
<i>abdominal A (abdA)</i>	3
<i>Abdominal B (AbdB)</i>	3
<i>columbus (clb)</i>	15
<i>heartless (htl)</i>	4
<i>fear-of-intimacy (foi)</i>	3
<i>tinman (tin)</i>	1
<i>trithorax (trx)</i>	17
<i>trithoraxgleich (trg)</i>	4
<i>zinc finger homeodomain-1 (zfh-1)</i>	5
Class 2: pattern formation mutants	
gut development	
<i>huckebein (hkb)</i>	4
<i>serpent (srp)</i>	11
dorsal/ventral polarity	
<i>shrew (srw)</i>	8
<i>tolloid (tld)</i>	19
gap	
<i>hunchback (hb)</i>	4
<i>forkhead (fkh)</i>	2
<i>knirps (kni)</i>	5
<i>tailless (tll)</i>	3
pair-rule	
<i>fushi-tarazu (ftz)</i>	2
<i>odd-paired (opa)</i>	8
segment polarity	
<i>hedgehog (hh)</i>	7
neurogenic	
<i>Delta (Dl)</i>	4
<i>Delta-like (Dl-1)</i>	3
dorsal open	
<i>canoe (cno)</i>	1
cellular differentiation	
<i>crumbs (crb)</i>	2
cellularization	
<i>thread (th)*</i>	6

*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).

that either could not be attributed to third chromosome lesions or that failed to show a phenotype in the secondary screen.

Complementation analysis suggests a high degree of saturation

70 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; Cumberland et al., 1992; Warrior, 1994). Complementation tests between our mutants with germ cell migration defects similar to those reported for *abdA*, *AbdB* and *tin* mutants revealed that we isolated alleles of all loci, demonstrating our screen's success in identifying genes required for the process.

We also conducted complementation tests between Class 2 alleles and many of the mutants identified in previous screens for defects in pattern formation (Table 2; 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 1 and Class 2 mutants, we have isolated an average of 5.8 alleles per locus (see also Materials and methods). This allele frequency is similar to that obtained in the saturation screens for defects in embryonic patterning (Jürgens et al., 1984). Given the results of our complementation analysis of both Class 1 and Class 2 mutants, we are confident to have thoroughly screened the third chromosome for zygotic mutations affecting germ cell migration and gonad formation. Although it is possible that genes required for overall embryonic patterning could also play a role in germ cell migration, we have chosen to focus the remainder of our phenotypic analysis on those complementation groups having relatively specific effects on germ cell migration and gonad formation.

Placing genes on the chromosomal map

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

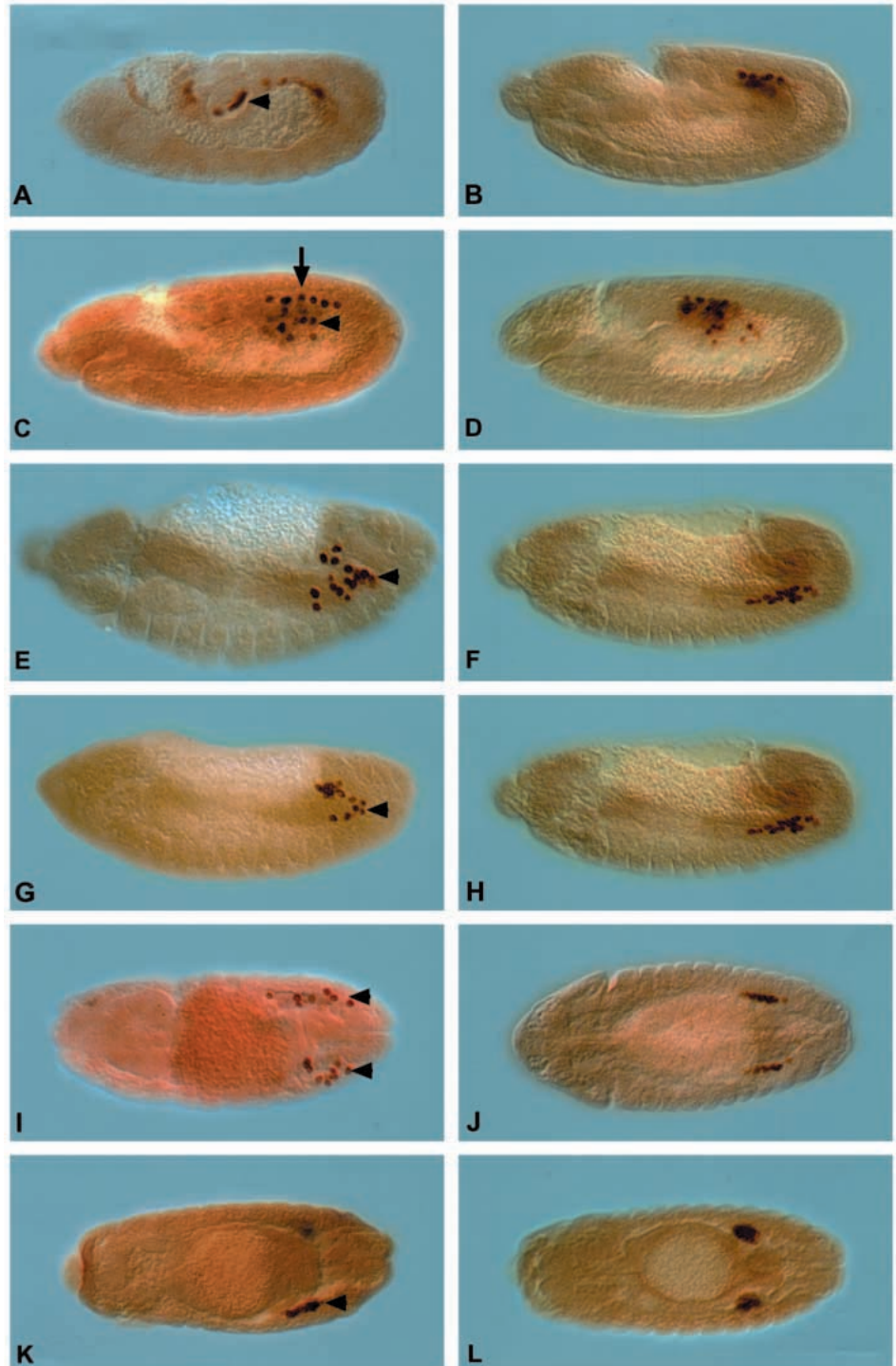


Fig. 3. Genes required for germ cell migration act during discrete steps in development. Anterior left in all panels. (A-L) Germ cells visualized using an anti-Vasa antibody. (A-H) lateral views; (I-L) dorsal views. (A,C,E,G,I,K) Mutant embryos displaying their characteristic phenotypes. (B,D,F,H,J,L) 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.

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 that 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 that 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 RNA 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

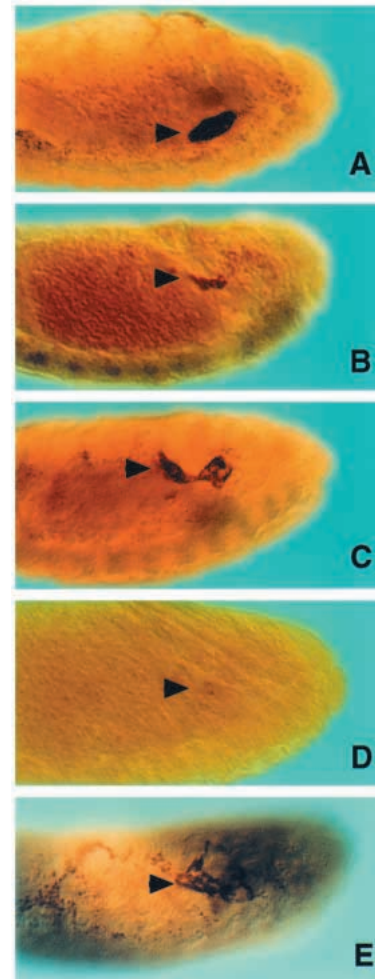


Fig. 4. Mutations affecting gonadal mesoderm development. Anterior left in all panels; lateral views. (A-E) Gonadal mesoderm development assayed by expression of the 412 retrotransposon (arrowheads). All embryos are at approximately stage 14 [Campos-Ortega and Hartenstein (1985)]. (A) Wild type; (B-E) Mutants. (B) *zfh-1*⁻. The number of gonadal mesoderm cells is drastically reduced compared to wild type. (C) *htl*⁻. Both the number and morphology of gonadal mesoderm cells is affected. However, more cells are present than in *zfh-1* mutants (compare with panel B). (D) *tin*⁻. Gonadal mesoderm cells are virtually abolished by this stage in development. (E) *foi*⁻. SGPs show aberrant morphology. Finger-like protrusions are seen, and they fail to show the tight cell-cell interactions characteristic of a coalesced gonad. However, SGP number appears normal. This embryo has been stained longer than the embryos in A-D, revealing low levels of 412 expression in the fat body.

AbdB are required for gonad assembly (Boyle and DiNardo, 1995; Cumberland 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*^{mx1} allele (see Materials and methods), which is a translocation breaking within the coding

region and fails to express a protein that is detectable by existing anti-AbdA antibodies (Karch et al., 1990). Earlier studies focused on a mutation in the *abdA* regulatory region, *iab4*, that affects *abdA* function in a subset of abdominal segments and, perhaps, in a subset of tissues (Cumberledge et al., 1992; Warrior, 1994). In mutants lacking most or all *abdA* function, germ cells are able to move through the PMG and initially find lateral mesoderm. However, germ cells fail to maintain their specific association with the mesoderm, and disperse in the posterior of the embryo. Earlier work has shown that *abdA* is required in the soma for gonad formation (Boyle and DiNardo, 1995; Cumberledge et al., 1992). This defect appears to be the result of a failure of gonadal mesoderm development, since the expression of 412 is severely reduced in these mutants (data not shown), in a manner similar to that seen in embryos lacking the Bithorax-Complex (Brookman et al., 1992).

Whereas *abdA* is required for the development of all gonadal mesoderm cells, mutations in *AbdB* appear to only affect the posterior component of these cells. In these mutants, many germ cells are able to coalesce along with gonadal mesoderm to form a gonad; however, some germ cells are excluded from this gonad, presumably due to the reduction in number of SGPs (Boyle and DiNardo, 1995; Brookman et al., 1992; our observations). We also identified mutations in a regulator of homeotic gene expression, *trx*, that has a germ cell migration phenotype very similar to that seen in *AbdB* mutants (Fig. 3G). The 'lost' germ cells in *trx* and *AbdB* mutants remain in an area ventral and posterior to the gonad until after coalescence. The *trx* gene is known to be required for maintaining the expression of homeotic genes including *abdA* and *AbdB* (Breen and Harte, 1993; Mazo et al., 1990). Several lines of evidence suggest that the defect seen in *trx* mutants is due to reduced function of *AbdB*, including the result that a *hs-AbdB* construct can partially rescue the *trx*⁻ germ cell migration defect (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 *trx*, 412 appears to be expressed at normal levels (data not shown), whereas in *AbdB* mutants, fewer SGPs express high levels of 412 than the number seen in wild type (Brookman et al., 1992). However, embryos that lack both maternal and zygotic *trx* show 412 expression levels identical to those seen in *AbdB* mutants (data not shown). These results suggest that *trx*, like *AbdB*, is required for a subset of SGPs to maintain their identity and, as a result, to maintain their association with germ cells (Boyle and DiNardo, 1995).

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

Mutations in the *tin* locus have a unique effect on germ cell migration. Germ cells are able to migrate through the gut epithelium to find their target mesodermal cells and remain associated with SGPs throughout germ band retraction. The germ cells attempt to line up, but do not achieve the organized

nature that 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 *tin* is required for proper development of gonadal mesoderm (Boyle et al., 1997). We have found that expression of 412 is virtually abolished in embryos lacking *tin* function (Fig. 4D). This result is consistent with previous studies demonstrating that expression of another SGP marker, *cli*, is drastically reduced in *tin* mutants (Boyle et al., 1997). It is unclear why *tin* mutants show such a relatively late germ cell migration defect, given *tin*'s striking effect on expression of gonadal mesoderm markers (Boyle et al., 1997; Broihier et al., 1998; see Discussion).

Gonad coalescence

Mutations in a novel gene, *foi*, specifically affect the ability of the germ cells and gonadal mesoderm to coalesce into the embryonic gonad. The hallmark of this phenotype is the appearance of very late stage embryos with germ cells and SGPs remaining in a line, instead of the characteristic round shape normally found in gonads by stage 14 (Fig. 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 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.

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 midgut 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.

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 that we have come close to saturation in this screen. Thus, the genes and phenotypes that 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 that 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* are all 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 PS10-12, and some continue to migrate into other parasegments, as well as other tissues. This result suggests that, in wild-type embryos, at the time that 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).

Mutations affecting gonadal mesoderm development

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

We and others have found the *tin* gene to be required for development of gonadal mesoderm, as exemplified by the lack of expression of gonadal mesoderm-specific markers in *tin* mutants (Boyle et al., 1997; Fig. 4D). Although the drastically

reduced expression of *cli* in *tin* mutants can be seen as early as stage 11 (Boyle et al., 1997), the resulting germ cell migration defect cannot be detected until stage 13 (Fig. 3I). More recent work has shown that most SGPs are at least partially specified in *tin* mutants, but fail to maintain this specification during later developmental stages (Broihier et al., 1998). This may explain how germ cells are initially able to associate with SGPs, but lose this association as SGPs fail to maintain their identity.

It has been recently shown that the *htl* gene, which encodes a *Drosophila* fibroblast growth factor receptor (DFR1/DFGF-R2), is involved in the dorsolateral migration of the invaginating mesodermal layer along the overlying ectoderm. Loss-of-function mutations in this locus affect the development of a number of dorsal mesodermal cell types, including midgut visceral mesoderm, cardiac mesoderm, and some somatic mesodermal derivatives. These studies further indicate that the number of precursors corresponding to the affected mesodermal cell types is significantly reduced in *htl* mutant embryos (Beiman et al., 1996; Gisselbrecht et al., 1996). Our phenotypic analysis of both germ cell migration and gonadal mesoderm defects in *htl* embryos demonstrates that this gene is required at an early stage in the development of yet another mesodermal cell type, the gonadal mesoderm. *htl* is also necessary for the Dpp-dependent maintenance of *tin* expression in dorsal regions of the mesoderm (Gisselbrecht et al., 1996). Therefore, *htl* could be acting through *tin* to specify SGPs, since *tin* is required for gonadal mesoderm development (see above). Conversely, *htl* could be required for a signaling process that is independent of its role in maintaining *tin*'s dorsal expression pattern. Further experiments are necessary to distinguish between these two possibilities, but the finding that *htl* has an additional requirement in gonadal mesoderm morphological differentiation suggests that the latter theory could prove correct. Moreover, recent studies demonstrate that *tin*'s role in gonadal mesoderm development is independent of Dpp signaling (Broihier et al., 1998).

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

Origin of the gonadal mesoderm

We have found that the segmentation genes *ftz*, *hh* and *opa* are all required for germ cell migration and gonadal mesoderm development. Furthermore, in a preliminary screen to identify patterning genes required

for germ cell migration, we have found that mutations in *even-skipped* (*eve*) and *engrailed* (*en*) also have a drastic effect on the development of gonadal mesoderm (Broihier, Moore, and Lehmann, unpublished results). These segmentation genes identified in our screens have been previously shown to play a role in the patterning of a component of mesoderm that 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 that they affect midgut visceral mesoderm and fat body.

Recent studies suggest that each parasegment of the mesoderm is initially subdivided into two domains. *hh* and *en* are positively regulated by pair-rule gene action in the 'eve-domain' of the mesoderm, whereas *wg* is a target in the 'slp domain' (Azpiazu et al., 1996; Riechmann et al., 1997). Our observations of the loss of gonadal mesoderm in *hh* and *en* mutants support the model that SGP origin lies within the *eve* domain of the mesoderm (Fig. 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

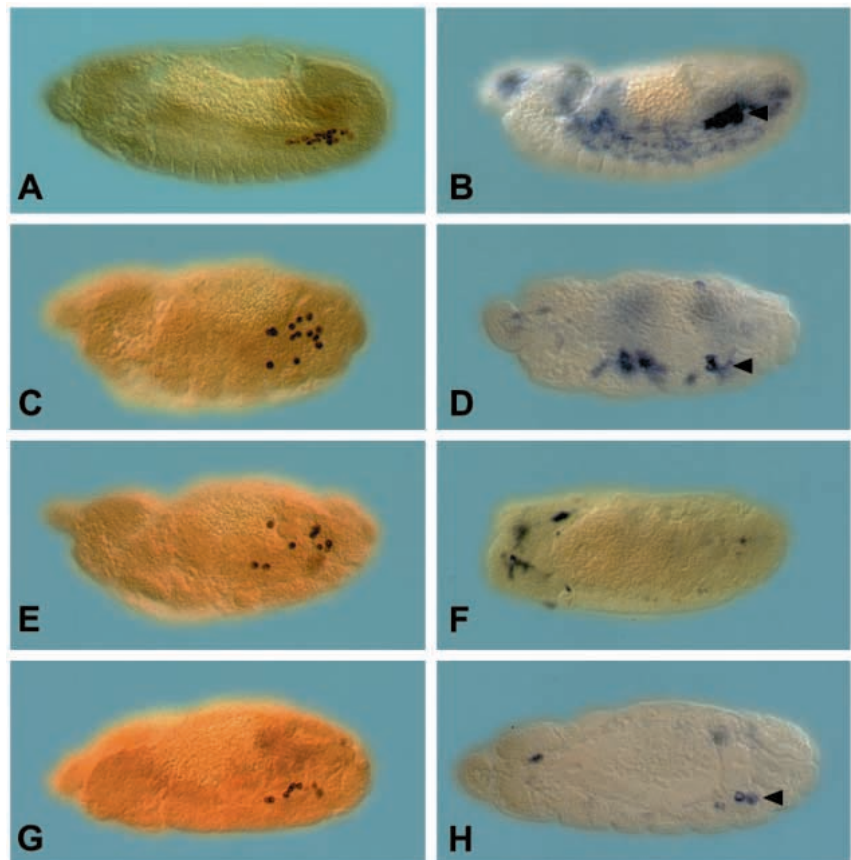
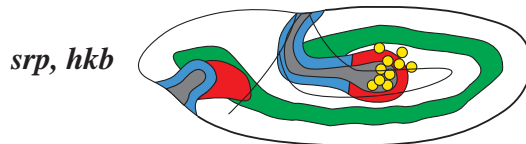
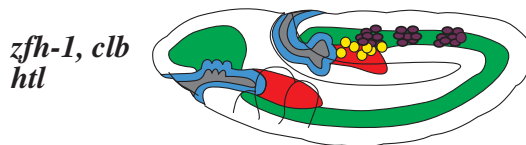
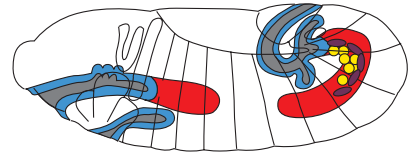


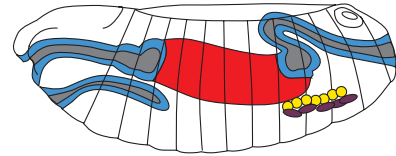
Fig. 5. Pair-rule and segmentation genes affecting germ cell migration and gonadal mesoderm development. Anterior left in all panels; lateral views. Embryos are at approximately stage 13-14. (A,C,E,G) Germ cells visualized using an anti-Vasa antibody; (B,D,F,H) gonadal mesoderm development assayed by expression of the 412 retrotransposon (arrowheads). (A,B) Wild type; (C,D) *ftz*⁻; (E,F) *opa*⁻; (G,H) *hh*⁻. (C,E,G) Mutations in *ftz*, *opa*, and *hh* all result in the failure of germ cells to associate with mesodermal cells. (D,F,H) The number of gonadal mesoderm cells is severely reduced in all mutants shown.

Migration through MidgutAttachment to MesodermAlignment with Gonadal Mesoderm

abdA, AbdB
trx, trg



tin

Gonad Coalescence

foi

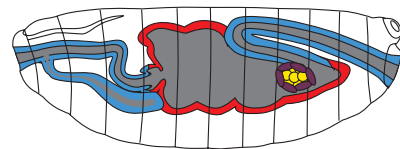


Fig. 6. Genetic summary of germ cell migration: third chromosome. Embryo drawings after V. Hartenstein. Blue, foregut and hindgut; red, anterior and posterior midgut; gray, gut lumen; green, mesoderm; purple, SGPs; yellow, germ cells. Phenotypic analysis of mutants identified in our screen shows that 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.

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 that 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 with each other (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 that 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 a key to identifying the missing germ-line cues that act in concert with genes that we have identified that are essential for germ cell migration, gonadal mesoderm development and gonad coalescence.

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