Identification of X-Linked Genes Required for Migration and Programmed Cell Death of *Drosophila melanogaster* Germ Cells

Clark R. Coffman,*,[1] Rachel C. Strohm,* Fredrick D. Oakley,* Yukiko Yamada,* Danielle Przychodzin[†] and Robert E. Boswell[†]

**Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011-3260 and [†]Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309*

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ABSTRACT

Drosophila germ cells form at the posterior pole of the embryo and migrate to the somatic gonad. Approximately 50% of the germ cells that form reach their target. The errant cells within the embryo undergo developmentally regulated cell death. Prior studies have identified some autosomal genes that regulate germ cell migration, but the genes that control germ cell death are not known. To identify X-linked genes required for germ cell migration and/or death, we performed a screen for mutations that disrupt these processes. Here we report the identification of *scattershot* and *outsiders*, two genes that regulate the programmed death of germ cells. The *scattershot* gene is defined by a mutation that disrupts both germ cell migration and the death of germ cells ectopic to the gonad. Maternal and zygotic expression of *scattershot* is required, but the migration and cell death functions can be genetically uncoupled. Zygotic expression of wild-type *scattershot* rescues germ cell pathfinding, but does not restore the programmed death of errant cells. The *outsiders* gene is required zygotically. In *outsiders* mutant embryos, the appropriate number of germ cells is incorporated into the gonad, but germ cells ectopic to the gonad persist.

CELL migration and programmed cell death play critical roles in animal development, immune system function, wound healing, angiogenesis, and metastasis (Lauffenburger and Horwitz 1996; Jacobson *et al.* 1997; Holder and Klein 1999; Meier *et al.* 2000). Many cells are formed in one part of the body and must migrate to their ultimate locations to function. A common strategy is to overproduce cells and then eliminate those that are no longer needed or that are potentially dangerous to the animal. The control of cell movements and cell death in an organism must be precisely regulated, and these processes require the integration of a wide range of signals between and within cells.

The germ cells of Drosophila provide an excellent system for the study of cell migration and cell death. The movements of the germ cells have been well documented, and the elimination of ectopic and/or supernumerary germ cells occurs with great efficiency (Sonnenblick 1941, 1950; Counce 1963; Fullilove and Jacobson 1978; Underwood *et al.* 1980; Technau and Campos-Ortega 1986; Hay *et al.* 1988; Lasko and Ashburner 1990; Smith *et al.* 1992; Jaglarz and Howard 1994, 1995; Warrior 1994; Williamson and Lehmann 1996; Campos-Ortega and Hartenstein 1997; Moore *et al.* 1998; Wylie 1999, 2000; Starz-Gaiano and Lehmann 2001). Selected features of germ cell movements and the elimination of germ cells ectopic to the gonad are shown in Figure 1. Prior studies have shown that ~50% of the primordial germ cells that form successfully complete migration and are incorporated into the somatic gonad (Sonnenblick 1950; Underwood *et al.* 1980; Technau and Campos-Ortega 1986). Primordial germ cells that have been labeled using horseradish peroxidase or radioactive thymidine do not transdifferentiate, and very few transplanted cells persist outside of the gonads (Underwood *et al.* 1980; Technau and Campos-Ortega 1986). Collectively, these studies demonstrate that germ cells ectopic to the gonad die and that a mechanism regulating germ cell survival must exist within the embryo.

To date, no X-linked genes with roles in germ cell migration or programmed cell death have been reported. Screens of the second and third chromosomes identified some genes necessary for germ cell migration in Drosophila, but their roles remain enigmatic (Kobayashi *et al.* 1996; Zhang *et al.* 1996; Boyle *et al.* 1997; Zhang *et al.* 1997; Asaoka *et al.* 1998; Broihier *et al.* 1998; Forbes and Lehmann 1998; Moore *et al.* 1998; Van Doren *et al.* 1998; Deshpande *et al.* 1999, 2001; Starz-Gaiano *et al.* 2001). Our understanding of the regulation of germ cell development is far from complete, as this process must involve the functions of additional genes. Therefore, we conducted a screen of the X chromosome for genes that are required for normal germ cell development. When mutated, all of the genes identified in our screen altered the distribution of germ

[1]*Corresponding author:* Department of Zoology and Genetics, 3258 Molecular Biology Bldg., Iowa State University, Ames, IA 50011-3260. E-mail: ccoffman@iastate.edu

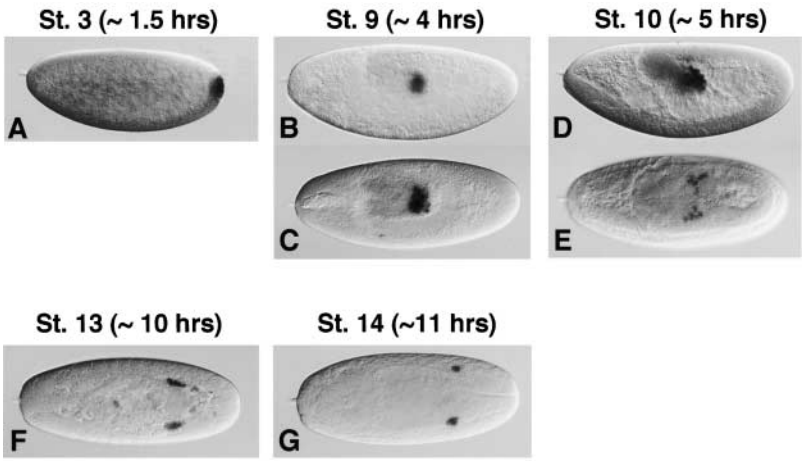

FIGURE 1.—Dynamics of germ cell development. Some of the stages of germ cell development are shown. The germ cells are labeled utilizing a *fat facets-lacZ* reporter transgene. Anterior is to the left in all panels. (A, B, and D) Lateral views. (C, E, F, and G) Dorsal views. (A) Germ cells form at the posterior pole of the embryo. (B and C) Germ band extension is complete and the germ cells are contained within the posterior midgut epithelium. (D and E) Germ cell migration through the posterior midgut epithelium is almost complete, and the germ cells are separating laterally into two populations. (F) Germ cells have migrated to and are coalescing with the somatic gonad precursor cells in a germ band retracted embryo. (G) Germ cell migration is complete. Note the absence of labeled cells outside of the gonads.

cells in the early Drosophila embryo. These mutations are informative to the study of germ cell migration and/or cell death, as they do not map to loci with previously described roles in these processes.

We report the identification and initial characterization of two genes. The first, *scattershot* (*sctt*), is maternally and zygotically required. This gene, when mutated, severely disrupts both germ cell migration and developmental cell death. In embryos from *sctt/sctt* mutant mothers, very few of the germ cells successfully migrate to the gonad. In addition, those germ cells ectopic to the gonad retain germ cell characteristics. Although the germ cell migration in *sctt* mutants is severely altered, germ cell formation, somatic gonad development, and body pattern appear normal. Homozygous female and hemizygous male *sctt* mutant embryos are viable and fertile, although fertility is greatly reduced. The *sctt* gene is only the third gene identified in Drosophila with a demonstrated role in germ cell migration whose product is maternally contributed. The other two are *polar granule component* (*pgc*) and *nanos* (*nos*; KOBAYASHI *et al.* 1996; NAKAMURA *et al.* 1996; ASAOKA *et al.* 1998; FORBES and LEHMANN 1998; DESHPANDE *et al.* 1999). Interestingly, zygotic expression of a wild-type copy of *sctt* rescues the germ cell migration defect, but fails to rescue the programmed cell death phenotype of *sctt* mutant embryos. A second gene, *outsiders* (*out*), must be expressed zygotically and has a function in the death of germ cells that fail to reach the gonad. The number of germ cells that reach the gonad is the same in *out/out* embryos and in unmutagenized controls. However, many additional cells outside of the gonad continue to express germ cell markers. Thus, both *sctt* and *out* are necessary for the execution of a cell death program, and *sctt* has an additional role in germ cell pathfinding.

MATERIALS AND METHODS

Mutagenesis and screening: To isolate X-linked mutations affecting germ cell development, 3475 independent mutagenized lines were screened. A P[w^+, *fat facets-lacZ*] transgene that is expressed specifically in the germ cells of the embryo was used to determine the location of the germ cells (FISCHER-VIZE *et al.* 1992). The screen is diagrammed in Figure 2. Three- to 4-day-old w^{1118}, P[w^+, *fat facets-lacZ*]/Y males were mutagenized using 25 mM ethyl methane sulfonate (EMS) in 1% sucrose (LEWIS and BACHER 1968) and crossed to *y w* hnt^{1142}, P[ry^+, FRT^{101}]/FM7, Df(1)KA14/FM7c, or Df(1)HA32/FM7c, P[$ry^{+t7.2}$, *ftz-lacZ*] (FM7Z) virgin females. After 4 days, the adults were cleared from the bottles. The resulting female offspring carrying the balancer chromosome were then individually crossed to FM7/Y, FM7c/Y, or FM7Z/Y males to establish stocks. The stocks were then expanded, and the embryos were collected using 50-ml conical tubes with apple juice-agar medium in the caps. Chorion removal, fixation, and staining were carried out in 48-well custom staining trays. The germ cells were labeled using X-Gal as the substrate (SIMON *et al.* 1985; HOLMES *et al.* 1998).

The nonmutagenized w^{1118}, P[w^+, *fat facets-lacZ*] parental stock had a very low percentage of embryos with any germ cells outside the gonad. During the screen and subsequent analyses, embryos were scored as mutant if they had four or more germ cells ectopic to the gonad. For brevity, the parental w^{1118}, P[w^+, *fat facets-lacZ*], mutagenized w^{1118} *sctt*, P[w^+, *fat facets-lacZ*], and w^{1118} *out*, P[w^+, *fat facets-lacZ*], chromosomes will be referred to as *faf-lacZ*, *sctt*, and *out*, respectively.

Mapping and complementation analyses: To determine the location of the *sctt* and *out* mutations, the mutant lines were tested for complementation using the X chromosome deletion stocks available from the Bloomington and Umeå stock centers (FLYBASE 1999). Since *sctt* is required maternally, *sctt*/Df(1) females were generated and their offspring were tested for abnormal germ cell development. The location of *out* was established by crossing Df(1)/balancer virgin females to *out*/Y males. The germ cell phenotype of the progeny of the Df(1)/*out* females was then assayed.

Recombination mapping to localize *sctt* was performed using the *sctt* mutant chromosome and w^{1118} cv^1 wy^{74i} f^1, sc^1 ec^1 cv^1 wy^{74i} f^1, or y^1 w^1 cv^1 ct^6 v^1 marker chromosomes. The insertion site of the P[w^+, *fat facets-lacZ*] was determined to be 18F-19A by *in situ* hybridization of a digoxygenin-labeled *white* probe (a kind gift from Joseph Heilig) to salivary gland chromosomes (O'HARE *et al.* 1984; JOHNSON-SCHLITZ and LIM 1987; TAUTZ and PFEIFLE 1989; KLINGLER and GERGEN 1993). Virgin females homozygous for the *sctt* mutant chromosome were crossed to males carrying the marker X chromosome. Recombination occurred in the F_1 females, which were crossed to FM7c/Y males. Individual F_2 males from this cross that carried

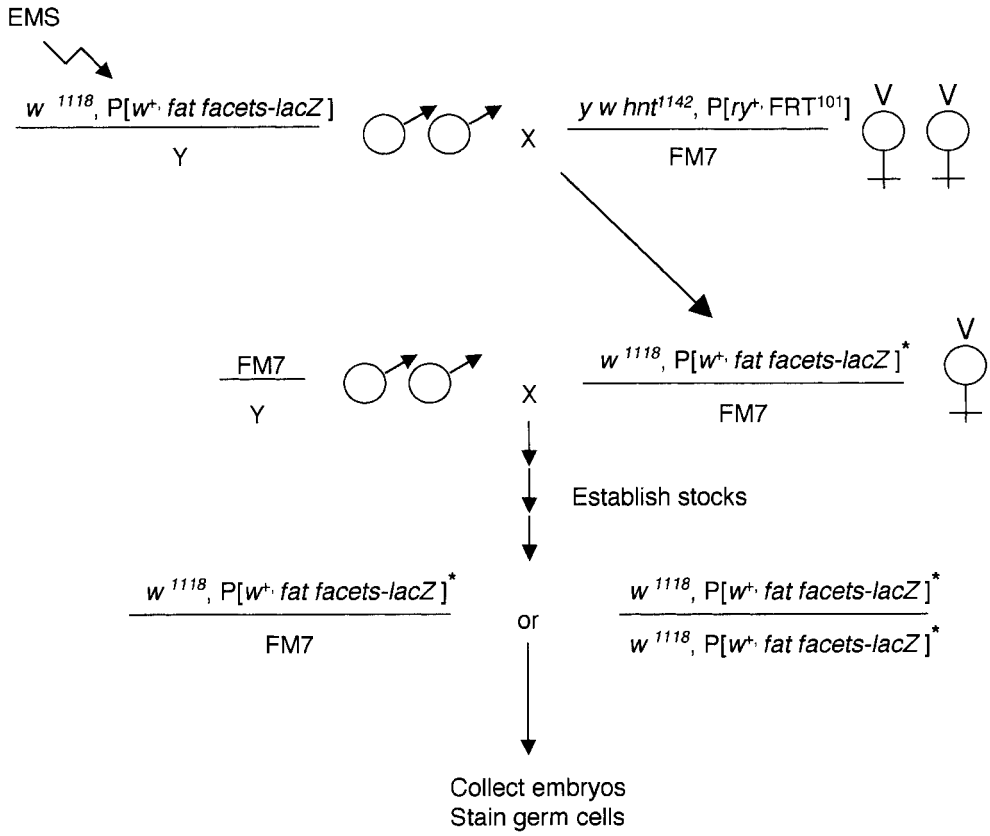

FIGURE 2.—Diagram of a screen for X-linked mutations affecting germ cell development. Males were mutagenized using EMS. The mutagenized X chromosome (*) was isolated over an FM7 balancer chromosome. Depending upon the viability and fertility of the mutagenized X chromosome, either homozygous or balanced stocks were established. Overnight collections of embryos from these stocks were used for staining.

a recombinant chromosome were then crossed to *y w hnt*^{1142}, P[*ry*$^{+}$, FRT^{101}]/FM7c, Df(1)KA14/FM7c, or Df(1)HA32/FM7Z virgin females. Each individual recombinant X chromosome was then isolated over a balancer chromosome to establish a stock. The balancer chromosome was removed to yield an independent homozygous recombinant line. Embryos collected from F_5 or later mothers homozygous for the recombinant X chromosome were scored for the sctt phenotype using X-Gal staining of 12- to 15-hr embryos.

Complementation tests between *sctt*, *out*, and the other lines obtained in the screen were also performed. To test for complementation of the maternal-effect germ cell migration phenotype of *sctt*, virgin females from the remaining lines were crossed to *sctt*/Y males. The offspring from *trans*-heterozygous *sctt*/test chromosome F_1 females were then assayed for complementation. The *out* complementation group was determined by testing *out*/FM7Z and/or *out/out* females crossed to males from the other lines, if the test line produced viable males. If the X chromosome carried a lethal mutation, heterozygous test chromosome/FM7Z virgin females were crossed to *out*/Y males. The germ cell phenotype of the progeny was then assayed by β-galactosidase (β-Gal) staining.

Fertility assay and ovary dissections: To test fertility, individual male and female offspring of *sctt*/*sctt*, *sctt*/FM7, or *faf-lacZ*/*faf-lacZ* females were crossed to Ore-R flies and placed in vials for 5–7 days. For the crosses testing male fertility, vials were scored for the presence of larvae or pupae. A vial that contained no offspring was scored as sterile only if adults of both sexes were still alive in the vial and unhatched eggs were present. In the crosses that tested female fertility, vials were scored for the presence of eggs or offspring. In these cases, a vial that did not contain eggs was scored as sterile only if the test female was still alive at the end of the trial period.

Ovaries from some of the test females were dissected in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.3% Triton X-100, pH 7.2; SAMBROOK *et al.* 1989) to assay the status of ovary development. Ovaries containing late-stage oocytes were scored as normal (KING 1970; MAHOWALD and KAMBYSELLIS 1980; SPRADLING 1993). Agametic ovaries had no detectable oocytes. Rudimentary ovaries contained early oogenic stages (younger than stage 9), but no late-stage oocytes. The data presented include only those females from which both ovaries were recovered in the dissection.

Germ cell counts: To determine the number of β-Gal-positive germ cells present in 11- to 12-hr *faf-lacZ* control, *sctt*, or *out* embryos, embryos were collected for 1 hr on apple juice caps and then aged 11 hr at 25°. β-Gal staining was performed as above and germ cells were counted using differential interference contrast microscopy. All of the cells counted were positive for β-Gal activity and exhibited the spherical nucleus and large cell size characteristic of germ cells at this stage of development (RABINOWITZ 1941; POULSON 1950; UNDERWOOD *et al.* 1980). The gonadal sheath cells were used to determine the boundary of the gonad. Statistical analyses were performed using Minitab Statistical Software (Minitab, State College, PA) or JMP version 4.0 (SAS Institute, Cary, NC).

RESULTS

Screen for mutants defective in germ cell development: X-linked genes with functions required for germ cell development were identified in an EMS mutagenesis screen of 3475 independent lines. Germ cells ectopic to the gonad were observed in 39 lines. Seventeen of these lines exhibited >40% penetrance and were kept

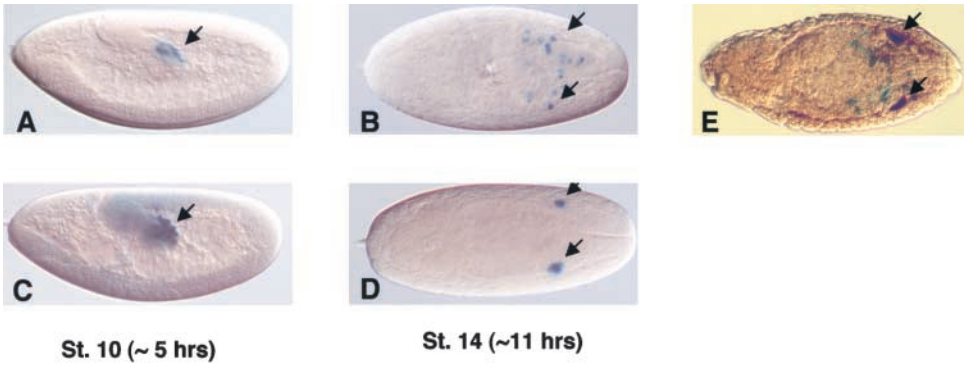

FIGURE 3.—Mutation of *sctt* results in aberrant germ cell migration and retention of germ cell marker expression in germ cells outside of the gonad. Lateral views of (A) *sctt* mutant and (C) wild-type embryos showing germ cells migrating across the posterior midgut epithelium (arrows). Germ cell formation and migration are indistinguishable from a wild-type embryo. Dorsal views of stage 14 *sctt* (B) and wild-type (D) embryos are shown. Note that very few germ cells, if any, in the *sctt* embryo are located in the regions of the gonad (arrows). Also, many germ cells outside of the gonad continue to express Faf-β-Gal. The embryos shown in A–D were stained in parallel. (E) An *sctt* mutant embryo double labeled for the 412 retrotransposon, a marker of somatic gonadal mesoderm, and for the Faf-β-Gal germ cell marker. The somatic gonadal mesoderm (arrows, brown) shows a wild-type staining pattern. The germ cells are blue and are seen scattered throughout the posterior of the embryo.

for further analyses. In the four most severe mutants, the germ cells are scattered throughout the posterior half of the embryo and very few, if any, germ cells populate the gonad. This scattered germ cell phenotype is observed in two homozygous viable lines and two lines that are homozygous lethal. A second class of 13 lines exhibit less severe phenotypes that are characterized by visible clusters of germ cells in the position of the gonad and four or more germ cells located outside of the gonads. This group consists of eight homozygous viable alleles and five chromosomes with lethal mutations. Complementation analyses of these 17 lines reveal that the mutations we isolated represent at least four complementation groups. Detailed analyses of the *sctt* and *out* genes are described here.

Genetic characterization of sctt: A single recessive allele of *sctt* was isolated (Figure 3). This allele is highly penetrant (>94%), and disruption of the germ cell staining pattern requires that the mother be homozygous mutant (Table 1). The offspring of *sctt/sctt* females will be referred to as *sctt* mutant embryos unless otherwise specified. This allele is complemented by all of the X chromosome deletion stocks available from the Bloomington and Umeå stock centers, which represent ~80% of the X chromosome. Since a noncomplementing deletion was unavailable, we determined the genetic map position by recombination mapping of *sctt* using marked X chromosomes. We analyzed 32 lines with a recombination event between *cv* and *wy* and 21 lines with a recombination event between *y* or *sc* and *cv*. These analyses place *sctt* within 1 map unit of *cv*. The *cv* gene maps to 1-13.7 on the genetic map and is predicted to lie within polytene chromosome bands 5A13–5B1 (LINDSLEY and ZIMM 1992; FLYBASE 1999). The Dp(1:Y)dx^{+}5 chromosome carrying polytene chromosome bands 4C11;6D8 zygotically rescues the sctt germ cell migration phenotype. This indicates that *sctt* is within the 4C11:6D8 interval. Three gaps in the deletion stocks fall within the region delimited by our recombination mapping. It is likely that *sctt* is located in one of these regions not represented by the deletion stocks. It is formally possible that the sctt phenotype is the result of two independent mutations. If this is the case, they are very tightly linked. The sctt phenotype is complemented by all *P*-element insertions and previously identified mutations tested to date.

TABLE 1

The scattershot phenotype is maternal effect and highly penetrant

Female	Male	*N*	% mutant
sctt/sctt	*sctt*	697	97.8
sctt/sctt	Ore-R	653	94.2[a]
sctt/sctt	FM7Z[b]	218	99.5[c]
sctt/FM7	*sctt*	1148	4.2
sctt/FM7	Ore-R	440	3.0
sctt/FM7	FM7	561	4.5
faf/faf	*faf*	787	6.5

[a] 366 with a scattershot phenotype, 249 with a less severe phenotype; see Figure 4.
[b] FM7c, P[*ry*$^{+t7.2}$, *ftz-lacZ*].
[c] 215 with scattershot phenotype; only *sctt*/Y embryos were scored.

Genetic characterization of out: The *out* complementation group consists of six recessive alleles (Table 2). In *out* mutants, clusters of Faf-β-Gal-expressing germ cells are clearly localized to each gonad, but germ cells ectopic to the gonad are also prominent (Figure 4). Three alleles are homozygous viable (*out*^{1}, *out*^{2}, and *out*^{3}), and three alleles are homozygous lethal (*out*^{4}, *out*^{5}, and *out*^{6}). The product of the *out* gene is required zygotically. Penetrance of the mutant phenotype ranges from 65.6% for *out*^{6} to 93.7% for *out*^{1}. The three viable alleles, *out*^{1}, *out*^{2}, and *out*^{3}, fail to complement the germ cell mutant phenotype of each other and, for each of these alleles, this phenotype is uncovered by Df(1)JA27, which is reported to be a deletion of polytene chromosome bands

TABLE 2

The *out* complementation group consists of six recessive alleles

Female	Male	*N*	% mutant embryos
out^1/*out*^{1}	*out*^{1}	175	93.7
out^1/FM7Z	*out*^{1}	212	89.2[a]
out^1/FM7Z	*out*^{2}	237	74.3[a]
out^1/FM7Z	*out*^{3}	216	71.8[a]
out^4/FM7Z	*out*^{1}	94	75.5[a]
out^5/FM7Z	*out*^{1}	144	92.4[a]
out^6/FM7Z	*out*^{1}	137	70.8[a]
out^1/FM7Z	*faf*	300	55.0[b]
out^2/*out*^{2}	*out*^{2}	654	93.4
out^2/*out*^{2}	*out*^{3}	72	81.9
out^4/FM7Z	*out*^{2}	635	83.1[a]
out^5/FM7Z	*out*^{2}	592	91.2[a]
out^6/FM7Z	*out*^{2}	450	39.6[a]
out^2/*out*^{2}	*faf*	79	62.0
out^3/*out*^{3}	*out*^{3}	620	78.4
out^3/*out*^{3}	*out*^{2}	545	68.3
out^4/FM7Z	*out*^{3}	45	80.0[a]
out^5/FM7Z	*out*^{3}	83	74.7[a]
out^6/FM7Z	*out*^{3}	85	74.1[a]
out^3/*out*^{3}	*faf*	338	44.1
out^4/FM7Z	FM7Z	144	82.6[c]
out^4/FM7Z	*faf*	30	66.7[b]
out^5/FM7Z	FM7Z	230	92.2[c]
out^5/FM7Z	*faf*	271	53.9[b]
out^6/FM7Z	FM7Z	186	65.6[c]
out^6/FM7Z	*faf*	87	34.5[b]
faf/*faf*	*faf*	787	6.5

[a] Only *out*/*out* and *out*/Y embryos were scored.
[b] Only *out*/*faf* and *out*/Y embryos were scored.
[c] Only *out*/Y male embryos were scored.

18A5;18D1 (FLYBASE 1999). The three viable alleles fail to complement *out*^{4} and *out*^{5}. Interestingly, *out*^{6} is not complemented by *out*^{1} and *out*^{3} but is complemented by *out*^{2} (Table 2). Embryos from the three lethal lines appear to have normal segmentation; however, the *out*^{4} and *out*^{5} lines exhibit increased pupal lethality. The phenotypes of Df(1)JA27/Y males and Df(1)JA27/*out*^{1} hemizygous females are more severe than any combination of *out* alleles from our screen. The germ cell phenotype associated with the Df(1)JA27 chromosome is recessive, as Df(1)JA27/+ embryos have a wild-type germ cell staining pattern. The out phenotype is complemented by all *P*-element and known mutations within the 18A5;18D1 interval that we have tested. We have not determined whether the lethality associated with *out*^{4}, *out*^{5}, and *out*^{6} is due to the *out* mutation or is due to a second mutation present on these chromosomes.

Mutation of *sctt* disrupts germ cell migration and developmentally regulated cell death: In *sctt* mutant embryos, germ cell development, as visualized by the Faf-β-Gal marker, appears normal prior to migration. Germ cells form at the posterior pole, and they are incorporated into the posterior midgut (PMG) pocket at the completion of germ band extension (stage 10; CAMPOS-ORTEGA and HARTENSTEIN 1997). The germ cells then transit the epithelium of the PMG (JAGLARZ and HOWARD 1994, 1995; WARRIOR 1994; CALLAINI *et al.* 1995; Figure 3A). However, very few, if any, of the germ cells in *sctt* mutant embryos successfully migrate to and become incorporated into the developing gonad. Instead, the germ cells disperse throughout the posterior half of the embryo and continue to express the Faf-β-Gal marker (compare Figures 3, B and D, and 5A). This phenotype reveals that this *sctt* mutation disrupts two processes. First, cell migration is severely impaired. Second, the cells that do not reach the gonad fail to undergo cell death. Despite the severity of the germ cell phenotype, homozygous *sctt* mutants are viable, and these individuals do not display any detectable morphological abnormalities as embryos or adults, with the exception of agametic gonads (see below).

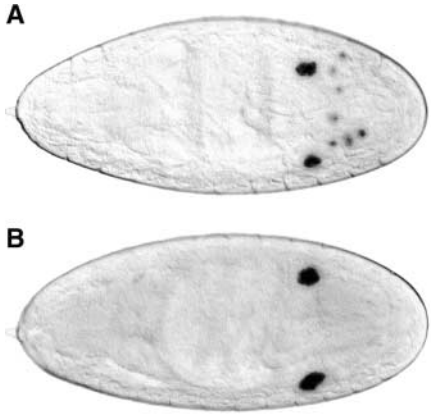

FIGURE 4.—The *out* mutants are deficient in the elimination of ectopic germ cells. (A) A dorsal view of a stage 14 *out/out* mutant embryo shows two gonads. However, many cells outside of the gonad continue to stain for Faf-β-Gal. (B) Dorsal view of a wild-type embryo stained in parallel with the embryo shown in A.

In the homozygous *sctt* stock, 97.8% of the embryos display the extremely severe phenotype shown in Figure 3B. In contrast, only 3–4.5% of embryos from *sctt*/FM7 mothers and 6.5% of embryos from the unmutagenized *faf-lacZ*/*faf-lacZ* stock have four or more labeled germ cells located outside of the gonad (Table 1). The germ cell migration defect is observed only among the offspring of homozygous *sctt* mutant mothers, and it is not evident in the *sctt*/*sctt* or *sctt*/Y offspring of *sctt*/FM7 females crossed to *sctt*/Y males. Therefore, the *sctt* mutation isolated in this screen is recessive and a maternal effect. Also, the phenotypes of *sctt*/*sctt* and *sctt*/Y embryos from *sctt*/*sctt* mutant mothers are equivalent.

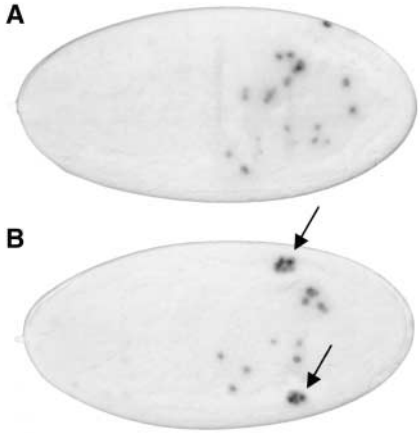

FIGURE 5.—The sctt germ cell migration phenotype can be paternally rescued. Zygotic expression of a wild-type copy of *sctt* rescues the germ cell migration defect, but cells outside of the gonad continue to express germ cell markers. Two embryos from *sctt*/*sctt* mutant mothers are shown. (A) The phenotype observed in homozygous *sctt*/*sctt* or hemizygous *sctt*/Y individuals. (B) An *sctt*/+ embryo. Note the small clusters of germ cells that have successfully migrated to the gonad (arrows in B).

The pathfinding and cell death defects in sctt embryos can be genetically uncoupled: To better understand the relationship between germ cell migration and programmed cell death, we performed additional genetic and phenotypic analyses of *sctt* mutant embryos. We reasoned that if *sctt* function is required at different times for either germ cell migration or the elimination of ectopic germ cells, then we might be able to separate differing temporal requirements through paternal contribution of a wild-type copy of *sctt*. If *sctt* function were required both maternally and zygotically for germ cell migration, then an embryo expressing a wild-type copy of *sctt* would display a less severe germ cell migration phenotype. Alternatively, if the cell death phenotype were rescued by zygotic expression of a wild-type copy of *sctt*, then only those germ cells that successfully migrated to the gonad would continue to express Faf-β-Gal, while those cells outside of the gonad would undergo programmed cell death.

When *sctt*/*sctt* females are crossed to Ore-R males, the offspring of this cross fall into two phenotypic classes (Table 1, line 2; Figure 5). The first class displays the severe germ cell migration defect seen in the homozygous *sctt* line, and the germ cells outside the gonad continue to express Faf-β-Gal (Figure 5A). In these embryos, so few germ cells successfully migrate that the gonads cannot be easily identified in a β-Gal-stained embryo. This phenotype was observed in 60% ($n = 653$) of the embryos. In the remaining embryos, an increased number of germ cells successfully migrate to the gonad, but many cells outside of the gonad continue to express Faf-β-Gal (Figure 5B). To test the hypothesis that the less severe phenotype is the result of paternal rescue, we crossed *sctt*/*sctt* females to FM7Z/Y males. This hypothesis predicts that the more severe phenotype will be observed in *sctt*/Y embryos, while the less severe phenotype will be observed in *sctt*/FM7Z embryos. The FM7Z chromosome carries a wild-type *sctt* gene. The *sctt*/FM7Z female embryos resulting from this cross stain blue due to the presence of the *ftz-lacZ* transgene. The severe sctt phenotype was observed in 217/218 *sctt*/Y embryos (Table 1, line 3). Among the lightly stained *sctt*/FM7Z female embryos, β-Gal-stained germ cells within the gonad as well as germ cells located outside of the gonad were observed. Therefore, zygotic expression of wild-type *sctt* from the paternal X chromosome improves germ cell migration. The recessive maternal effect, the recessive zygotic phenotypes, and the zygotic rescue data collectively suggest that this *sctt* allele is loss of function.

We counted the number of germ cells that successfully migrated to the gonad in embryos that were born to *sctt*/*sctt* mothers but received a wild-type paternal copy of *sctt*. Interestingly, the same numbers of germ cells were observed within the gonads of control and paternally rescued embryos at 11–12 hr of development (Table 3). We counted an average of 13.7 germ cells within the two gonads of *faf-lacZ* embryos ($n = 50$) and 12.7 germ cells incorporated into the two gonads of *sctt*/+ rescued embryos ($n = 50$). These values are not statistically different ($P = 0.09$, Student's *t*-test). The average of 6–7 germ cells per gonad is within the range reported for other Drosophila lines (SONNENBLICK 1941, 1950; UNDERWOOD *et al.* 1980; HAY *et al.* 1988). These data support the conclusion that zygotic expression of a wild-type copy of *sctt* rescues the germ cell migration defect associated with *sctt* mutant embryos. However, an average of 7.7 germ cells was observed outside of the gonads in the rescued embryos. The range was 4–16 germ cells ectopic to the gonad, and most of the Faf-β-Gal positive cells retained a characteristic germ cell morphology (RABINOWITZ 1941; POULSON 1950; UNDERWOOD *et al.* 1980). Thus, the developmental cell death of ectopic germ cells is not rescued by zygotic expression of wild-type *sctt*. The average total number of germ cells observed is 17.7 ($n = 50$) in *sctt*/*sctt* or *sctt*/Y embryos and 20.4 in *sctt*/+ embryos ($n = 50$; Table 3). Both of these means are significantly higher than the total germ cell number of 14.0 observed in controls ($P \leq 0.0001$, Student's *t*-test).

Fertility is greatly reduced in *sctt* mutants: The sctt phenotype cosegregates with reduced fecundity. To quantitate this phenotype, we performed fertility tests. Male and female offspring of *faf-lacZ*/*faf-lacZ* control, *sctt*/FM7, and *sctt*/*sctt* females were assayed for fertility. When individual test males or females from *faf-lacZ*/*faf-lacZ* or *sctt*/FM7 mothers were crossed to Ore-R flies of the opposite sex, the fertility rate was >90% (Table 4). However, *sctt* males from *sctt*/*sctt* mutant mothers were fertile in only 33.7% of the crosses ($n = 285$).

TABLE 3

Germ cell numbers observed in *out* and *sctt* mutants

		No. of germ cells in the gonads			Total no. of germ cells		
Genotype	*N*	Range	Mean	SEM	Range	Mean	SEM
faf/faf	50	9–19	13.7	0.3	9–20	14.0	0.4
sctt/sctt	50	ND	ND	ND	7–30	17.7	0.8
sctt/+	50	6–20	12.7	0.5	10–31	20.4	0.6
out/out	50	8–21	13.0	0.3	15–32	21.6	0.6

ND, not determined.

When *sctt/sctt* females from *sctt/sctt* mutant mothers were mated to Ore-R males, 41.7% of the females produced offspring (n = 163). The remaining females failed to lay any eggs. Interestingly, *sctt/+* females from *sctt/sctt* mothers were as fertile as wild-type animals (100%, n = 135). Therefore, the zygotic rescue of the germ-cell-migration defect associated with the *sctt* mutation restores fertility to wild-type levels. This is consistent with the observation that wild-type numbers of germ cells are incorporated into the gonad of these paternally rescued embryos. The ovaries from these individuals were also normal (see below).

Agametic ovaries occur at a high frequency in *sctt* mutants: The sterility associated with the *sctt* mutation suggested a lack of germ-line stem cells within the gonad, an arrest of oogenesis, or abnormal somatic gonad development. We investigated these possibilities by analyzing ovarian development in different mutant backgrounds. An absence of germ-line stem cells or an early disruption of oogenesis would result in agametic ovaries, while abnormal somatic gonad development would lead to a lack of or malformation of somatically derived ovarian structures.

The formation of a functional gonad requires the interaction of the germ cells with the somatic gonadal mesoderm. To test whether the somatic gonad cell fate is specified in *sctt* mutant embryos, we used the 412 retrotransposon as a molecular marker for somatic gonad differentiation. The 412 retrotransposon probe labels gonadal mesoderm cells even in the absence of germ cells (Brookman 1992). The *in situ* hybridization staining pattern in *sctt* mutant embryos is indistinguishable from wild type (Figure 3E). In addition, the fact that *sctt* embryos produce viable and fertile adults, albeit at a lower frequency, demonstrates that the gonad can function if populated by germ cells.

TABLE 4

Decreased fertility is associated with the *sctt* mutation

Maternal genotype	Genotype of progeny: Female[a]	Genotype of progeny: Male[a]	*N*	% fertile
faf/faf				
		faf/Y	103	96.1
	faf/faf		123	90.2
sctt/FM7				
		sctt/Y	110	98.2
	sctt/FM7		122	98.4
	sctt/sctt		104	95.2
sctt/sctt				
		sctt/Y	285	33.7
	sctt/sctt		163	41.7
	sctt/+		135	100.0

[a] Individuals were assayed by mating to Ore-R.

The dissection of ovaries from control and *sctt* mutants reveals that the sterility associated with the *sctt* mutation is the result of a lack of germ-line stem cells or a very early arrest of oogenesis. Of the ovaries dissected from 87 offspring of *faf-lacZ/faf-lacZ* or *sctt*/FM7 females, 99% were normal (n = 174 ovaries; Table 5; Figure 6B). Two ovaries from the *sctt*/FM7 test lines were scored as rudimentary because they did not contain oocytes older than stage 9 (King 1970; Spradling 1993). In contrast, among the *sctt/sctt* female offspring of *sctt/sctt* mutant mothers, 57% of the ovaries were agametic, 11% were rudimentary, and 32% were normal (n = 122). An example of agametic ovaries from an *sctt/sctt* mutant is shown in Figure 6A. The gonadal defects correlate with fecundity. We dissected 32 females that did not lay eggs. Of these, 24 had two agametic ovaries, 6 had two rudimentary ovaries, and 2 had one agametic ovary and one rudimentary ovary. We examined an additional 29 *sctt* females that did lay eggs. All of these had at least one normal ovary and, in 10 of them, both ovaries appeared normal. Interestingly, 97% (n = 120) of the ovaries from *sctt/+* females from *sctt/sctt* mothers were wild type. Therefore, zygotic rescue of the germ cell migration defect leads to nearly normal ovary formation and restoration of wild-type fertility levels.

The *out* mutation impairs cell death of errant germ cells: An example of an out^1/out^1 mutant embryo is shown in Figure 4A. Somatic gonad development as assayed by the 412 retrotransposon is normal (data not shown). The *out* gene product is required zygotically, and the six *out* alleles result in many β-Gal-labeled germ

TABLE 5

The *sctt* mutation causes a high percentage of agametic ovaries

Maternal genotype	Zygotic genotype	No. of ovaries scored	Ovary phenotype			% agametic
			Normal	Rudimentary	Agametic	
faf/faf	*faf/faf*	66	66	0	0	0
sctt/FM7	*sctt*/FM7	108	106	2	0	0
sctt/sctt	*sctt/sctt*	122	39	14	69	57
sctt/sctt	*sctt/+*[a]	120	116	0	4	3

[a] Ore-R was used as wild type.

cells outside of the gonad. These β-Gal-positive cells can be seen outside of the gonad until at least 15 hr of development. At this time, cuticle secretion inhibits effective staining (Campos-Ortega and Hartenstein 1997). Thus, errant germ cells persist in *out* mutants and continue to express the *faf-lacZ* marker.

To determine whether this defect in cell death is linked to a migration defect, we determined the number and location of germ cells present in 11- to 12-hr embryos. The results are presented in Table 3. Control embryos had an average of 13.7 germ cells ($n = 50$) in the gonads compared to an average of 13.0 germ cells in out^1/out^1 mutants ($n = 50$). These numbers are not statistically different ($P = 0.16$, Student's *t*-test). An average of 8.6 germ cells was observed ectopic to the gonad in the mutants, compared to 0.3 β-Gal-positive germ cells outside of the gonads in controls. The range was 4–18 ectopic germ cells in the *out* mutants. The average total number of germ cells in out^1/out^1 embryos is 21.6.

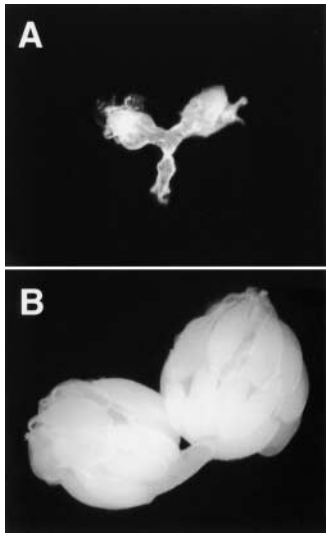

Figure 6.—The sterility associated with the *sctt* mutation is due to an absence of germ cells within the gonad. (A) Agametic ovaries from an *sctt* mutant have a normal complement of somatic gonad-derived structures, but lack germ cells. (B) Wild-type ovaries. Both photographs are taken at the same magnification.

This number is statistically different from the average of 14.0 observed in controls ($P < 0.0001$, Student's *t*-test).

As an additional test of whether the Faf-β-Gal-positive cells outside of the gonad are continuing to express the germ cell fate, we analyzed the expression of Vasa-GFP, another germ cell-specific marker (Breitwieser *et al.* 1996). The Vasa-GFP germ cell labeling pattern is indistinguishable from the Faf-β-Gal staining pattern in out^1/out^1 embryos (data not shown). In addition, the Faf-β-Gal-labeled cells are larger than neighboring cells and possess the distinct spherical nucleus typical of germ cells (Rabinowitz 1941; Poulson 1950; Underwood *et al.* 1980). Therefore, the mislocalized cells in *out/out* embryos continue to display three independent germ cell traits. These data suggest that mutations of the *out* gene disrupt the programmed cell death of the germ cells.

DISCUSSION

We report the identification of two X-linked genes, *sctt* and *out*. When mutated, *sctt* severely disrupts germ cell migration, and the germ cells ectopic to the gonad fail to undergo programmed cell death. The *sctt* mutant flies are often sterile, and the females have a high percentage of agametic ovaries. Zygotic expression of a wild-type copy of *sctt* rescues germ cell migration, but not the germ cell death defect. Thus, the *sctt* mutation disrupts at least two developmental processes: the pathfinding mechanisms that enable the germ cells to migrate to the gonad and the regulation of cell survival. In *out* mutant embryos, the same numbers of germ cells are incorporated into the gonads as in wild-type embryos. Thus, germ cell migration is normal. However, germ cells ectopic to the gonads do not undergo programmed cell death. We propose two models for *out* function. The function of *out* may be part of a cell death mechanism responsible for the elimination of germ cells ectopic to the gonad. Alternatively, a mutation in *out* may result in increased activity of a survival factor that allows germ cells ectopic to the gonad to persist.

Maternal *sctt* regulates cell migration: The sctt phenotype has several noteworthy features. First, it requires

that the mother be homozygous mutant. To date, only the products of the *nos* and *pgc-1* genes are known to be required in the germ cells for normal migration (Kobayashi *et al.* 1996; Nakamura *et al.* 1996; Forbes and Lehmann 1998). Both are maternally contributed. Mutation of *pgc-1*, an untranslatable RNA, results in decreased *nos* RNA levels after the cellular blastoderm stage. Therefore, the germ cell phenotype of *pgc-1* embryos may be the result of its effect on *nos* expression. Nos, a zinc-finger protein, is necessary to suppress transcription and mitosis in the germ cells (Kobayashi *et al.* 1996; Asaoka *et al.* 1998; Forbes and Lehmann 1998; Deshpande *et al.* 1999). Zygotic expression of *nos* does not rescue the germ cell migration defect (Forbes and Lehmann 1998). Since the germ cell migration defect of *sctt* mutants can be rescued zygotically, *sctt* may function in germ cell migration later than *nos*. In fact, the nos germ cell migration phenotype is clearly distinct from the sctt phenotype. Germ cells deprived of maternal *nos* expression exit the posterior midgut and then cluster close to the posterior midgut rudiment (Forbes and Lehmann 1998). The *sctt* mutant germ cells cross the midgut epithelium and disperse individually throughout the posterior of the embryo.

Since the germ cell migration phenotype of *sctt* mutant embryos is so severe, we favor the hypothesis that the agametic ovaries are caused by a failure of germline stem cells to populate the gonads. A similar agametic ovary phenotype has been reported for *wunen* mutants (Zhang *et al.* 1996). Paternal rescue of germ cell migration and the concomitant restoration of fertility by a wild-type copy of *sctt* are consistent with this interpretation. However, it is possible that the *sctt* mutation disrupts a later developmental event within the ovary, resulting in a degeneration of germ-line stem cells or a very early arrest of oogenesis.

It is likely that additional alleles of *sctt* were generated in our screen, but they were not successfully retained. To observe the germ cell defects resulting from a mutation of *sctt*, a homozygous mutant female must be viable and fertile. Therefore, lethal or sterile chromosomes would not have been recovered. In addition, *sctt* males are only 33.7% fertile, and females are only 41.7% fertile. Homozygous viable lines may have been lost because of a failure to reproduce. Noncomplementation screens specifically designed to isolate additional alleles of *sctt* will be required for a more thorough genetic analysis of the *sctt* locus.

Cell migration coupled to cell survival: Another interesting feature of *sctt* is that it affects both germ cell migration and germ cell survival. However, the temporal requirements for *sctt* in these two processes are different. The effect on germ cell death is not rescued by zygotic expression of a wild-type copy of the gene. In some experimental systems, migration guidance cues have been shown to be integrally linked to or to have additional functions as survival factors (Fleischman 1993; Goetzl and An 1998; Kunisada *et al.* 1998; Wakamatsu *et al.* 1998; Ashman 1999; De Felici 2000; Goetzl *et al.* 2000; Swarthout and Walling 2000; Fukushima *et al.* 2001; Kierszenbaum and Tres 2001; Miller and Kaplan 2001). For example, in avian and mouse embryos, the receptor tyrosine kinase, c-Kit, and its ligand, Stem cell factor, have been shown to act as both cell migration and anti-apoptotic factors in both neural crest and germ cell lineages (Fleischman 1993; Kunisada *et al.* 1998; Wakamatsu *et al.* 1998; Ashman 1999; De Felici 2000; Kierszenbaum and Tres 2001). In neurons, neurotrophins can act as both prosurvival and proapoptotic signals, depending upon the developmental context and receptor configuration of the receiving cell (Miller and Kaplan 2001).

Recently, Deshpande *et al.* (2001) demonstrated that ectopic expression of *hedgehog* could act as an attractive signal for Drosophila germ cells. In avian embryos, Charrier *et al.* (2001) have shown that Sonic hedgehog can act as an anti-apoptotic factor in neural tube formation. Considering the proposed role of Hedgehog as an attractive signal for germ cell migration in Drosophila, it is possible that Hedgehog may be involved in both pathfinding and cell survival in germ cell development.

The lysophospholipid class of molecules, including lysophosphatidic acid and sphingosine 1-phosphate, has been shown to mediate both survival and cell migration behaviors (Goetzl and An 1998; Goetzl *et al.* 2000; Swarthout and Walling 2000; Fukushima *et al.* 2001). In zebrafish, a sphingosine-1-phosphate receptor has been shown to be necessary for heart precursor cell migration (Kupperman *et al.* 2000). The recently identified roles of various lipid-modifying enzymes in Drosophila germ cell migration, including a phosphatidic acid phosphatase and 3-hydroxy-3-methylglutaryl coenzymeA reductase, make lipid-based signals likely candidates for germ cell guidance cues (Zhang *et al.* 1996, 1997; Van Doren *et al.* 1998; Starz-Gaiano *et al.* 2001). Overexpression of *wunen* can act at a distance to decrease germ cell number (Starz-Gaiano *et al.* 2001). Deciphering the potential role of the lysophospholipid class of signaling molecules in cell migration and/or cell survival will be a particularly interesting area of study.

It is possible that inappropriate cell division may contribute to the sctt and out cell death phenotypes. In *nos* mutant germ cells and *patched* mutant embryos, germ cells reenter the cell cycle prematurely and the number of germ cells is increased (Deshpande *et al.* 1999, 2001). Germ cells ectopic to the gonad are frequently observed in these embryos. However, the total number of germ cells we observe in *out* and *sctt* mutant embryos is less than that observed in either the *nos* or the *patched* mutant backgrounds. Other observations suggest that the extra germ cells observed in the *sctt* and *out* mutants are insufficient to override the embryo's ability to eliminate these additional cells. When a 39% increase in germ

cell number is caused by the overexpression of *osk* in 6X *osk* embryos, ectopic germ cells are not seen after gastrulation (Smith *et al.* 1992). In our *faf-lacZ* genetic background, a mutation that causes a 29% increase in the number of germ cells forming at the posterior pole does not result in an increase in germ cells ectopic to the gonads (C. R. Coffman and R. E. Boswell, unpublished results). Therefore, the Drosophila embryo appears to have some additional capacity for destroying germ cells ectopic to the gonads, and the generation of additional germ cells cannot completely account for the out and sctt phenotypes.

Programmed cell death and Drosophila germ cells: There are multiple types of programmed cell death. Programmed cell death via apoptosis and autophagy has been reported in Drosophila (Abrams *et al.* 1993; Foley and Cooley 1998; Abrams 1999; Bangs and White 2000; Lee and Baehrecke 2000, 2001). While apoptosis and autophagy have distinct morphologies, they utilize some of the same molecular machinery and therefore cannot always be distinguished using molecular markers.

Over 60 years ago, Drosophila biologists demonstrated that the number of primordial germ cells exceeds the number of germ cells incorporated into the gonads (Rabinowitz 1941; Sonnenblick 1941). These authors and others have suggested that this reduction in germ cell number is likely to occur via an active and highly regulated process (Sonnenblick 1950; Technau and Campos-Ortega 1986; Starz-Gaiano *et al.* 2001). However, a direct demonstration of the cellular mechanism or mechanisms responsible for the elimination of these germ cells has not been reported. Starz-Gaiano *et al.* (2001) make the interesting observation that forced expression of *Wrinkled* [*W*, a.k.a. *head involution defective* (*hid*)] or *reaper* (*rpr*) in germ cells results in their death. This argues that the machinery for apoptotic or autophagic cell death is present in the germ cells, as both of these programmed cell death mechanisms utilize *W/hid* or *rpr* functions (Lee and Baehrecke 2001; Thummel 2001).

High levels of p53 RNA expression are observed in Drosophila primordial germ cells. This may reflect a function for this pro-apoptotic gene in the elimination of germ cells that have experienced DNA damage. In support of this hypothesis, p53 has been shown to activate the expression of *rpr*, a cell death activator, in cells exposed to radiation-induced DNA damage (Brodsky *et al.* 2000; Ollmann *et al.* 2000). Determining whether the elimination of germ cells ectopic to the gonad utilizes the caspase-mediated pathways activated by the cell death inducers *rpr*, *grim*, *sickle*, and *W* (Abrams *et al.* 1993; Abrams 1999; Bangs and White 2000; Lee and Baehrecke 2000, 2001; Christich *et al.* 2002; Srinivasula *et al.* 2002) or by some alternative cell death pathway such as the one reported for nurse cells (Foley and Cooley 1998), will require further investigation. Future studies may also reveal roles for genes that affect the expression and/or regulation of cell death inhibitors such as *iap1/thread*, *iap2*, or *Deterin* (Abrams 1999; Vernooy *et al.* 2000). None of the known cell death genes identified to date map to the *sctt* or *out* regions.

The presence of a large number of germ cells outside of the gonads in both *sctt* and *out* mutants indicates that mutations in these genes affect the elimination of these germ cells and that the death of germ cells ectopic to the gonads occurs via an active, developmentally regulated process. Both phenotypes are recessive, and in the case of *out*, germ cells ectopic to the gonad are observed when *out* is placed over a deletion. This indicates that these *out* alleles are loss of function. However, we have not demonstrated whether apoptosis, autophagy, or some other mechanism of programmed death is disrupted in *sctt* and *out* mutants. We also cannot rule out the formal possibility that necrosis is responsible for some germ cell loss during development. It has been proposed that the lost germ cells may lack sufficient germ plasm (Sonnenblick 1950).

The germ cells of Drosophila are an excellent model for studying cell death, and there is great potential for identifying additional genes necessary for this process. Since only ~50% of the germ cells successfully reach the gonad, ~10–30 germ cells normally undergo developmental cell death in the embryo (Sonnenblick 1941, 1950; Underwood *et al.* 1980; Technau and Campos-Ortega 1986).

Mutations that affected germ cell survival occurred very frequently in our screen. There are at least two explanations for this. First, the ability to detect germ cells outside the gonad is requisite for a screen like the one reported here. If the "lost" germ cells immediately ceased expression of germ cell-specific markers, then the phenotype of a germ cell migration defective mutant would be a reduction in the number of germ cells within the gonad and might go undetected. Therefore, our screen was very effective at identifying mutations that affect germ cell death as well as germ cell migration.

Second, the mechanisms regulating germ cell survival and death are likely to have many components. Therefore, there are many potential targets for mutagenesis screens. Since very few genes have been identified with roles in Drosophila germ cell migration and death, additional screens will be required to achieve a more complete understanding of these processes. In addition, it will be interesting to examine potential cell death defects in other germ cell migration mutants identified and to investigate possible interactions between these genes and *sctt* and/or *out*. Further, it will be important to determine whether *sctt* and *out* function in the same pathway or in distinct pathways to regulate germ cell survival.

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