Programmed cell death of primordial germ cells in *Drosophila* is regulated by p53 and the Outsiders moncarboxylate transporter

Yukiko Yamada, Keri D. Davis and Clark R. Coffman*

Primordial germ cell development uses programmed cell death to remove abnormal, misplaced or excess cells. Precise control of this process is essential to maintain the continuity and integrity of the germline, and to prevent germ cells from colonizing locations other than the gonads. Through careful analyses of primordial germ cell distribution in developing *Drosophila melanogaster* embryos, we show that normal germ cell development involves extensive programmed cell death during stages 10-12 of embryogenesis. This germ cell death is mediated by *Drosophila p53* (*p53*). Mutations in *p53* result in excess primordial germ cells that are ectopic to the gonads. Initial movements of the germ cells appear normal, and wild-type numbers of germ cells populate the gonads, indicating that *p53* is required for germ cell death, but not migration. To our knowledge, this is the first report of a loss-of-function phenotype for *Drosophila p53* in a non-sensitized background. The *p53* phenotype is remarkably similar to that of *outsiders (out)* mutants. Here, we show that the *out* gene encodes a putative monocarboxylate transporter. Mutations in *p53* and *out* show nonallelic noncomplementation. Interestingly, overexpression of *p53* in primordial germ cells of *out* mutant embryos partially suppresses the out germ cell death phenotype, suggesting that *p53* functions in germ cells either downstream of *out* or in a closely linked pathway. These findings inform models in which signaling between *p53* and cellular metabolism are integrated to regulate programmed cell death decisions.

**KEY WORDS:** *p53, outsiders, Programmed cell death, Germ cells, Monocarboxylate transporter, Drosophila*

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**INTRODUCTION**

Germine precursor cells in a wide variety of animal species initially form in an extraembryonic location. To serve as the basis for the next generation, they must migrate across epithelial layers, move back into the embryo, and migrate to their target tissue, the somatic gonad precursor cells (Starz-Gaiano and Lehmann, 2001; Raz, 2004; Santos and Lehmann, 2004). Developing primordial germ cells (PGCs) share many characteristics in common with metastatic cells. These include invasive movements across epithelial cell layers, migration from their site of origin to distant target tissues, and establishing colonies at secondary locations. An essential feature of both germ cell development and metastasis is the ability to survive amidst a gauntlet of signals that would normally result in the elimination of these migrating cells through the activation of intrinsic cell death programs. The ability to thwart cell death mechanisms is a hallmark of metastatic cells and is often a major factor in tumors that are resistant to traditional cancer therapies (Jin et al., 2007; Rubinsztein et al., 2007). Many clinical pathologies result from abnormal programmed cell death (PCD). One example involving the PCD of germ cells is that over 50% of germ-line-derived tumors in children are believed to be the result of impaired PCD (Göbel et al., 2000; Schneider et al., 2001; de Silva et al., 2004; Lee, 2004; Schultz et al., 2005).

In *Drosophila melanogaster*, PGCs undergo efficient PCD during embryogenesis (Underwood et al., 1980; Technau and Campos-Ortega, 1986; Coffman et al., 2002; Coffman, 2003; Sano et al., 2005). However, the molecular machinery responsible for regulating germ cell PCD is poorly understood. Both extracellular cues and cell-autonomous determinants are thought to regulate germ cell migration and death. Maternally provided wun2 in germ cells is necessary and sufficient for germ cell survival, and overexpression of lipid phosphate phosphatases Wun or Wun2 in somatic tissues is sufficient to trigger germ cell death (Starz-Gaiano and Lehmann, 2001; Burnett and Howard, 2003; Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005). The downstream effectors of Wun/Wun2 action are not known. In wild-type, wun or wun2 mutant embryos, germ cell PCD does not require the function of the proapoptotic genes grim, reaper or head involution defective (hid). Germ cell death is not affected by the expression of the inhibitor-of-apoptosis proteins DIAP1, DIAP2 or p35, nor is it altered by the expression of a dominant negative form of the initiator caspase Nc/Dronc (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005) (Y.Y., unpublished results). Therefore, caspase-mediated apoptosis is not the predominant mechanism of PCD in *Drosophila* germ cells.

The relatively small number of PGCs produced in the *Drosophila* embryo allows the detection of subtle differences in cell death phenotypes, providing a powerful system to study the molecular mechanisms regulating these processes. In addition, the movements of PGCs through the developing embryo are well characterized. PGCs form at the posterior pole of the embryo and divide 0-2 times to produce ~30-40 cells (Rabinowitz, 1941; Sonnenblick, 1941; Underwood et al., 1980; Technau and Campos-Ortega, 1986; Hay et al., 1988; Williamson and Lehmann, 1996). Soon after these divisions, wild-type PGCs enter mitotic arrest and remain nonproliferative until the end of embryogenesis (Sonnenblick, 1941; Deshpande et al., 1999). Another feature of wild-type PGCs is that transcription is repressed. The germ cells remain transcriptionally quiescent until stages 8-9 of embryogenesis, a point just before the germ cells begin migrating (Zalokar, 1976; Van Doren et al., 1998).
Therefore, germ cell development requires both maternally and zygotically supplied gene products. Approximately 50% of PGCs initially formed successfully migrate and are incorporated into the gonads. Classic studies have shown that the remaining PGCs do not transdifferentiate but are eliminated (Sonnenblick, 1950; Underwood et al., 1980; Technau and Campos-Ortega, 1986).

In previous studies, we used a mutagenic screen to identify genes required for Drosophila germ cell development (Coffman et al., 2002). This screen isolated multiple alleles of out, a gene that, when mutated, disrupts germ cell death, but not migration. Embryos mutant for out had wild-type numbers of germ cells within the gonads plus 10-15 germ cells in ectopic locations. Ectopic germ cells were rare in wild-type embryos.

To elucidate more key components of germ cell development, we studied the literature to identify central regulators of PCD. The p53 tumor suppressor gene was of particular interest because it had demonstrated roles in multiple forms of PCD in diverse organisms. Extensive studies have shown that p53 plays pivotal roles in genome integrity and stability (reviewed by Sutherland and Brehm, 2004). Mutations in p53 are present in approximately 50% of tumors (Greenblatt et al., 1994). Remarkably, loss-of-function mutations of Drosophila p53 alone do not result in any obvious phenotypic defects. In sensitized backgrounds, p53 has been shown to be involved in DNA-damage-induced PCD and in growth arrest associated with tissue damage (Brodsky et al., 2000; Ollmann et al., 2004). Remarkably, loss-of-function mutations of p53–ns

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Here we show that PGC elimination occurs between stages 10 and 12. Loss-of-function p53 and out embryos exhibit abnormal cell death with ectopic germ cells persisting outside the gonads. Germ cell migration in p53 mutants is normal with a wild-type number of germ cells reaching the gonads. The phenotype of p53 mutants is normal with a wild-type number of germ cells within the gonads, but not migrating. Embryos mutant for out had wild-type numbers of germ cells within the gonads plus 10-15 germ cells in ectopic locations. Ectopic germ cells were rare in wild-type embryos.

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Removal of lethality through chromosomal recombination

The original out and out X chromosomes were lethal (Coffman et al., 2002) owing to second mutations on these chromosomes. Recombination was performed to remove the lethality. Heterozygous out and out females were crossed to w; w; f; c. Recombination took place in a region of 300 bp from the original out locus. The entire translated region of 3200 bp was sequenced. Sequence analyses revealed nonsense mutations in out, out', and out. These mutations were confirmed through repeated sequencing of both strands. No sequence changes that would affect protein coding of regions were observed in out, out', and out.

Reverse transcriptase PCR

To study expression of the out transcript, total RNA was isolated from 0-15 hour out, out', out', out, out, and KG07784 and wild-type embryos using Trizol (Invitrogen). RNA samples were treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contamination. First-strand cDNA synthesis was performed using AffinityScript™ QPCR cDNA Synthesis Kit (Stratagene) using an oligo (dT) primer. To detect the presence of the out cDNA in the samples, the following primers were used for PCR: 5'-gatgccagaaacagac-3' (forward) and 5'-gctccgcaagatgtaggc-3' (reverse) to amplify a 634 bp fragment spanning exons 3-4 of CG8062. As a positive control, constitutively expressed ribosomal protein 49 (rp49)-specific primers 5'-ggcagcaacagccatc-3' (forward) and 5'-ggcagcagctggtagtac-3' (reverse) were used to ensure the quality of the cDNA templates (O’Connell and Rosbash, 1984). To distinguish cDNA from genomic contamination, all primer pairs spanned introns. PCR was performed using Taq DNA polymerase (Eppendorf) using 35 cycles of DNA amplification.

RESULTS

Most PGC death occurs by stage 12 in wild-type embryos

To define the window of time in which germ cells are eliminated and to gain more insight into the mechanisms of germ cell death, we performed careful and extensive analyses of germ cell numbers during defined stages of development (Campos-Ortega and Hartenstein, 1997). Using antibodies to Vasa as a marker for germ cells, we conducted counts of PGCs during stages 10-14 (Fig. 1 and see Table S1 in the supplementary material). During stage 10, PGCs...
traversed the posterior midgut epithelium (Fig. 2A) (Warrior, 1994; Callaini et al., 1995; Jaglarz and Howard, 1995; Moore et al., 1998). They subsequently attached to overlying mesoderm. As stage 11 proceeds, the PGCs separated into two bilateral clusters and moved toward the somatic gonadal precursor cells (SGPs) (Fig. 2B). Beginning stage 12, PGCs associated with SGPs, which are specified in parasegments 10-12 (Brookman et al., 1992; Boyle and DiNardo, 1995; Boyle et al., 1997) (Fig. 2C and Fig. 3A). By stage 13, the three SGP-PGC clusters became contiguous, forming a band of cells on both sides of the embryo (Fig. 2D, Fig. 3B, Fig. 4A,B). At stage 14, PGCs and SGPs coalesced in parasegment 10 forming the early embryonic gonads (Fig. 2E and Fig. 3C).

As shown in Fig. 1 (and see Table S1 in the supplementary material), the number of Vasa-labeled germ cells in wild-type embryos dropped dramatically between stages 10 and 12. The average number of PGCs in control embryos at stage 10 was 33.7. This observation agrees with previous reports stating the numbers of germ cells present at the beginning of gastrulation, the point when the PGCs cease mitotic divisions, is in the order of 30-40 (Rabinowitz, 1941; Sonnenblick, 1941; Underwood et al., 1980; Technau and Campos-Ortega, 1986; Hay et al., 1988). Notably, the average number of PGCs at stage 11 was down to 22.6, a 33% decrease. We found that by stage 12, the average number of germ cells in the embryo was 16.6, less than 50% of the number of PGCs observed at stage 10. These observations indicate that extensive germ cell PCD took place between stages 10 and 11 as PGCs crossed the midgut epithelial layer and then transitioned into the mesoderm and moved toward the SGPs. Very little reduction in PGC numbers was observed subsequent to stage 12. We obtained similar results using a different marker for PGCs, fat facets-lacZ (Fischer-Vize et al., 1992) (data not shown).

**p53 mutants are defective in germ cell PCD**

The molecular mechanisms responsible for germ cell PCD in wild-type *Drosophila* embryos are largely unknown, but multiple observations suggested that *p53* might have a role in *Drosophila* germ cell development. First, *p53* has been shown to mediate programmed cell death in other systems (Jin, 2005; Crighton et al., 2006; Green and Chipuk, 2006). In *Drosophila*, *p53* has been shown to mediate DNA-damage-induced PCD (Jaklevic and Su, 2004; Qi et al., 2004). However, the role of *p53* in germ cell development has not been explored. In this study, we used *p53* mutants to investigate the role of *p53* in germ cell PCD.

**Fig. 1. Programmed cell death of migrating germ cells in wild-type, p53 and out mutant embryos.**

Total numbers of PGCs in embryos between stages 10-14 were determined. In wild-type embryos, germ cell death was essentially complete by stage 12. In *p53* and *out* embryos, germ cell death was disrupted. Similar numbers of PGCs were observed with a gradual loss of germ cells between stages 10 and 14. Error bars represent s.d.

**Fig. 2. Mutants initiate migration and form bilateral clusters in a similar manner to wild-type embryos.**

(A-O) Dorsal views of wild-type, *p53* and *out* mutant PGC development at stages 10-14. Anterior is left in all figures. PGCs are labeled with an anti-Vasa antibody. (A) At stage 10, wild-type PGCs moved out of the posterior midgut. (F,K) The initial PGC movements in *p53* and *out* appear normal. (B) At stage 11, bilateral segregation of PGCs occurred in the wild-type embryos. (G,L) PGCs in *p53* and *out* mutants form bilateral clusters. However, there are occasionally PGCs left in the midline. (C) During stage 12, wild-type PGCs form clusters. (H,M) Bilateral clusters are also seen in *p53* and *out* mutants. Note that some PGCs remained in the midline of some *p53* embryos. (D) At stage 13, PGCs form tightly associated linear arrays of cells. (I,N) In both *p53* and *out* embryos, most PGCs were aligned. However, isolated PGCs were observed. (E) At stage 14, PGCs in wild-type embryos coalesce with SGPs. (J,O) *p53* and *out* mutant embryos exhibit very similar phenotypes. PGCs are able to migrate to the gonads; however, many PGCs persisted ectopic to the gonads.
et al., 2004). Second, p53 RNA expression during early Drosophila embryogenesis coincides with the timing and location of PGC death. p53 maternal transcripts and zygotic expression is high in mesoderm, gut (stage 10), and PGCs (stages 10-16) (Ollmann et al., 2000; Tomancak et al., 2002).

Germ cell development was examined in three recessive loss-of-function alleles of p53: p535A-1-4, p5311-1B-1, and p53–ns (Rong et al., 2002; Sogame et al., 2003). In these p53 mutants, PGCs are not appropriately eliminated during migration across the midgut epithelium and subsequent movements towards the somatic gonad precursor cells. To determine whether these extra PGCs resulted from overproduction of germ cells, PGC counts were conducted at different time points during germ cell migration (Fig. 1 and see Table S1 in the supplementary material). At stage 10, the average number of germ cells, PGC counts were conducted at precursor cells. To determine whether these extra PGCs resulted from overproduction of germ cells, PGC counts were conducted at different time points during germ cell migration (Fig. 1 and see Table S1 in the supplementary material).

Subsequent to exiting the PMG the germ cells separate into two clusters of cells. It had been shown that the functions of wunen/wunen2 (wun/wun2) expressed in the central nervous system were necessary and sufficient to direct bilateral segregation of PGCs during stage 11 (Sano et al., 2005). We investigated whether the PGCs in p53 mutants, including those that failed to become ectopic PGCs, were able to respond to wun/wun2 guidance cues and form bilateral clusters. To address this issue, we counted PGCs that failed to respond to midline repulsive signaling and remained in the middle of the embryo (see Table S2 in the supplementary material). We found averages of 1.9 PGCs in p535A-1-4, 2.5 PGCs in p5311-1B-1 and 0.3 PGCs in p535A-1-4/p53–ns left in the midline of stage 11 embryos. These numbers were similar to the wild-type average of 1.8. Thus, PGCs in p53 mutants successfully migrated away from the midline. Counts of PGCs at the midline in stage 12 and 13 p53 embryos showed that PGCs continued to stay organized in two elongated bilateral clusters, largely avoiding the midline. Notably, between stages 11 and 13, the midline PGCs in wild-type embryos appeared to undergo PCD, the average number of midline PGCs declined from 1.8 to 0.4 between stages 11 and 12. This reduction in PGC number at the midline did not occur in p53 mutants.

Next, we examined the requirements for maternal and zygotic expression of p53. Penetrance of the mutant phenotype in homozygous mutant stocks was 93% and 96% for the p535A-1-4 and p5311-1B-1 alleles, respectively (Table 1). When p53 homozygous mutant mothers were crossed to wild-type males, 50-35% of these heterozygous (p53+/+) embryos displayed abnormal germ cell death. When heterozygous p53+/+ mothers were crossed to homozygous mutant p53/p53 males, half of the embryos were homozygous for the mutant p53. The penetrance of the mutant phenotype in these embryos was 42%. Thus although there was a maternal effect for p53, the role of zygotic expression of the gene accounted for most of the PGC phenotype.

### Table 1. p53 mutant embryos are defective in germ cell PCD

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![Fig. 3. Germ cells initiate migration, but those ectopic to the gonads fail to die.](image)

(A-I) Lateral views of wild-type, p53 and outsiders mutant PGC development at stages 12-14. Anterior is left and dorsal up in all figures. PGCs are labeled with an anti-Vasa antibody. (A,D,G) PGCS in stage 12 embryos migrate into the mesoderm. (B) At stage 13, PGCs form a band of cells and are associated with one another in wild-type embryos. (E,H) In p53 and out embryos, some PGCs form clusters, but isolation of several PGCs becomes apparent. (C) At stage 14, wild-type PGCs reach the gonads. (F,I) In the mutants, PGCs successfully migrated to the gonads whereas errant PGCs are found ectopic to the gonads.
To investigate whether p53 mutant PGCs could correctly migrate towards and associate with SGPs, we double labeled embryos with a SGP marker (anti-EYA-antibody) (Boyle et al., 1997) and a PGC marker (anti-VASA-antibody). SGPs are specified at stage 11 in bilateral clusters of 9–12 cells in parasegments 10 to 12. We found that PGCs in stage 11 p53 mutants were able to properly form clusters moving towards the SGPs (Fig. 4E,F). Also, at stage 13, double labeling for PGCs and SGPs showed alignment of PGCs with SGPs (Fig. 4G,H). However, isolation of some PGCs was also noted (Fig. 4G arrows).

PGC incorporation into the gonads was similar in wild-type and p53 mutants (Fig. 5 and see Table S3 in the supplementary material). PGC counts of stage 14 embryos showed wild-type numbers of germ cells were successfully incorporated into the gonads in p53 mutants. The average numbers of intragonadal germ cells were 18.9 in p53 mutants. In the wild type, the average was 16.5. The average numbers of germ cells ectopic to the gonads were 9.1 (p535A-1-4), 9.4 (p53H1-1B-1) and 6.5 (p535A-1-4/p53−/−), whereas the average in wild-type embryos was 0.4.

Collectively, these data support the conclusion that mutations in p53 result in survival of additional germ cells ectopic to the gonads because of impaired PCD rather than delays in the initiation of migration, an inability of the germ cells to leave the midline of the embryo or because of a failure of normal numbers of germ cells to associate with SGPs.

**out germ phenotypes are strikingly similar to p53**

In previous studies, we isolated six alleles of the out gene (Coffman et al., 2002). These preliminary studies indicated that the programmed cell death of out germ cells was disrupted. The similarities between the germ cell phenotypes of out and p53 prompted us to investigate the out phenotype in greater depth. We examined germ cell development patterns in five out alleles (out1, out2, out4, out5 and KG07784), four from our original screen plus an amorphic P-element-containing line (see below) (Crosby et al., 2007), to look for similarities and differences between out and p53 mutants.

As shown in Fig. 1 and see Table S1 in the supplementary material, the total numbers of germ cells observed in out and p53 mutants overlapped, and PGC elimination followed a parallel pattern during stages 10 to 14. As shown in Figs 2 and 3, the general features of germ cell development were the same in out and p53 embryos. Double labeling of PGCs and SGPs (Fig. 4A–D) showed that in out mutants, the somatic gonad cells formed normally, and germ cells were able to coalesce with SGPs. However, there were subtle differences. All of the out alleles had slightly more germ cells ectopic to the gonads at stage 14 than the p53 alleles (Fig. 5 and see Table S3 in the supplementary material). This may reflect differences in genetic background because we observed strain-specific differences in germ cell numbers. Interestingly, the out1, out2 and out5 alleles may represent an allelic series that reflects the severity of the amino acid truncation of the protein (see below) with out2 being the most severe. This was apparent when the number of germ cells ectopic to the gonads (Fig. 5 and see Table S3 in the supplementary material) and the number of germ cells that failed to migrate away from the midline (see Table S2 in the supplementary material) were compared.

**p53 and out interact genetically and overexpression of p53 suppresses the out phenotype**

The similarities between the p53 and out phenotypes were intriguing. This prompted us to test the hypothesis that p53 and out were involved in common PCD signaling networks. To do this, we tested for genetic interactions by creating embryos that were mutant for both p53 and out.

First, we asked whether p53 and out were required for the death of the same or different subsets of germ cells. If p53 and out function in a common PCD signaling pathway, the number of germ cells that fail to die in double mutants would be very similar to that of either p53 or out single mutants. Alternatively, if p53 and out function in separate pathways responsible for elimination of different germ cells, the number of surviving germ cells ectopic to the gonads would be greater in double mutant embryos. In order to address this possibility, out1/p535A-1-4 double mutants were assayed (Fig. 6E and see Table S3 in the supplementary material). Germ cell counts showed that the number of germ cells ectopic to the gonads was only slightly lower in out1/p53 double mutants (12.0) compared with out1 mutants (13.4). Wild-type numbers of germ cells were incorporated into the gonads: 13.2 and 14.8 in the double mutants and out1.
mutants, respectively. This evidence suggested that p53 and out functions are required for the elimination of the same subset of germ cells.

When two genes regulate common signaling pathways, transheterozygous combinations of the mutant alleles will sometimes show nonallelic noncomplementation. We reasoned that if p53 and out were part of the same PCD signaling network, p53 mutations might fail to complement out mutations. To test this, we produced transheterozygous embryos. Interestingly, when out1/ out2; +/+ females were crossed to +/Y; p535A-1-4 males, the offspring (out/-; p535A/+ and out/Y; p535+/-) were 77% and 84% mutant for out1 and out2 alleles, respectively (Table 1). When out1/ out2 or out2/ out2 females were crossed to wild-type males, 66% and 67% (out1 and out2, respectively) of the offspring displayed abnormal germ cell PCD (see Table S4 in the supplementary material). When wild-type females were crossed to +/Y; p535A-1-4/ p535A-1-4 males, only 3% of the offspring were scored as having more than three germ cells ectopic to the gonads. Therefore, the transheterozygous combination of out and p53 increased the penetrance of the PCD phenotype observed.

Finally, we examined the hypothesis that p53 functions downstream of out in signaling the death of germ cells that are ectopic to the gonads. To do this, we overexpressed p53 specifically in PGCs in out mutant embryos using the nos-Gal4:VP16 germ-cell-specific driver (Van Doren et al., 1998). The results showed that p53 expression in PGCs can partially rescue the defective germ cell PCD in out mutants (Table 2). When there was no p53 expression in PGCs, 47% of the embryos displayed the mutant phenotype. When p53 expression was driven in PGCs, 32% of the embryos had more than three germ cells ectopic to the gonads. p53 expression in wild-type embryos did not affect survival of PGCs in the gonads. These data support a model where p53 functions downstream of, or in parallel to out to induce PCD when expressed in PGCs.

Mapping of out
To gain further understanding of out- and p53-mediated germ cell PCD, we determined the molecular identity of out. In previous studies we had determined that the out mutant germ cell phenotype was uncovered by a deletion Df(1)JA27, which removed the 18A-18D region of X-chromosome (Coffman et al., 2002). To more narrowly define the region, we performed recombination mapping, resolving the location of out the 18B-C interval. With this information, we tested for non-complementation of out with P-element lines containing inserts in 18B-C (Crosby et al., 2007). Among the P-element lines tested, KG07784, a P-element insertion in 18C, failed to complement out (see Table S4 in the supplementary material). Test crosses showed that KG07784 was recessive to the wild type, and crosses between KG07784 and the out alleles resulted in over 90% mutant embryos.

The KG07784 P-element was reported to be inserted into the first intron of the gene CG8062 (Crosby et al., 2007). We confirmed the location of the P-element insertion by performing inverse PCR. Recovered flanking sequences were located in the first intron of CG8062 (Fig. 7A). These results provided preliminary evidence that disruption of CG8062 caused defective PCD of the ectopic germ cells.

Predicted molecular function of the Out protein
The CG8062 sequence is predicted to encode a protein of 655 amino acids encoding a potential monocarboxylate transporter (MCT) (Fig. 7B). Secondary structure prediction and domain analysis programs indicated 12 potential transmembrane domains.

![Fig. 6. The p53 germ cell phenotype is similar to the out; p53 double mutant. Dorsal views of the p53 mutants at stage 14 are shown. (A, B, C) p53 mutants displayed germ cells ectopic to the gonads. (D) A transheterozygous p535A-1-4/p535-70 mutation embryo is shown. (E) The out1/ p535 double mutant appears very similar to the p53 mutants. The germ cells in the double mutant are labeled with an anti-β-gal antibody.](image)

![Fig. 5. Germ cells ectopic to the gonads persist in p53 and out mutants. For each embryo examined, PGCs in the gonads (blue bars) and PGCs ectopic to the gonads (orange bars) were determined. PGC incorporation into the gonads was similar in wild-type, p52 and out embryos. In both p53 and out mutants, many ectopic PGCs were observed. In wild-type embryos, an average of 0.4 PGCs were found ectopic to the gonads. Error bars represent s.d.](image)

### Table 2. Partial rescue of out mutant phenotype through PGC expression of p53

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>n</th>
<th>% mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+; nos-GAL4/nos-GAL4*</td>
<td>W1118/Y</td>
<td>219</td>
<td>8</td>
</tr>
<tr>
<td>+/+; nos-GAL4/nos-GAL4</td>
<td>UAS-p53/UAS-p53</td>
<td>69</td>
<td>3</td>
</tr>
<tr>
<td>out1/ out2; nos-GAL4/nos-GAL4</td>
<td>W1118/Y</td>
<td>133</td>
<td>47†</td>
</tr>
<tr>
<td>out1/ out2; nos-GAL4/nos-GAL4</td>
<td>UAS-p53/UAS-p53</td>
<td>90</td>
<td>32†</td>
</tr>
</tbody>
</table>

* nos-GAL4:VP16.
† out is X-linked: 50% of offspring are out/+ and 50% are out/Y.
A

Fig. 7. Molecular identification of out. (A) A P-element insertion in CG8062 disrupts germ cell PCD. This allele fails to complement other out mutants. The P-element lies between exons 1 and 2. (B) Amino acid sequence of OUT. Three out alleles introduce premature stop codons in the CG8062 transcript. The wild-type OUT peptide comprises 655 amino acids. The positions of the stop codons are indicated by the bold underlined letters. (C) Schematic of the predicted OUT protein. Positions of the out nonsense mutations are indicated by arrows. The introduced stop codons would result in truncated proteins of 224, 276 and 310 amino acids for outA, outB and outC, respectively.

Molecular characterization of out alleles reveals premature stop codons in out1, out2 and out5

We predicted that significant out mutations should be present in CG8062 to give such a severe germ cell PCD defect. out1, out2, out3, out4, out5, KG07784 and wild-type genomic templates were PCR amplified and sequenced. The amplified 3200 bp included exons 2-5, the translated regions of the gene. We found nonsense mutations in out1, out2 and out5 (Fig. 7B,C). The predicted proteins encoded by the out5 mutants would be 224 amino acids in out1, 276 amino acids in out2, and 310 amino acids in out5. In out5, a single basepair change from G to A at a splice donor site preceding the conserved GT-intron border is predicted to significantly reduce its likelihood of being a splicing junction (Crosby et al., 2007). No significant basepair changes were found in exons 2-5 of KG07784. The P-element insertion in the intronic region of CG8062 may interfere with transcription or processing of the transcript. To detect the presence of the CG8062 transcript in KG07784, we used reverse-transcriptase PCR. The CG8062 transcript was detected in KG07784, out5 and the wild type, but was absent in KG07784 mutants (see Fig. S1A in the supplementary material). As a control, we assayed for the presence of a transcript from the ribosomal protein 49 gene (rp49) to ensure the quality of the cDNA. The rp49 transcript was detected in KG07784. We concluded that KG07784 is a transcript null allele of CG8062.

DISCUSSION

Drosophila PGC death is mediated by p53 and out

We provide evidence that p53 and out are required for the elimination of excess PGCs in the early stages of Drosophila development. Loss-of-function alleles of both genes result in PGCs that persist in ectopic locations, whereas wild-type numbers of PGCs are incorporated into the gonads. Germ cell movements are not delayed, and the PGCs appear to respond to midline repulsive signals, separating into two bilateral clusters. We conclude that p53 and out are necessary for PCD, but not for migration. Interestingly, p53 and out appear to mediate common PCD signaling networks to eliminate the same populations of ectopic germ cells. Although the molecular mechanisms are not known, our observation that overexpression of p53 in germ cells is able to suppress the effects of mutations in out suggests that p53 may be acting downstream of out. To our knowledge, this is the first report of a phenotype associated with loss-of-function alleles of Drosophila p53 in a nonsensitized background.

Roles for p53 in germ cell development and PCD

Studies in other metazoans have shown significant roles of p53 in germline development. In C. elegans, the p53 homolog cep-1 is required for proper chromosome segregation during meiosis and DNA-damage-induced germ cell death (Derry et al., 2001). In mice, p53 acts to maintain the integrity of the germ line. Mice lacking p53 exhibited reduced spontaneous germ cell death and increased levels of abnormal sperm (Beumer et al., 1998; Yiu et al., 1998). Furthermore, murine p53 has been shown to positively regulate PGC apoptosis associated with loss of Connexin 43, a gap junction component expressed in PGCs (Francis and Lo, 2006). PGCs in Connexin-43-knockout mice exhibit abnormally increased levels of activated p53 and apoptosis. This increased PGC death can be rescued by injections with a p53 inhibitor. These observations, together with our work, clearly show that p53 is an essential mediator of germ cell PCD.

Our observations indicate that PGCs in wild-type embryos undergo extensive PCD between stages 10-12 of embryogenesis. However, the mechanisms of Drosophila PGC death are not understood. Accumulating evidence suggests that Drosophila germ cell PCD is context dependent, and both apoptotic and non-apoptotic cell death can occur. Embryos homozygous for the Df (3L)H99 deletion, which removes the potent apoptotic inducers grim, rpr and hid, show normal germ cell PCD (Sano et al., 2005) (Y.Y., unpublished results). Altered expression of the caspase inhibitors p35, DIAP1, DIAP2 or a dominant negative form of Nc/Dronc did not affect Wun/Wun2-mediated PGC death (Hanyu-Nakamura et al., 2004; Renault et al., 2004). Cells dying in response to Wun/Wun2-mediated signals were negative for TUNEL staining and did not label for another marker of apoptosis, cleaved caspase 3. These observations argue that during normal development, PGC death does not occur by apoptosis. However, it is important to note that germ cells are capable of undergoing caspase-mediated PCD. Expression of hid or rpr in PGCs induces extensive PCD (Sano et al., 2005) (Y.Y., unpublished). In addition, germ cells mutant for nanos fail to maintain the germ cell fate and undergo apoptosis (Hayashi et al., 2004).
There are reports demonstrating that p53 has roles in caspase-independent modes of PCD (Feng et al., 2005; Coureuil et al., 2006; Crichton et al., 2006). For example, death of terminally differentiating murine germ cells induced by p53 overexpression is mediated by calpains rather than caspases (Coureuil et al., 2006).

There is accumulating evidence that PCD is context dependent and that cell death does not always occur by a single mechanism (Edinger and Thompson, 2004; Lockshin and Zukeri, 2004). Therefore, the possibility of crosstalk between different cell death pathways and hybrid forms of cell death need to be considered. For example, when apoptosis is blocked by caspase inhibition, mammalian neurons can still undergo PCD via autophagy (Lang-Rollin et al., 2003). This raises an intriguing possibility that PGCs use multiple cell death mechanisms. In support of this hypothesis, ectopic PGCs, which normally undergo Bax-mediated apoptosis, still undergo PCD in Bax-deficient mouse embryos suggesting that there must be Bax-independent PCD mechanisms (Stalloch et al., 2003).

**p53 expression in PGCs eliminates errant PGCs in out mutants**

Our data show that p53 genetically interacts with out, a predicted MCT, in germ cell PCD. Additionally, out and p53 show nonallelic noncomplementation suggesting that they may be involved in closely linked functions. Partial rescue of the defective germ cell PCD by overexpression of p53 in out mutants suggests that p53 may function downstream of out. This incomplete zygotic rescue may reflect the fact that p53 shows a maternal effect. Alternatively, p53 and out may function in parallel pathways. Importantly, PGC expression of p53 in wild-type embryos does not affect PGCs within the gonads. Forced expression of p53 induces PCD only in the errant PGCs in out mutants. These observations implicate potential mechanisms that distinguish between subpopulations of germ cells. Such mechanisms may involve activation of downstream antagonists of p53-mediated PCD in surviving PGCs.

The MCT family, of which out is a member, consists of eighteen predicted *Drosophila* genes (Crosby et al., 2007). Very little is known about their cellular functions. The mammalian MCT family of proteins includes 14 members, 4 of them with experimentally demonstrated functions for catalysis of the proton-linked transport of monocarboxylates (Halestrap and Meredith, 2004). It has been shown that MCTs localize at the plasma membrane and/or mitochondrial membranes, and their substrates include major factors in cellular metabolism such as lactate and pyruvate (Halestrap and Price, 1999).

Some recent reports suggest interesting possibilities for links between MCTs, cell metabolism, p53 and programmed cell death (Bensaad and Voussen, 2007; Danial et al., 2003; Feng et al., 2007). First, low nutrient levels negatively regulate mTOR and promote autophagy (Kamada et al., 2004; Lunn et al., 2005). mTOR, together with insulin-like growth factor 1, monitors levels of nutrients and mitogens to regulate cell growth and division. Downstream components of these regulators include Akt-1 kinase. Akt negatively regulates both autophagy and apoptosis (Rasoulpour et al., 2006; Quevedo et al., 2007). *Caenorhabditis elegans* homologs of Akt have been shown to suppress DNA-damage-induced germ cell death, involving cep-1, the p53 homolog (Quevedo et al., 2007). Germ cell death was significantly decreased in akt-1 gain-of-function mutants. Loss of cep-1/p53 completely blocks apoptotic hypersensitivity in akt-1 loss-of-function mutants.

Second, the functions of the SLC5A8 Na+-coupled MCT were linked to induction of pyruvate-dependent inhibition of histone deacetylases (HDAC), elevated levels of p53 and apoptosis in tumor cell cultures, suggesting pro-PCD functions of this MCT within the dying cells (Thangaraju et al., 2006). Although the exact mechanism as to how pyruvate uptake leads to cell death is unknown, the correlation with upregulated p53 expression and HDAC inhibition is intriguing. Interestingly, studies in *Drosophila* wing development show that cell death mechanisms require functions of a histone acetyltransferase (HAT). This activity is antagonized by a HDAC (Miotto et al., 2006). Additionally, p53 function is linked to histone acetylation. It has been shown that p53 is important for maintenance of histone H3 acetylation after irradiation (Rebollar et al., 2006).

*Drosophila* PGCs appear capable of undergoing multiple forms of PCD. Our demonstration that p53 is involved in some form of PGC death suggests interesting hypotheses to test. Our observations of pro-cell death roles for out, an MCT that can be suppressed by p53 overexpression, provide tantalizing clues. Clearly p53 and out are two pieces of a much larger puzzle potentially linking cell metabolism to cell death or survival signaling.


