Deciphering molecular mechanisms that regulate programmed cell death of primordial germ cells in *Drosophila melanogaster*

by

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CHAPTER 1. GENERAL INTRODUCTION

Programmed cell death (PCD) is a genetically regulated process that is central to life of multicellular organisms. It serves to shape tissues, remove transient structures, regulate sizes of cell populations, and eliminate unnecessary or harmful cells (Jacobson et al., 1997). In humans, deregulation of PCD can lead to either cell proliferative or degenerative conditions such as cancer or neurodegenerative disorders. Therefore, proper regulation of this process is essential. Molecular studies in model organisms have revealed that core components of PCD machineries are conserved from nematodes to mammals. My research project aimed to elucidate molecular mechanisms that regulate PCD using Drosophila germ cells as a model system. In this introductory chapter, I will outline the following: PCD and human disorders, Drosophila germ cells as a model system to study PCD, Drosophila germ cell development, monocarboxylate transporters, and the overview of up-coming chapters. Further background information on PCD is provided in Chapter 6, a published literature review.

PCD and human disorders

Understanding of molecular mechanisms regulating PCD is significant because many human disorders are linked to deregulation of PCD machineries. Here are some examples.

a. Cancer

   Characteristics of cancer cells include overproliferation of cells and/or decreased removal of cells (King and Cidlowski, 1998). Suppression of pro-PCD genes plays key roles in carcinogenesis and tumor progression. For instance, deficiency of pro-PCD gene, Bax, combined with inactivation of the retinoblastoma susceptibility (RB) gene promotes tumorigenesis in mice (Yin et al., 1997). Additionally, Bax is often mutated in leukemias (Kitada et al., 1998) , gastric and colorectal cancers (Ouyang et al., 1998) in humans.

b. Infertility

Functions of both pro- and anti-PCD proteins are essential for successful reproduction. Spermatogenesis in mice, for example, requires tightly regulated interaction of the pro-PCD Bax and Bad with anti-PCD Bcl-2 family proteins Bcl-xL and Bcl-w (Yan et al., 2000). Deregulation of PCD can result in infertility (Russell et al., 2002).

c. Neurodegeneration

Parkinson's disease (PD) is a progressive neurodegenerative disease. It is caused by the selective loss of dopaminergic neurons. Among several identified PD genes, DJ-1 is an anti-PCD gene that plays an important role in maintaining the dopamine neurons in humans (Bonifati et al., 2003). Loss of DJ-1 expression increases susceptibility of the neurons to stress and leads to upregulation of pro-PCD genes *p53* and Bax in Zebrafish dopaminergic neurons (Bretaud et al., 2007). Inhibition of both DJ-1 and a *p53* negative regulator, mdm2, leads to substantial loss of the neurons.

d. Immunosuppression

Human immunodeficiency virus (HIV) infection causes massive depletion of CD4(+) T cells, leading to immunosuppression. HIV infected CD4(+) T cells upregulate expression of pro-PCD genes, such as Fas ligand (Badley et al., 1996; Katsikis et al., 1997) and TRAIL (Herbeuval et al., 2005; Lum et al., 2001), resulting in excessive death of T cells.

As these examples illustrate, deregulation of PCD machineries are associated with serious medical conditions. Therefore, it is important to pursue deeper insights into molecular mechanisms that regulate PCD.

Drosophila germ cells as a model system to study PCD

Drosophila serves as a powerful model organism to dissect molecular pathways that regulate PCD. Many essential components of PCD mechanisms are conserved across the animal phyla. For example, *p53*, a tumor suppressor that is mutated in 50% of human cancers, has counterparts in animals such as mouse (Frenkel et al., 1999), Drosophila (Brodsky et al., 2000; Ollmann et al., 2000), and *C. elegans* (Derry et al., 2001). Drosophila genetics can be used to improve our understanding of complex molecular interactions in vertebrate systems. Both forward and reverse genetic approaches work efficiently to study PCD, and numerous mutations have already been investigated in great detail (Crosby et al., 2007). In addition, transparency of the embryos allows analyses of development using both fixed and living specimens.

Our laboratory uses Drosophila germ cells as a model system to study PCD. Drosophila germ cells undergo very efficient PCD during migration, reducing the cell population by half in just several hours (Coffman, 2003; Coffman et al., 2002; Sano et al., 2005; Technau, 1986; Underwood et al., 1980; Yamada et al., 2007). In addition, using germ cells as a model provides another advantage since germ cells and metastasizing cancer cells share some characteristics. Like metastasizing cancer cells, germ cells invasively migrate from their origin and proliferate to form colonies in distant tissues. Due to these shared behaviors, studies of germ cells can provide insights into metastasis. Importantly, it has been shown that 50% of human pediatric extragonadal germ cell tumors are thought to have derived from misplaced germ cells that fail to undergo PCD during embryogenesis (de Silva et al., 2004; Gobel et al., 2000; Lee, 2004; Schneider et al., 2001; Schultz et al., 2005). Mechanisms by which germ cells transform into tumors are virtually unknown. Therefore using the Drosophila germ cell system, one can start to address some important questions: What genes regulate germ cell PCD? How do they interact with one another?

Drosophila germ cell development

In Drosophila, formation of the germ cells involves assembly of the specialized RNAs and proteins, called germ plasm. The RNAs and proteins are deposited maternally and asymmetric intracellular localization is achieved during oogenesis. Oskar, Vasa, Fat-

facets are among several components of germ plasm that have been identified to have roles in germ cell development (Williamson and Lehmann, 1996). They have been shown to be important for localization, translation, and stabilization of maternal RNAs and proteins (Liu et al., 2003). During early syncytial blastoderm stage, germ cell formation is initiated within the germ plasm. They divide once or twice to produce 30-40 germ cells (stage 5) (Hay et al., 1988; Rabinowitz, 1941; Sonnenblick, 1941; Technau, 1986; Underwood et al., 1980; Williamson and Lehmann, 1996). Soon after these divisions, germ cells enter mitotic arrest and remain nonproliferative until the end of embryogenesis (Deshpande et al., 1999). Germ cells are transcriptionally quiescent until stages 9-10 of embryogenesis (Kobayashi et al., 1996). *nanos*, a germ plasm component, has been shown to be important for the transcriptional silencing in germ cells (Deshpande et al., 2005). Upon gastrulation, as the underlying posterior midgut primordium moves, germ cells are carried into the posterior midgut primordium (stages 6-9). Active migration begins at stage 10 when germ cells move through the midgut epithelium (Callaini et al., 1995; Jaglarz and Howard, 1995). Germ cells sort bilaterally away from the midline as they exit the midgut and move to the mesoderm (stage 11) (Sano et al., 2005). Germ cells form bilateral clusters and migrate toward where somatic gonadal precursors (SGPs) are formed (stage 12) (Warrior, 1994). During germ band retraction, germ cells and SGPs make contacts and start to intermingle (stage 13). They coalesce into gonads (stage 14), and by this time the total number of germ cells in the embryos is reduced by half (~ 15 germ cells) (Coffman et al., 2002; Yamada et al., 2007). Importantly, prior studies have shown that germ cells do not transdifferentiate (Underwood, et al. 1980; Technau and Campos-Ortega 1986). This was demonstrated by tracing radioactively labeled transplanted germ cell movement throughout embryogenesis. Germ cells that fail to migrate to the gonads were eliminated and very few extragonadal germ cells remained in other tissues.

It is clear from the previous observations that germ cell elimination takes place between stages 6-14. However, how germ cells are eliminated in this time frame remains virtually unknown. Some outstanding questions addressed by my research include:

1. At what stages do germ cells undergo PCD?
2. What are some of the genes involved in regulating germ cell PCD?
3. Do surrounding tissues produce signals that trigger germ cell death?

Monocarboxylate transporters

One of the main focuses of this study is the *outsiders* (*out*) gene that has been predicted to encode a monocarboxylate transporter (MCT) (Yamada et al., 2007). *out* has been shown to be involved in germ cell PCD (Coffman et al., 2002). The molecular mechanisms by which *out* mediates germ cell PCD remain to be explored. In order to gain insights into *out*-mediated germ cell PCD, it is important to understand cellular functions of MCTs. There are 18 predicted MCTs in Drosophila (Crosby et al., 2007). However, very little is documented on their functions. Studies in mammalian systems provide useful information on MCTs. Nine members of the MCT family have been identified in mammals (Halestrap and Price, 1999). It has been shown that MCTs localize at plasma membrane and mitochondrial membranes. They move metabolically important molecules such as lactate, pyruvate, and ketone bodies (Poole and Halestrap, 1993). Therefore, MCTs play major roles in cellular metabolism. Extensive studies involving MCT1, 2, and 4 have shown that each MCT has unique developmental expression patterns. MCT1 has weak expression that is global and high expression in heart and red muscle tissues (Juel and Halestrap, 1999; McCullagh et al., 1996; Pilegaard et al., 1999). MCT2 is expressed predominantly in kidney, liver, and neurons (Halestrap and Price, 1999). MCT4 is highly expressed in tumor cells and white muscle cells (Wilson et al., 1998). In addition, proton-linked lactate and pyruvate transport has been demonstrated for MCTs 1-4. MCT1 and MCT4 have also been shown to interact with an accessory protein, CD147 (Kirk et al., 2000). CD147 is an extracellular matrix metalloproteinase inducer. Importantly, increased expression of CD147 results in upregulation of MCT4. Furthermore, loss of CD147 leads to defective plasma membrane localization of MCT4 (Gallagher et al., 2007). Drosophila genome contains two genes that show amino acid sequence similarities to CD147, *roughest* and *basigin* (Crosby et al., 2007). Whether either of these gene products interacts with Out remains to be investigated. Based on the

studies in mammalian systems, interesting hypotheses can be raised to explore potential molecular functions of *out* and how it may regulate germ cell PCD.

Overview of the up-coming chapters

The following chapters provide new insights into germ cell PCD in Drosophila.

Ch. 2 The purpose was to identify when and where germ cells undergo PCD during migration. We also determined the requirement of the *p53* tumor suppressor gene for germ cell PCD and its interaction with the *outsiders* (*out*) monocarboxylate transporter gene. (PCR of *out* alleles and statistical analysis were performed by Keri Andersen)

Ch. 3 The aim was to study temporal and spatial expression of *out* during embryogenesis and to analyze tissue-specific requirements of *out*. (In situ work was performed by Keri Andersen.)

Ch. 4 The purpose was to investigate movement of ectopic germ cells in *out*. In *out* mutants, only the wild-type numbers of germ cells were incorporated into the gonads and excess germ cells failed to migrate to the gonads. Potential molecular mechanisms were examined to address this interesting observation.

Ch. 5 The aim was to elucidate involvement of other known components of cell death machineries in germ cell PCD. *p53* has been shown to mediate the two prominent cell death programs, apoptosis and autophagy. We examined whether germ cells undergo PCD via either apoptsis or autophagy.

Ch. 6 This chapter is a published literature review on DNA damage-induced PCD machineries. It discusses potential involvement of DNA damage-induced PCD machineries in germ cell development.

Ch. 7 It is a summary of some main findings of this study and discussion of future directions.

References

Badley, A. D., McElhinny, J. A., Leibson, P. J., Lynch, D. H., Alderson, M. R. and Paya, C. V. (1996). Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. *J Virol* **70**, 199-206.

Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O., Breedveld, G. J., Krieger, E., Dekker, M. C., Squitieri, F., Ibanez, P., Joosse, M. et al. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* **299**, 256-9.

Bretaud, S., Allen, C., Ingham, P. W. and Bandmann, O. (2007). p53-dependent neuronal cell death in a DJ-1-deficient zebrafish model of Parkinson's disease. *J Neurochem* **100**, 1626-35.

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M. and Abrams, J. M. (2000). Drosophila p53 binds a damage response element at the reaper locus. *Cell* **101**, 103-13.

Callaini, G., Riparbelli, M. G. and Dallai, R. (1995). Pole cell migration through the gut wall of the Drosophila embryo: analysis of cell interactions. *Dev Biol* **170**, 365-75.

Coffman, C. R. (2003). Cell migration and programmed cell death of Drosophila germ cells. *Ann N Y Acad Sci* **995**, 117-26.

Coffman, C. R., Strohm, R. C., Oakley, F. D., Yamada, Y., Przychodzin, D. and Boswell, R. E. (2002). Identification of X-linked genes required for migration and programmed cell death of Drosophila melanogaster germ cells. *Genetics* **162**, 273-84.

Crosby, M. A., Goodman, J. L., Strelets, V. B., Zhang, P. and Gelbart, W. M. (2007). FlyBase: genomes by the dozen. *Nucleic Acids Res* **35**, D486-91.

de Silva, K. S., Kanumakala, S., Grover, S. R., Chow, C. W. and Warne, G. L. (2004). Ovarian lesions in children and adolescents--an 11-year review. *J Pediatr Endocrinol Metab* **17**, 951-7.

Derry, W. B., Putzke, A. P. and Rothman, J. H. (2001). Caenorhabditis elegans p53: Role in Apoptosis, Meiosis, and Stress Resistance, vol. 294 (ed., pp. 591-595.

Deshpande, G., Calhoun, G., Jinks, T. M., Polydorides, A. D. and Schedl, P. (2005). Nanos downregulates transcription and modulates CTD phosphorylation in the soma of early Drosophila embryos. *Mech Dev* **122**, 645-57.

Deshpande, G., Calhoun, G., Yanowitz, J. L. and Schedl, P. D. (1999). Novel functions of nanos in downregulating mitosis and transcription during the development of the Drosophila germline. *Cell* **99**, 271-81.

Frenkel, J., Sherman, D., Fein, A., Schwartz, D., Almog, N., Kapon, A., Goldfinger, N. and Rotter, V. (1999). Accentuated apoptosis in normally developing p53 knockout mouse embryos following genotoxic stress. *Oncogene* **18**, 2901-7.

Gallagher, S. M., Castorino, J. J., Wang, D. and Philp, N. J. (2007). Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. *Cancer Res* **67**, 4182-9.

Gobel, U., Schneider, D. T., Calaminus, G., Haas, R. J., Schmidt, P. and Harms, D. (2000). Germ-cell tumors in childhood and adolescence. GPOH MAKEI and the MAHO study groups. *Ann Oncol* **11**, 263-71.

Halestrap, A. P. and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* **343 Pt 2**, 281-99.

Hay, B., Ackerman, L., Barbel, S., Jan, L. Y. and Jan, Y. N. (1988). Identification of a component of Drosophila polar granules. *Development* **103**, 625-40.

Herbeuval, J. P., Boasso, A., Grivel, J. C., Hardy, A. W., Anderson, S. A., Dolan, M. J., Chougnet, C., Lifson, J. D. and Shearer, G. M. (2005). TNF-related apoptosis-inducing ligand (TRAIL) in HIV-1-infected patients and its in vitro production by antigen-presenting cells. *Blood* **105**, 2458-64.

Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* **88**, 347-54.

Jaglarz, M. K. and Howard, K. R. (1995). The active migration of Drosophila primordial germ cells. *Development* **121**, 3495-503.

Juel, C. and Halestrap, A. P. (1999). Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter. *J Physiol* **517 (Pt 3)**, 633-42.

Katsikis, P. D., Garcia-Ojeda, M. E., Torres-Roca, J. F., Tijoe, I. M., Smith, C. A., Herzenberg, L. A. and Herzenberg, L. A. (1997). Interleukin-1 beta converting enzyme-like protease involvement in Fas-induced and activation-induced peripheral blood T cell apoptosis in HIV infection. TNF-related apoptosis-inducing ligand can mediate activation-induced T cell death in HIV infection. *J Exp Med* **186**, 1365-72.

King, K. L. and Cidlowski, J. A. (1998). Cell cycle regulation and apoptosis. *Annu Rev Physiol* **60**, 601-17.

Kirk, P., Wilson, M. C., Heddle, C., Brown, M. H., Barclay, A. N. and Halestrap, A. P. (2000). CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *Embo J* **19**, 3896-904.

Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M. et al. (1998). Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. *Blood* **91**, 3379-89.

Kobayashi, S., Yamada, M., Asaoka, M. and Kitamura, T. (1996). Essential role of the posterior morphogen nanos for germline development in Drosophila. *Nature* **380**, 708-11.

Lee, S. D. (2004). Epidemiological and clinical behavior of prepubertal testicular tumors in Korea. *J Urol* **172**, 674-8.

Liu, N., Dansereau, D. A. and Lasko, P. (2003). Fat facets interacts with vasa in the Drosophila pole plasm and protects it from degradation. *Curr Biol* **13**, 1905-9.

Lum, J. J., Pilon, A. A., Sanchez-Dardon, J., Phenix, B. N., Kim, J. E., Mihowich, J., Jamison, K., Hawley-Foss, N., Lynch, D. H. and Badley, A. D. (2001). Induction of cell death in human immunodeficiency virus-infected macrophages and resting memory CD4 T cells by TRAIL/Apo2l. *J Virol* **75**, 11128-36.

McCullagh, K. J., Poole, R. C., Halestrap, A. P., O'Brien, M. and Bonen, A. (1996). Role of the lactate transporter (MCT1) in skeletal muscles. *Am J Physiol* **271**, E143-50.

Ollmann, M., Young, L. M., Di Como, C. J., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W. W., Buchman, A. et al. (2000). Drosophila p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* **101**, 91-101.

Ouyang, H., Furukawa, T., Abe, T., Kato, Y. and Horii, A. (1998). The BAX gene, the promoter of apoptosis, is mutated in genetically unstable cancers of the colorectum, stomach, and endometrium. *Clin Cancer Res* **4**, 1071-4.

Pilegaard, H., Terzis, G., Halestrap, A. and Juel, C. (1999). Distribution of the lactate/H+ transporter isoforms MCT1 and MCT4 in human skeletal muscle. *Am J Physiol* **276**, E843-8.

Poole, R. C. and Halestrap, A. P. (1993). Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol* **264**, C761-82.

Rabinowitz, M. (1941). Studies on the cytology and early embryology of the egg of Drosophila melanogaster. *J Morphol* **69**, 1-49.

Russell, L. D., Chiarini-Garcia, H., Korsmeyer, S. J. and Knudson, C. M. (2002). Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. *Biol Reprod* **66**, 950-8.

Sano, H., Renault, A. D. and Lehmann, R. (2005). Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen 2. *J Cell Biol* **171**, 675-83.

Schneider, D. T., Schuster, A. E., Fritsch, M. K., Hu, J., Olson, T., Lauer, S., Gobel, U. and Perlman, E. J. (2001). Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. *Cancer Res* **61**, 7268-76.

Schultz, K. A., Sencer, S. F., Messinger, Y., Neglia, J. P. and Steiner, M. E. (2005). Pediatric ovarian tumors: a review of 67 cases. *Pediatr Blood Cancer* **44**, 167-73.

Sonnenblick, B. P. (1941). Germ Cell Movements and Sex Differentiation of the Gonads in the Drosophila Embryo. *Proc Natl Acad Sci U S A* **27**, 484-9.

Technau, G. M. a. J. A. C.-O. (1986). Lineage analysis of transplanted individual cells in embryos of Drosophila melanogaster. III. Commitment and proliferative capabilities of pole cells and midgut progenitors. *Roux's Arch. Dev. Biol.* **195**, 489-498.

Underwood, E. M., Caulton, J. H., Allis, C. D. and Mahowald, A. P. (1980). Developmental fate of pole cells in Drosophila melanogaster. *Dev Biol* **77**, 303-14.

Warrior, R. (1994). Primordial germ cell migration and the assembly of the Drosophila embryonic gonad. *Dev Biol* **166**, 180-94.

Williamson, A. and Lehmann, R. (1996). Germ cell development in Drosophila. *Annu Rev Cell Dev Biol* **12**, 365-91.

Wilson, M. C., Jackson, V. N., Heddle, C., Price, N. T., Pilegaard, H., Juel, C., Bonen, A., Montgomery, I., Hutter, O. F. and Halestrap, A. P. (1998). Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. *J Biol Chem* **273**, 15920-6.

Yamada, Y., Andersen, D. K. and Coffman, C. R. (2007). Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monocarboxylate transporter. *Development*, Accepted for publication, minor revisions pending.

Yan, W., Suominen, J., Samson, M., Jegou, B. and Toppari, J. (2000). Involvement of Bcl-2 family proteins in germ cell apoptosis during testicular development in the rat and pro-survival effect of stem cell factor on germ cells in vitro. *Mol Cell Endocrinol* **165**, 115-29.

Yin, C., Knudson, C. M., Korsmeyer, S. J. and Van Dyke, T. (1997). Bax suppresses tumorigenesis and stimulates apoptosis in vivo. *Nature* **385**, 637-40.

CHAPTER 2. PROGRAMMED CELL DEATH OF PRIMORDIAL GERM CELLS IN DROSOPHILA IS REGULATED BY P53 AND THE OUTSIDERS MONOCARBOXYLATE TRANSPORTER

(Accepted by Development, pending minor revisions)

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Summary

Primordial germ cell development utilizes 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. 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. We have cloned *out* and identified it as a gene encoding 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 downstream of *out*. We discuss possible links between monocarboxylate transporters and *p53* in mediating programmed cell death.

Introduction

Germline precursor cells in a wide variety of animal species initially form in an extraembryonic location. In order 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 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, 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 Wun or Wun2, lipid phosphate phosphatases, 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 functions of the proapopotic 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) (Chapter 5). 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 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 up to two times to produce ~30-40 cells (Rabinowitz, 1941; Sonnenblick, 1941; Underwood et al., 1980; Technau, 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, rather, they are eliminated (Sonnenblick, 1950; Underwood et al., 1980; Technau, 1986).

In prior studies, we employed a mutagenic screen to identify genes required for Drosophila germ cell development. This screen isolated multiple alleles of *out*, a gene that, when mutated, disrupts germ cell death, but not migration. Embryos that were 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 (Coffman et al., 2002).

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 in (Sutcliffe 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., 2000; Rong et al., 2002; Sogame et al., 2003; Brodsky et al., 2004; Jaklevic and Su, 2004; Wells et al., 2006). *In situ* data of *p53* transcripts reveal global maternal expression followed by zygotic expression in the PGCs and hindgut cells about half way through embryogenesis (Ollmann et al., 2000; Tomancak et al., 2002). The extensive roles of *p53* in programmed cell death and development along with its germ cell expression pattern prompted us to investigate potential roles for *p53* in PGC death.

Here we show that PGC elimination occurs between stages 10 and 12. Loss-of-function *p53* 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 remarkably similar to *out*. We have identified *out* as a gene encoding a putative monocarboxylate transporter. Genetic analyses suggest that *p53* and *out* may function in a common pathway to eliminate a subset of PGCs during embryogenesis. We discuss possible PCD mechanisms that are mediated by *p53* and its potential interactions with *out* during PGC development.

Materials & Methods

Fly stocks and breeding conditions

Flies were maintained on standard media at 25 °C. The out^{1}, out^{2}, out^{4}, and out^{5} alleles were generated in an EMS mutagenesis screen described in a previous publication (Coffman et al., 2002). For a wild-type control, we used w^{1118}, P{w^{+}, *fat facets-lacZ*}, the parental strain used in the mutagenesis (Fischer-Vize et al., 1992). The KG07784 strain was generated in the Berkeley Drosophila Genome Project (Crosby et al., 2007). The *p53* alleles assayed were $p53^{5A\text{-}1\text{-}4}$and $p53^{11\text{-}1B\text{-}1}$ (Rong et al., 2002). The following transgenic

lines were used: P{GAL4::VP16-nos.UTR} (Van Doren et al., 1998) and P{UAS-p53} (Ollmann et al., 2000).

Immunocytochemistry

Immunostaining was performed following established methods (Johansen, 2003). The embryos were fixed in a 4 % paraformaldehyde fixative. The following primary antibodies were used for immunostaining of embryos: Chicken anti-Vasa (a gift from K. Howard, 1/10,000), mouse anti-ß-galactosidase (40-1a Developmental Studies Hybridoma Bank, 1/50), mouse-anti-clift (Eya10H6 Developmental Studies Hybridoma Bank, 1/25) (Bonini et al., 1993). The secondary antibodies used were as follows: biotinylated anti-mouse IgG; biotinylated anti-chicken, Alexa Fluor 488–conjugated goat anti–mouse (Invitrogen,1/500) and Alexa Fluor 568–conjugated goat anti–chicken antibodies (Invitrogen,1/500). The ABC Elite Kit (Vector Labs) was applied to complex the biotinylated secondary antibodies with avidin-conjugated to horseradish peroxidase. Peroxidase activity was visualized using diaminobenzidine as a substrate.

Germ cell counts

Germ cells were labeled using an anti-Vasa antibody. Germ cells were counted using differential interference contrast microscopy. Staging of embryos was done based on morphological criteria (Campos-Ortega and Hartensein, 1997). For bilateral segregation assays, PGCs were scored as middle cells when the cells remained close to the midline while other PGCs had moved laterally forming bilateral clusters. For stage 14 embryos, the gonadal sheath cells were used to determine whether germ cells were inside or outside of the gonads. Our criterion for a mutant phenotype in stage 14 embryos was more than three germ cells ectopic to the gonads.

Sequencing

out^{1}, *out*^{2}, *out*^{4}, *out*^{5}, KG07784^{a}, and wild-type (*w*^{1118}, P{*w*$^{+}$, *fat facets-lacZ*}) genomic templates were PCR amplified using the TripleMaster Taq system (Eppendorf). The following primers were used to amplify exons 2-5 of CG8062: 5'-

caagttggtatatgggctcacc-3' (forward) and 5'-caagccctcgaatttctgg-3' (reverse). We sequenced the entire translated region of 3200 basepairs in *out* mutants and wild type. Sequence analyses revealed nonsense mutations in out^1, out^2, and out^5. These mutations were confirmed through repeated sequencing of both strands. No sequence changes that would affect protein coding of regions were observed in out^4 and KG07784.

Removal of lethality through chromosomal recombination

The original out^4 and out^5 X-chromosomes were lethal (Coffman et al., 2002), due to second mutations on these chromosomes from the EMS mutagenesis. Recombination was performed to separate the lethality away from the *out* mutations. out^4 and out^5 heterozygous females were crossed to w^{1118} cv^1 wy^{74i} f^1/Y males. Recombination events were allowed to take place in F1 (w^{1118} cv^+ wy^+ *out-* f^+ P{w^+, *fat facets-lacZ*} /w^{1118} cv^1 wy^{74i} f^1) females. These females were then crossed to FM7Z/Y males. Subsequently, viable P{w^+, *fat facets-lacZ*} / Y males were collected. Stocks were established using these recombined chromosomes and tested for retention of the *out* mutations.

Reverse transcriptase polymerase chain reaction

To study expression of the *out* transcript, out^1, out^2, out^4, out^5, KG07784[a], and wild-type flies (w^{1118}, P{w^+, *fat facets-lacZ*}) were assayed. Total RNA was isolated from 0-15 hour embryos using Trizol (Invitrogen). All RNA samples were treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contamination. First-strand cDNA synthesis was performed on the DNase-treated RNA using AffinityScript™ QPCR cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions using an oligo(dT) primer. To detect presence of the *out* cDNA in the samples, the following primers were used for PCR: 5'-gatgccaagcaaaccacg-3' (forward) and 5'-gcctccgtcaagataccaag-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 were also used to ensure the quality of the cDNA templates (O'Connell and Rosbash, 1984). The following rp49 primers were used: 5'-gcgcaccaagcacttcatc-3' (forward) and 5'-gacgcactctgttgtcgatacc-3' (reverse). To distinguish cDNA amplified

product and genomic contamination, we used primer pairs that spanned introns. All PCR was performed using Taq DNA polymerase (Eppendorf) using 35 cycles of DNA amplification.

Results

In wild-type embryos most primordial germ cell death occurs by stage 12

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 Hartensein, 1997). Using antibodies to Vasa as a marker for germ cells, we conducted counts of PGCs during stages 10-14 (Fig. 2.1 and Supplemental Table 2.1). During stage 10, PGCs traverse the posterior midgut epithelium (Fig. 2.2, A) (Warrior, 1994; Callaini et al., 1995; Jaglarz and Howard, 1995; Moore et al., 1998). They subsequently attach to overlying mesoderm. As stage 11 proceeds, the PGCs separate into two bilateral clusters and move toward the somatic gonadal precursor cells (SGPs) (Fig. 2.2, B). Beginning stage 12, PGCs associate with SGPs, which are specified in parasegments 10-12 (Brookman et al., 1992; Boyle and DiNardo, 1995; Boyle et al., 1997) (Fig 2.2, C, Fig. 2.3, A). By stage 13, the three SGP/PGC clusters become contiguous, forming a band of cells on both sides of the embryo (Fig. 2.2, D, Fig. 2.3, B). At stage 14, PGCs and SGPs coalesce in parasegment 10 to form the early embryonic gonads (Fig. 2.2, E, Fig. 2.3, C).

As shown in figure 2.1 (and Supplemental Table 2.1), the number of Vasa-labeled germ cells in wild-type embryos drops 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, a point when the PGCs cease mitotic divisions, is on the order of 30-40 (Rabinowitz, 1941; Sonnenblick, 1941; Underwood et al., 1980; Technau, 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. An additional 18% of PGCs were eliminated between stages 11 and 12, and very little reduction in PGC numbers was observed subsequent to stage 12. To independently assay germ cell numbers, we used a different marker for PGCs, *fat facets-lacZ* (Fischer-Vize et al., 1992). We obtained nearly identical results in these parallel experiments (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 a variety of 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). 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 two recessive loss-of-function alleles of *p53*, $p53^{5A\text{-}1\text{-}4}$ and $p53^{11\text{-}1B\text{-}1}$ (Rong et al., 2002). 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 result from overproduction of germ cells, PGC counts were conducted at different time points during germ cell migration (Fig. 2.1 and Supplemental Table 2.1). At stage 10, the average total number of PGCs in the wild-type embryos was 33.7. This number was not significantly different from the average of 35.0 and 36.0 observed in $p53^{5A\text{-}1\text{-}4}$ in $p53^{11\text{-}1B\text{-}1}$ respectively. During stage 11, an average of 1 or 2 PGCs were eliminated in *p53* mutants. This was significantly different from what was observed in the wild type where an average of 11 germ cells was eliminated ($P < 0.0001$, Student's t-test). Over the

course of PGC migration, a gradual reduction of PGCs occurred in *p53* mutants. By stage 14, the average total numbers of PGCs in the embryo were 27.8 in *p53* $^{5A\text{-}1\text{-}4}$ and 28.0 in *p53*$^{11\text{-}1B\text{-}1}$. The wild-type average was 17.0. Therefore, the initial numbers of germ cells were the same in wild-type and *p53* mutants, and since the total number of germ cells slowly decreased over time, it seems unlikely that the additional PGCs observed were due to premature resumption of mitoses.

Next, we examined the requirements for maternally-contributed and embryonically expressed *p53* during germ cell development. We determined that there were both maternal and zygotic requirements for *p53* in the germ cells. Penetrance of the mutant phenotype in homozygous mutant stocks was 93% and 96% for the *p53* $^{5A\text{-}1\text{-}4}$ and *p53*$^{11\text{-}1B\text{-}1}$ alleles respectively (Table 2.1). When *p53* homozygous mutant mothers were crossed to wild-type males, 30-35% of these heterozygous (*p53*/+) embryos display abnormal germ cell death. When heterozygous mothers were crossed to homozygous mutant males, half of the embryos were homozygous for the mutant *p53*. The penetrance of the mutant phenotype was 42% in these embryos. Thus although there was maternal effect for *p53*, the role of zygotic expression of the gene accounted for most of the PGC phenotype.

Initial phases of PGC migration are not disrupted in *p53* mutants

We observed PGCs ectopic to the gonads at stage 14 (Fig. 2.2 compare E and J, and Fig. 2.3 compare C and F). We considered several models to explain the presence of these PGCs ectopic to the gonads of *p53* mutants. First, it could represent a defect in the ability of germ cells to cross the posterior midgut epithelium and transition into the surrounding mesoderm as in *tre1* and *slam* mutants (Kunwar et al., 2003; Stein et al., 2002). Second, *p53* mutant germ cells might fail to respond to midline repulsive signals such as those mediated by *wun* and *wun2* (Sano et al., 2005). Third, the PGCs in *p53* mutants might not successfully coalesce with somatic gonad precursor cells. Fourth, the *p53*-defective PGCs may not respond to death signals that eliminate errant germ cells in wild-type animals. We examined each of these models.

Because the first indication of defective germ cell PCD was noted at stage 11, we inspected where PGCs are positioned at this stage. A failure of germ cells to exit the posterior midgut and transition to the mesoderm results in a phenotype where the germ cells remain associated with the endoderm. Analyses of PGC locations during stages 11 and 12 showed that *p53* mutant germ cells had exited the PMG (Fig. 2.2, G, Fig. 2.3, D).

Subsequent to exiting the PMG the germ cells separate into two clusters of cells, one on each side of the embryo. 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*, including those fated 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 (Table 2.2). We found averages of 1.9 PGCs in $p53^{5A\text{-}1\text{-}4}$ and 2.5 PGCs in $p53^{11\text{-}1B\text{-}1}$ left in the midline of stage 11 embryos. These numbers were similar to the wild-type average of 1.8 PGCs. Thus, PGCs in *p53* mutants successfully migrated away from the midline and were able to form bilateral clusters. They appeared to respond initially to the midline repulsive signaling. 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.

To investigate whether *p53* mutant PGCs could correctly migrate towards and associate with somatic gonad precursor cells (SGPs), we double-labeled the 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 through 12. We found that PGCs in stage 11 *p53* mutants were able to properly form clusters moving towards the SGPs (Fig. 2.4, A, B). Also, at stage 13, double labeling for PGCs and SGPs showed an alignment of PGCs with SGPs (Fig. 2.4, C, D). However, isolation of some PGCs was also noted (Fig. 2.4, C arrows).

PGC incorporation into the gonads was similar in both wild type and *p53* mutants (Fig. 2.5 and supplemental table 2.2). PGC counts of stage 14 embryos showed that wild-type numbers of germ cells were successfully incorporated into the gonads in *p53* mutants. The average numbers of intragonadal germ cells were 18.7 in *p53* mutants. In wild type, the average was 16.5. The average numbers of germ cells ectopic to the gonads were 9.1 in $p53^{5A\text{-}1\text{-}4}$ and 9.4 in $p53^{11\text{-}1B\text{-}1}$ while 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 due to impaired PCD and not due to delays in the initiation of migration, an inability of the germ cells to leave the midline of the embryo, or by a failure of normal numbers of germ cells to associate with SGPs.

out germ phenotypes are strikingly similar to _p53_

In previous studies, we performed forward genetic screens for mutations affecting germ cell development. We isolated six alleles of the *out* gene (Coffman et al., 2002). These preliminary studies indicated that germ cell migration in *out* mutants was normal, but the programmed cell death of 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 (out^{1}, out^{2}, out^{4}, out^{5}, and KG07784), four from our original screen plus an amorphic P-element-containing line (see below) from the Berkeley Drosophila Genome Disruption Project (Crosby et al., 2007) to look for similarities and differences between *out* and *p53* mutants.

As shown in figure 2.1 and supplemental table 2.1, 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 figures 2.2 and 2.3, the general features of germ cell development were the same in *out* and *p53* embryos. Double labeling of PGCs and SGPs (Fig. 2.6) showed that in *out* mutants, the somatic gonad cells formed normally and that 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. 2.5 and Supplemental Table 2.2). This may reflect differences in genetic background as we observed strain-specific differences in germ cell numbers. Interestingly, the *out* ^{1}, *out* ^{4}, and *out* ^{5} alleles may represent an allelic series that reflects the severity of the amino acid truncation of the protein (see below) with *out* ^{2} being the most severe. This was apparent when the number of germ cells ectopic to the gonads (Fig. 2.2) and the number of germ cells that failed to migrate away from the midline (Table 2.2) 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. 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 similar to that of *out* mutants. Alternatively, if *p53* and *out* function in separate pathways responsible for elimination of different subsets of germ cells, the number of surviving germ cells ectopic to the gonads would be greater in double mutant embryos than either *out* or *p53* alone. In order to address this possibility, *out*^{1}; *p53* $^{\underline{5A\text{-}1\text{-}4}}$ double mutants were assayed (Fig. 2.7, C), and germ cell counts were performed (Supplemental Table 2.2). Germ cell counts of double mutant embryos showed that the number of germ cells ectopic to the gonads was only slightly fewer in *out*^{1} ; *p53* double mutants,12.0, than in the *out*^{1} mutants, 13.4. Wild-type numbers of germ cells were incorporated into the gonads, 13.2 and 14.8 in the double mutants and *out*^{1} respectively. This evidence suggested that *p53* and *out* functions were required for the elimination of the same subset of germ cells.

When two genes regulate the same signaling pathways, transheterozygous combinations of the mutant alleles will sometimes show nonallelic noncomplementation. We reasoned that if *p53* and *out* were part of the some PCD signaling network, *p53* mutations might fail to complement *out* mutations in some contexts. To test this, we

produced transheterozygous embryos. Interestingly, when *out* / *out* ; + / + females were crossed to + / Y ; *p53 / p53* males, the offspring (*out* / + ; *p53* / + and *out*/Y ; *p53* / +) were 77% mutant for out^{1} and 84% mutant for out^{2} (Table 2.1). When out^{1} / out^{1} or out^{2} / out^{2} females were crossed to wild-type males, 66% and 67% (out^{1} and out^{2} respectively) of the offspring displayed abnormal germ cell PCD (Table 2.3). When wild-type females were crossed to +/Y ; $p53^{5A\text{-}1\text{-}4}$ / $p53^{5A\text{-}1\text{-}4}$ males, only 3% of the offspring were scored as having more than 3 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 whether *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 2.4). 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 3 germ cells ectopic to the gonads. *p53* expression wild-type embryos did not affect survival of PGCs in the gonads. These data demonstrate that *p53* can function downstream of, or 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 identification 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). In order to more narrowly define the region, we performed recombination mapping. The mapping data indicated that *out* resided in a small region within 18B-C. With this information, we tested for non-complementation of *out* with P-element lines containing inserts in 18B-C generated by the Berkeley Drosophila Genome Project (BDGP) (Crosby et al., 2007). Among the P-element lines tested, KG07784, a P-element insertion in 18C, failed to

complement *out* (Table 2.3). Test crosses showed that KG07784 was recessive to wild type, and crosses between KG07784 and the *out* alleles resulted in over 90% mutant embryos. KG07784 homozygous embryos showed high penetrance of the mutant germ cell phenotype.

The KG07784 P-element was reported to be inserted into the first intron of the gene CG8062 (Crosby et al., 2007). To confirm the location of the P-element insertion, we performed inverse PCR on the KG07784 flies. Recovered flanking sequences were found to be in the first intron of CG8062 (Fig. 2.8). Together, 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 containing potential transmembrane domains and conserved domains for monocarboxylate transporters (Fig. 2.9). Secondary structure prediction and domain analysis programs indicated 12 potential transmembrane domains, a sugar transporter domain, and a carbohydrate transporter domain (Fig. 2.9, B) (Krogh et al., 2001; Juretic et al., 2002; Marchler-Bauer and Bryant, 2004). The Drosophila genome contains 18 genes predicted to encode monocarboxylate transporters (Crosby et al., 2007), but little is known about their molecular or developmental functions. Studies in other organisms have shown that monocarboxylate transporters localize in the plasma membrane and/or mitochondrial membrane, allowing trafficking of molecules such as lactate, pyruvate, and protons, major factors in cell metabolism (Halestrap and Price, 1999; Enerson and Drewes, 2003; Izumi et al., 2003; Halestrap and Meredith, 2004; Pierre and Pellerin, 2005).

Molecular characterization of *out* alleles reveals premature stop codons in *out*^{1}, *out*^{2}, and *out*^{5}

We predicted that significant *out* mutations should be present in CG8062 to give such a severe germ cell PCD defect. To look for mutations, *out*^{1}, *out*^{2}, *out*^{4}, *out*^{5},

KG07784, and wild-type genomic templates were PCR amplified and sequenced. The amplified 3200 basepairs included exons 2-5, the translated regions of the gene. We found nonsense mutations in *out*^{1}, *out*^{2}, and *out*^{5} (Fig. 2.9). The wild-type *out* peptide is 655 amino acids in length. The predicted proteins encoded by the *out* mutants would be 224 amino acids in *out*^{2}, 276 amino acids in *out*^{1}, and 310 amino acids in *out*^{5}. In *out*^{4}, a single basepair change from G to A at a donor site preceding conserved GT-intron 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 CG8062 transcript in KG07784, we employed reverse-transcriptase PCR. The CG8062 transcript was detected in *out*^{1}, *out*^{2}, *out*^{5}, and the wild-type, but was absent in KG07784 (Fig. 2.10). As a control, we assayed for 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*

Here 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, while wild-type numbers of PGCs are successfully incorporated into the gonads. The 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. 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., 2001b). In mice, *p53* performs similar functions 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; Yin 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 during development of these animals.

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 machineries exist. Embryos homozygous for the Df(3L)H99 deletion, which removes the potent apoptotic inducers *grim*, *rpr*, and *hid*, show normal PCD of germ cells (Sano et al., 2005 and Yamada unpublished). Altered expression of the caspase inhibitors p35, DIAP1, DIAP2, or a dominant negative form of 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. Collectively, 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 and Yamada

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 programmed cell death (Feng et al., 2005; Coureuil et al., 2006; Crighton 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 modes of cell death are context dependent and that cell death does not always occur by a single mechanism (Edinger and Thompson, 2004; Lockshin and Zakeri, 2004). Rather, the possibility of cross talk 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 utilize multiple cell death machineries for elimination of unwanted cells. 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 present (Stallock et al., 2003).

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

Our data show that *p53* genetically interacts with *out*, a predicted monocarboxylate transporter (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.* Alternatively, *p53* and *out* may function in parallel pathways to promote the death of the same subset of PGCs. Importantly, PGC expression of *p53* in the wild-type embryos does not induce massive PGC death. Forced expression of *p53* induces PCD only in the errant PGCs in *out* mutants. These observations implicate potential mechanisms that keep some PGCs alive to occupy the gonads. Such mechanisms may involve activation of downstream antagonists of *p53*-mediated PCD in surviving PGCs.

out is a member of the MCT family that consists of eighteen predicted Drosophila MCT genes (Crosby et al., 2007). Very little is known about their cellular functions. Studies in mammals offer some interesting insights. 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, p53, and programmed cell death (Danial et al., 2003; Feng et al., 2007). First, low nutrient levels negatively regulate mTOR and promote autophagy (Kamada et al., 2004; Lum 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). *C. 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 *akt-1* gain-of-function mutants. Importantly, loss of *cep-1*/*p53* completely blocks apoptotic hypersensitivity in *akt-1* loss-of-function mutants.

Second, the functions of a Na(+)-coupled monocarboxylate transporter, SLC5A8, 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). Though the exact mechanisms as to how pyruvate uptake leads to cell death is yet unknown, the correlation to upregulated *p53* expression and HDAC inhibition is intriguing. Interestingly, studies in Drosophila wing development show evidence for apoptosis regulated through histone modifications. In the wing, the developmental cell death mechanisms require functions of a histone aceltransferase (HAT) and 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 both apoptotic and non-apoptotic cell death. Our demonstration that *p53* is involved in with some form of PGC death suggests interesting hypotheses to test in the future. Our observations of pro-cell death roles for *out*, a 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/survival signaling.

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References

Beumer, T. L., Roepers-Gajadien, H. L., Gademan, I. S., van Buul, P. P., Gil-Gomez, G., Rutgers, D. H. and de Rooij, D. G. (1998). The role of the tumor suppressor p53 in spermatogenesis. *Cell death and differentiation* **5**, 669-677.

Bonini, N. M., Leiserson, W. M. and Benzer, S. (1993). The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. *Cell* **72**, 379-395.

Boyle, M. and DiNardo, S. (1995). Specification, migration and assembly of the somatic cells of the Drosophila gonad. *Development* **121**, 1815-1825.

Boyle, M., Bonini, N. and DiNardo, S. (1997). Expression and function of clift in the development of somatic gonadal precursors within the Drosophila mesoderm. *Development* **124**, 971-982.

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M. and Abrams, J. M. (2000). Drosophila p53 binds a damage response element at the reaper locus. *Cell* **101**, 103-113.

Brodsky, M. H., Weinert, B. T., Tsang, G., Rong, Y. S., McGinnis, N. M., Golic, K. G., Rio, D. C. and Rubin, G. M. (2004). Drosophila melanogaster MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. *Molecular and cellular biology* **24**, 1219-1231.

Brookman, J. J., Toosy, A. T., Shashidhara, L. S. and White, R. A. (1992). The 412 retrotransposon and the development of gonadal mesoderm in Drosophila. *Development* **116**, 1185-1192.

Burnett, C. and Howard, K. (2003). Fly and mammalian lipid phosphate phosphatase isoforms differ in activity both in vitro and in vivo. *EMBO reports* **4**, 793-799.

Callaini, G., Riparbelli, M. G. and Dallai, R. (1995). Pole cell migration through the gut wall of the Drosophila embryo: analysis of cell interactions. *Dev Biol* **170**, 365-375.

Campos-Ortega, A., J. and Hartensein, V. (1997). The Embryonic Development of Drosophila melanogaster. New York: Springer-Verlag.

Coffman, C. R. (2003). Cell migration and programmed cell death of Drosophila germ cells. *Ann N Y Acad Sci* **995**, 117-126.

Coffman, C. R., Strohm, R. C., Oakley, F. D., Yamada, Y., Przychodzin, D. and Boswell, R. E. (2002). Identification of X-linked genes required for migration and programmed cell death of Drosophila melanogaster germ cells. *Genetics* **162**, 273-284.

Coureuil, M., Fouchet, P., Prat, M., Letallec, B., Barroca, V., Dos Santos, C., Racine, C. and Allemand, I. (2006). Caspase-independent death of meiotic and postmeiotic cells overexpressing p53: calpain involvement. *Cell death and differentiation* **13**, 1927-1937.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P. R., Gasco, M., Garrone, O., Crook, T. and Ryan, K. M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* **126**, 121-134.

Crosby, M. A., Goodman, J. L., Strelets, V. B., Zhang, P. and Gelbart, W. M. (2007). FlyBase: genomes by the dozen. *Nucleic Acids Res* **35**, D486-491.

Danial, N. N., Gramm, C. F., Scorrano, L., Zhang, C. Y., Krauss, S., Ranger, A. M., Datta, S. R., Greenberg, M. E., Licklider, L. J., Lowell, B. B. et al. (2003). BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature* **424**, 952-956.

de Silva, K. S., Kanumakala, S., Grover, S. R., Chow, C. W. and Warne, G. L. (2004). Ovarian lesions in children and adolescents--an 11-year review. *J Pediatr Endocrinol Metab* **17**, 951-957.

Derry, W. B., Putzke, A. P. and Rothman, J. H. (2001). Caenorhabditis elegans p53: Role in Apoptosis, Meiosis, and Stress Resistance, vol. 294, pp. 591-595.

Deshpande, G., Calhoun, G., Yanowitz, J. L. and Schedl, P. D. (1999). Novel functions of nanos in downregulating mitosis and transcription during the development of the Drosophila germline. *Cell* **99**, 271-281.

Edinger, A. L. and Thompson, C. B. (2004). Death by design: apoptosis, necrosis and autophagy. *Current opinion in cell biology* **16**, 663-669.

Enerson, B. E. and Drewes, L. R. (2003). Molecular features, regulation, and function of monocarboxylate transporters: implications for drug delivery. *Journal of pharmaceutical sciences* **92**, 1531-1544.

Feng, Z., Zhang, H., Levine, A. J. and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8204-8209.

Feng, Z., Hu, W., de Stanchina, E., Teresky, A. K., Jin, S., Lowe, S. and Levine, A. J. (2007). The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer research* **67**, 3043-3053.

Fischer-Vize, J. A., Rubin, G. M. and Lehmann, R. (1992). The fat facets gene is required for Drosophila eye and embryo development. *Development* **116**, 985-1000.

Francis, R. J. and Lo, C. W. (2006). Primordial germ cell deficiency in the connexin 43 knockout mouse arises from apoptosis associated with abnormal p53 activation. *Development* **133**, 3451-3460.

Göbel, U., Schneider, D. T., Calaminus, G., Haas, R. J., Schmidt, P. and Harms, D. (2000). Germ-cell tumors in childhood and adolescence. . *Ann Oncol* **11**, 263-271.

Green, D. R. and Chipuk, J. E. (2006). p53 and metabolism: Inside the TIGAR. *Cell* **126**, 30-32.

Greenblatt, M. S., Bennett, W. P., Hollstein, M. and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer research* **54**, 4855-4878.

Halestrap, A. P. and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *The Biochemical journal* **343 Pt 2**, 281-299.

Halestrap, A. P. and Meredith, D. (2004). The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch* **447**, 619-628.

Hanyu-Nakamura, K., Kobayashi, S. and Nakamura, A. (2004). Germ cell-autonomous Wunen2 is required for germline development in Drosophila embryos. *Development* **131**, 4545-4553.

Hay, B., Ackerman, L., Barbel, S., Jan, L. Y. and Jan, Y. N. (1988). Identification of a component of Drosophila polar granules. *Development* **103**, 625-640.

Hayashi, Y., Hayashi, M. and Kobayashi, S. (2004). Nanos suppresses somatic cell fate in Drosophila germ line. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 10338-10342.

Izumi, H., Torigoe, T., Ishiguchi, H., Uramoto, H., Yoshida, Y., Tanabe, M., Ise, T., Murakami, T., Yoshida, T., Nomoto, M. et al. (2003). Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. *Cancer treatment reviews* **29**, 541-549.

Jaglarz, M. K. and Howard, K. R. (1995). The active migration of Drosophila primordial germ cells. *Development* **121**, 3495-3503.

Jaklevic, B. R. and Su, T. T. (2004). Relative contribution of DNA repair, cell cycle checkpoints, and cell death to survival after DNA damage in Drosophila larvae. *Curr Biol* **14**, 23-32.

Jin, S. (2005). p53, Autophagy and tumor suppression. *Autophagy* **1**, 171-173.

Jin, S., DiPaola, R. S., Mathew, R. and White, E. (2007). Metabolic catastrophe as a means to cancer cell death. *Journal of cell science* **120**, 379-383.

Johansen, K. M. a. J. J. (2003). Studying nuclear organization in embryos using antibody tools. In *Drosophila Cytogenetics Protocols* (ed. D. S. Henderson), pp. p. 215-234. Totowa, New Jersey: Humana Press.

Juretic, D., Zoranic, L. and Zucic, D. (2002). Basic charge clusters and predictions of membrane protein topology. *J Chem Inf Comput Sci* **42**, 620-632.

Kamada, Y., Sekito, T. and Ohsumi, Y. (2004). Autophagy in yeast: a TOR-mediated response to nutrient starvation. *Current topics in microbiology and immunology* **279**, 73-84.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567-580.

Lang-Rollin, I. C., Rideout, H. J., Noticewala, M. and Stefanis, L. (2003). Mechanisms of caspase-independent neuronal death: energy depletion and free radical generation. *J Neurosci* **23**, 11015-11025.

Lee, S. D. (2004). Epidemiological and clinical behavior of prepubertal testicular tumors in Korea. *J Urol* **172**, 674-678.

Lockshin, R. A. and Zakeri, Z. (2004). Apoptosis, autophagy, and more. *The international journal of biochemistry & cell biology* **36**, 2405-2419.

Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T. and Thompson, C. B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* **120**, 237-248.

Marchler-Bauer, A. and Bryant, S. H. (2004). CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* **32**, W327-331.

Miotto, B., Sagnier, T., Berenger, H., Bohmann, D., Pradel, J. and Graba, Y. (2006). Chameau HAT and DRpd3 HDAC function as antagonistic cofactors of JNK/AP-1-dependent transcription during Drosophila metamorphosis. *Genes Dev* **20**, 101-112.

Moore, L. A., Broihier, H. T., Van Doren, M., Lunsford, L. B. and Lehmann, R. (1998). Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila. *Development* **125**, 667-678.

O'Connell, P. O. and Rosbash, M. (1984). Sequence, structure, and codon preference of the Drosophila ribosomal protein 49 gene. *Nucleic Acids Res* **12**, 5495-5513.

Ollmann, M., Young, L. M., Di Como, C. J., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W. W., Buchman, A. et al. (2000). Drosophila p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* **101**, 91-101.

Pierre, K. and Pellerin, L. (2005). Monocarboxylate transporters in the central nervous system: distribution, regulation and function. *Journal of neurochemistry* **94**, 1-14.

Qi, D., Larsson, J. and Mannervik, M. (2004). Drosophila Ada2b is required for viability and normal histone H3 acetylation. *Molecular and cellular biology* **24**, 8080-8089.

Quevedo, C., Kaplan, D. R. and Derry, W. B. (2007). AKT-1 regulates DNA-damage-induced germline apoptosis in C. elegans. *Curr Biol* **17**, 286-292.

Rabinowitz, M. (1941). Studies on the cytology and early embryology of the egg of Drosophila melanogaster. *Journal of Morphology* **69**, 1-49.

Rasoulpour, T., DiPalma, K., Kolvek, B. and Hixon, M. (2006). Akt1 suppresses radiation-induced germ cell apoptosis in vivo. *Endocrinology* **147**, 4213-4221.

Raz, E. (2004). Guidance of primordial germ cell migration. *Current opinion in cell biology* **16**, 169-173.

Rebollar, E., Valadez-Graham, V., Vazquez, M., Reynaud, E. and Zurita, M. (2006). Role of the p53 homologue from Drosophila melanogaster in the maintenance of histone H3 acetylation and response to UV-light irradiation. *FEBS Lett* **580**, 642-648.

Renault, A. D., Sigal, Y. J., Morris, A. J. and Lehmann, R. (2004). Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science* **305**, 1963-1966.

Rong, Y. S., Titen, S. W., Xie, H. B., Golic, M. M., Bastiani, M., Bandyopadhyay, P., Olivera, B. M., Brodsky, M., Rubin, G. M. and Golic, K. G. (2002). Targeted mutagenesis by homologous recombination in D. melanogaster. *Genes Dev* **16**, 1568-1581.

Rubinsztein, D. C., Gestwicki, J. E., Murphy, L. O. and Klionsky, D. J. (2007). Potential therapeutic applications of autophagy. *Nature reviews* **6**, 304-312.

Sano, H., Renault, A. D. and Lehmann, R. (2005). Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen 2. *J Cell Biol* **171**, 675-683.

Santos, A. C. and Lehmann, R. (2004). Germ cell specification and migration in Drosophila and beyond. *Curr Biol* **14**, R578-589.

Schneider, D. T., Schuster, A. E., Fritsch, M. K., Hu, J., Olson, T., Lauer, S., Göbel, U. and Perlman, E. J. (2001). Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. *Cancer research* **61**, 7268-7276.

Schultz, K. A., Sencer, S. F., Messinger, Y., Neglia, J. P. and Steiner, M. E. (2005). Pediatric ovarian tumors: a review of 67 cases. *Pediatr Blood Cancer* **44**, 167-173.

Sogame, N., Kim, M. and Abrams, J. M. (2003). Drosophila p53 preserves genomic stability by regulating cell death. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 4696-4701.

Sonnenblick, B. P. (1941). Germ cell movements and sex differentiation of the gonads in the Drosophila embryo. *Proceedings of the National Academy of Sciences of the United States of America* **27**, 484-489.

Sonnenblick, B. P. (1950). The early embryology of Drosophila melanogaster. In *Biology of Drosophila* (ed. M. Demerec), pp. 62–167 New York: John Wiley & Sons.

Stallock, J., Molyneaux, K., Schaible, K., Knudson, C. M. and Wylie, C. (2003). The pro-apoptotic gene Bax is required for the death of ectopic primordial germ cells during their migration in the mouse embryo. *Development* **130**, 6589-6597.

Starz-Gaiano, M. and Lehmann, R. (2001). Moving towards the next generation. *Mech Dev* **105**, 5-18.

Sutcliffe, J. E. and Brehm, A. (2004). Of flies and men; p53, a tumour suppressor. *FEBS Lett* **567**, 86-91.

Technau, G. M. a. J. A. C.-O. (1986). Lineage analysis of transplanted individual cells in embryos of Drosophila melanogaster. III. Commitment and proliferative capabilities of pole cells and midgut progenitors. *Roux's Arch. Dev. Biol.* **195**, 489-498.

Thangaraju, M., Gopal, E., Martin, P. M., Ananth, S., Smith, S. B., Prasad, P. D., Sterneck, E. and Ganapathy, V. (2006). SLC5A8 triggers tumor cell apoptosis through pyruvate-dependent inhibition of histone deacetylases. *Cancer research* **66**, 11560-11564.

Tomancak, P., Beaton, A., Weiszmann, R., Kwan, E., Shu, S., Lewis, S. E., Richards, S., Ashburner, M., Hartenstein, V., Celniker, S. E. et al. (2002). Systematic determination of patterns of gene expression during Drosophila embryogenesis. *Genome Biol* **3**, RESEARCH0088.

Underwood, E. M., Caulton, J. H., Allis, C. D. and Mahowald, A. P. (1980). Developmental fate of pole cells in Drosophila melanogaster. *Dev Biol* **77**, 303-314.

Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol* **8**, 243-246.

Warrior, R. (1994). Primordial germ cell migration and the assembly of the Drosophila embryonic gonad. *Dev Biol* **166**, 180-194.

Wells, B. S., Yoshida, E. and Johnston, L. A. (2006). Compensatory proliferation in Drosophila imaginal discs requires Dronc-dependent p53 activity. *Curr Biol* **16**, 1606-1615.

Williamson, A. and Lehmann, R. (1996). Germ cell development in Drosophila. *Annu Rev Cell Dev Biol* **12**, 365-391.

Yin, Y., Stahl, B. C., DeWolf, W. C. and Morgentaler, A. (1998). p53-mediated germ cell quality control in spermatogenesis. *Dev Biol* **204**, 165-171.

Zalokar, M. (1976). Autoradiographic study of protein and RNA formation during early development of Drosophila eggs. *Dev Biol* **49**, 425-437.

CHAPTER 3. OUTSIDERS MOSAIC ANALYSES

Introduction

The *outsiders* monocarboxylate transporter gene has been shown to be required zygotically for PCD of germ cells (Coffman et al., 2002). Our studies have demonstrated that *out* functions upstream of or parallel to *p53* to regulate germ cell PCD (Yamada et al., 2007). Double mutants for *p53* and *out* show no increase in severity of the germ cell death defect compared to *p53* or *out* mutant alone. Neither *p53* nor *out* is haplo-insufficient for germ cell PCD. However, interallelic non-complementation is seen when mutations in these two genes are combined *in trans.* Moreover, *p53* is able to partially restore the defective PGC death in *out* mutants when expressed in germ cells. This observation provides evidence for PCD functions of *p53* in germ cells. However, when and where the actions of *out* are required remains to be investigated.

The pro-PCD function of *out* may be required in germline, soma, or both. The *wun* and *wun2* lipid phosphate phosphatase genes serve as an interesting example. *wun2* expression in germ cells has been demonstrated to be both necessary and sufficient for survival of PGCs (Hanyu-Nakamura et al., 2004; Renault et al., 2004). Interestingly, *wun*/*wun2* expression in soma is necessary and sufficient for elimination of PGCs (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). It is thought that the proper germ cell development requires balanced expression of *wun*/*wun2* between germline and soma (Renault et al., 2004).

In order to investigate spatial and temporal requirement of *out*, we studied *out* mRNA expression and also created embryos mosaic for *out*. Identifying where *out* is required would allow generation and testing of more accurate molecular models to better understand germ cell PCD.

Materials & Methods

Fly strains

The origins of *out* alleles are described in chapter 2. The following transgenic lines were used: P{UAS-*out*}2, P{UAS-*out*}3 (created in this study), P{GawB}how[24B] (Brand and Perrimon, 1993), P{GawB}34B (Ingham and Fietz, 1995), P{GawB}*sca*$^{109\text{-}68}$ (Baker et al., 1996), P{GawB}227 (Bossing et al., 2002), P{*daughterless*-GAL4} (Wodarz et al., 1995), and P{GAL4::VP16-*nos*.UTR} (Van Doren et al., 1998).

Fly crosses

Offspring from mothers homozygous for P{w^+, *fat facets-lacZ*} and P{GAL4::VP16-*nos*.UTR} crossed to P{UAS-*out*} males have mutant germ cell phenotypes, possibly due to all three P-element insertions in the genome disrupting genes important for embryogenesis. To alleviate this problem, the germ expression of *out* was achieved using heterozygous out^1/+ and out^2/+ females, and homozygous KG07784 females.

Whole mount *in situ* hybridization

In situ hybridization was performed according to the protocol described in (Tautz and Pfeifle, 1989). A full-length *out* cDNA clone LD28120 in pOT2 vector was obtained through the Drosophila Genome Resource Center (https://dgrc.cgb.indiana.edu/). The antisense probe was made with SP6 RNA polymerase from the EcoRI-linearized LD28120 template. As a negative control, the sense probe was made with T7 RNA polymerase from the XhoI-linearized LD28120 template. As a positive control *bicoid* RNA was used.

Generation of transgenic flies

The *out* cDNA was excised from the pOT2 vector and subcloned into the Drosophila transformation vector, pUAST (Brand and Perrimon, 1993). The resulting UAS-*out* plasmid was co-injected with the P helper plasmid *P{ϖ25.7wc}* (Karess and Rubin, 1984) into the host strain w^{1118} to generate transgenic flies. Standard P-element–

mediated transformation techniques as modified by Nicholas Gompel were used (Spradling and Rubin, 1982). We obtained 5 independent transgenic lines. The locations of the inserts were identified through inverse PCR (Bellen et al., 2004). For further genetic analyses, we used homozygous viable UAS-*out* on the 2rd (P{UAS-*out*}2) and the 3rd (P{UAS-*out*}3).

Results

In situ studies of *out* mRNA expression (work done by Keri Andersen)

To study the spatial and temporal expression patterns of *out*, we performed whole mount *in situ* hybridization. Since our previous studies have shown that *out* mutants are defective for extensive germ cell PCD that takes place between stages 10-12, we reasoned that *out* must be required before stage 12. Therefore, we focused our efforts on investigating *out* expression in early to mid-embryogenesis. In wild-type embryos, *out* mRNA expression is observed in ventral epidermis between stages 11-12 (Fig. 3.1, A). Low levels of expression are also seen in mesoderm. At stage 14, some expression is observed in the midgut and small clusters of cells in the anterior (Fig. 3.1, B). As a negative control, KG07784, *out* null mutant embryos were also stained. No positive stainings were observed. Our results are consistent with the *out* in situ data from the Berkeley Drosophila Genome Project (BDGP). According to the *in situ* pictures provided by BDGP, *out* has ubiquitous maternal expression. Between stages 4-10, *out* mRNA expression is not detected. Between stages 11-12, some expression is seen in somatic muscle primordium, ventral epidermis primordium, and atrium primordium. Between stages 13-16, *out* expression is observed in embryonic midgut and hypopharynx.

out expression in PGCs partially restores germ cell PCD

To further investigate *out* mRNA expression during development, we obtained data on *out* mRNA expression from microarray analyses of germ cell RNAs through personal communications with Drs. Satoru Kobayashi and Shuji Shigenobu at the Okazaki National Research Institutes, Okazaki, Aichi, Japan. According to the data

provided, *out* has maternal expression in germ cells and the mRNA levels drop as embryogenesis proceeds. To explore the possibility that *out* functions in germ cells, we attempted to rescue the mutant phenotype by germ cell-specific expression of *out*. When *out* was expressed in germ cells, PCD of germ cells was partially restored (Table 3.1). As a control, out^{1}/FM7Z; *nos*-GAL4 females were mated to w^{1118}/Y males (Table 3.1, A). We only scored embryos that did not carry the FM7Z chromosome. FM7Z embryos were identified by their *ftz*-lacZ staining patterns. Half of the embryos scored were expected to be hemizygous out^{1} /Y and the other half heterozygous out^{1} / w^{1118}. The frequency of mutant embryos was 44%. Next, we mated out^{1}/FM7Z; *nos*-GAL4 females to w^{1118}/Y; UAS-*out* males to test rescue of the defective germ cell PCD (Table 3.1, B). All of the offspring expressed the *out* rescue construct in germ cells, resulting in a mutant frequency of 30%. Though not complete, there was some PCD effect when *out* is expressed in germ cells. In addition, we tested out^{2}. The out^{2}/FM7Z; UAS-*out* stock had the mutant phenotype penetrance of 87% (Table 3.1, C). When *out* was expressed in this genetic background by crossing out^{2}/FM7Z; *nos*-GAL4/ UAS-*out* females to out^{2}/Y; UAS-*out* males, the frequency of mutant embryos was reduced to 74% (Table 3.1, D). (In both crosses, we did not score embryos carrying FM7Z.) Finally, we tested the effect of *out* rescue construct in the null mutant. KG07784/KG07784; *nos*-GAL4 stock had the mutant phenotype penetrance of 100% (Table 3.1, E). We expressed the *out* rescue construct in this genetic background by mating KG07784/KG07784; *nos*-GAL4 females to UAS-*out* males. Half of the offspring were hemizygous for KG07784 and the other half were heterozygous. All of the offspring expressed the rescue construct. This resulted in a mutant embryo frequency of 36% when using UAS-*out* on the 2nd chromosome and 35% using another rescue construct on the 3rd chromosome (Table 3.1, F, G). Taken together these observations indicate that *out* has some PCD functions in germ cells.

out expression in some somatic tissues does not restore germ cell PCD

To investigate the alternative hypothesis that out may be required in somatic tissues, we expressed *out* in somatic cells along the path PGCs migrate. Our previous studies have shown that massive PGC death takes place as germ cells exit the midgut and

move to mesoderm. Therefore, we expressed *out* in midgut to examine the effect (Table 3.1). When *out* was expressed in the midgut using the 34B-GAL4 driver, the germ cell PCD phenotype was not rescued. The KG07784/KG07784; UAS-*out* females were mated to w^{1118}/Y; 34B-GAL4 males. Half of the offspring were hemizygous for KG07784 and the rest were heterozygotes. Though all of the offspring expressed *out* in the midgut, the frequency of the mutant embryos was 63% (Table 3.1, L).

Next, we examined mesoderm expression of *out*. The *out* mRNA *in situ* data showed low levels of mesoderm expression at stages 11-12. Also, it has been demonstrated that *wun2* expression in mesoderm eliminates germ cells (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). This prompted us to test PCD effects of *out* expression in the mesoderm. When *out* was expressed in mesoderm by the 24B-GAL4 driver, it did not rescue the defective germ cell death phenotype. As a control, penetrance of the mutant phenotype was examined in the out^{1}/ FM7Z; 24B-GAL4 stock. It was 96% (Table 3.1, H). Also as a negative control, out^{1}/ FM7Z; 24B-GAL4 females were crossed to w^{1118}/Y males. Half of the scored offspring were hemizygous for $out^{1}$and the other half were heterozygotes. This resulted in 47% penetrance of the mutant phenotype (Table 3.1, I). When out^{1}/FM7Z; 24B-GAL4 females were mated to w^{1118}/Y; UAS-*out* males, 52% mutant frequency was observed (Table 3.1, J). To confirm the results, we also tested the effect in KG07784 embryos. KG07784/KG07784; UAS-*out* females were mated to w^{1118}/Y; 24B-GAL4 males, resulting in half of the offspring hemizygous for KG07784 and the other half heterozygous. Though all of the offspring expressed *out* in the mesoderm, the mutant frequency was 58% (Table 3.1, K). Combined, these observations show that *out* rescue construct expression in the mesoderm did not rescue the germ cell PCD defects of *out*.

Since expression of *out* in tissues that have direct contact with PGCs during germ cell development did not rescue the defective PCD of *out* mutants, we tested effects of *out* expression in other tissues. As a candidate, nervous system was selected based on the observation that *wun2* expression in nervous system can induce germ cell PCD (Starz-Gaiano et al., 2001). We assayed whether nervous system expression of *out* would induce germ cell PCD using *sca*-GAL4 driver. The sca-GAL4 driver expresses GAL4 in

proneural cells. Interestingly, the expression resulted in defective PGC migration. KG07784/KG07784; UAS-*out* females were mated to w^{1118}/Y; *sca*-GAL4. Half of the offspring were hemizygous for KG07784 and the other half heterozygous. All of the offspring expressed *out* in the nervous system. The resulting mutant frequency was 91% (Table 3.1, M). It appears that expression of *out* in proneural cells and sensory organs causes defective germ cell migration. Closer examination of the embryos revealed unusual germ cell development. At stage 12, bilateral segregation appears to be defective. There are many germ cells in the midline (Fig. 3.2, A). Lateral views of these embryos also revealed that many germ cells were in the epidermis on the dorsal surface instead of staying in the mesoderm (Figure 3.2, B). It appears *out* expression in proneural cells repels germ cells to the dorsal surface. At stage 14, many germ cells are found in the epidermis (Fig. 3.2, C, D, arrows).

In addition, we examined effects of *out* expression in embryonic epidermis and CNS. KG07784/KG07784; UAS-*out* females were mated to w^{1118}/Y; 227-GAL4 males. The 227-GAL4 driver expressed GAL4 in epidermis and CNS starting at embryonic stage 10. Half of the offspring were hemizygous for KG07784 and the other half were heterozygotes. Expression of *out* in epidermis of all of the offspring resulted in mutant embryo frequency of 67% (Table 3.1, N). It appears that *out* expression in epidermis and CNS from stage 10 was not sufficient to restore germ cell PCD in the mutants.

Finally, we expressed *out* in all somatic cells using the *daughterless*-GAL4 driver (*da*-GAL4). KG07784/KG07784; UAS-*out* females were mated to w^{1118}/Y; *da*-GAL4 males. Though only half of the embryos were hemizygous for KG07784 and the rest of the half were heterozygotes, it resulted in 100% mutant embryos (Table 3.1, O). Unlike CNS expression *out*, the mutant embryos look like *out* (Fig. 3.2, F,G, H). It is important to note that *da*-GAL4-driven expression of *out* causes 100% larval lethality.

Discussion

Investigation of *out* expression profiles has revealed some clues as to when and where *out* is highly expressed. In early stages, global maternal expression is observed. *out*

is required zygotically for proper germ cell PCD, but the maternal expression implies other important maternal roles of *out*. Although the most prominent effects are due to zygotically expressed *out*, we did observe a weak maternal effect in some *out* alleles (Yamada et al., 2007). In situ hybridization identified some somatic cell expression of *out*. Between stages 11-12, ventral epidermis primordium and mesoderm appear to express *out*. During stages 13-16, *out* expression is seen in midgut and hypopharynx. Our observations did not detect *out* expression between stages 4-10, possibly due low expression levels. Significance of these expression patterns is yet to be investigated.

Though *in situ* studies did not show *out* expression in germ cells, germ cell-specific microarray analyses of RNA expression profiles reveal expression of *out* in germ cells (Kobayashi and Shigenobu, unpublished data). It is likely that the relatively low levels of *out* germ cell expression were not detected using *in situ* hybridization assays. Expression of *out* in germ cells partially restores germ cell PCD in mutant embryos. The 13-14% rescue was observed. Importantly, germ cell expression of *wun2* in *wun2* maternal mutant partially restores germ cell survival by 7-12% (Renault et al., 2004). In the study, germ cell numbers were counted to examine the effect of *wun2* expression. It would be important to also count the numbers of germ cells in *out* mosaic analyses.

Tissue specific expression of *out* in soma did not rescue the mutant phenotype. Based on the *in situ* results, *out* has low levels of expressions in mesoderm. Expression of *out* in mesoderm did not rescue the defective germ cell PCD in the mutants. Interestingly expression in proneural cells in particular disrupted germ cell migration. When *out* was expressed in epidermis and CNS starting stage 10, it did not result in dominant defective migration. Additionally, *out* expression in the entire somatic cells of embryos did not rescue the out phenotype. This effect was very powerful since it resulted in 100% mutant embryo frequency and 100% larval lethality. It appears that both proneural cell driver *sca*-GAL4 and the global somatic cell driver *da*-GAL4 worked efficiently to drive *out* expression, giving strong germ cell phenotypes. It would be important to confirm the negative results obtained by using the other drivers. One approach would be to use whole mount in situ hybridization. to ensure *out* expression is induced at the appropriate times and locations during embryogenesis.

Germ cell expression of *out* only partially restored the defective germ PCD. It is possible that certain somatic expression of *out* may also be important to enhance germ cell elimination. So far tissue-specific expression of *out* using the somatic cell drivers did not rescue the mutant germ cell PCD. It would be important to test other drivers with different spatial and temporal expression patterns. *out* may be required in both germ cells and somatic cells for germ cell PCD. Therefore, it would be crucial to test combination of both germ cell and somatic cell drivers to test rescue of the mutant phenotype. Anti-Out antibody is in the process of production, and profiling of Out protein expressions will allow selection of more appropriate GAL4 drivers for further investigation.

The very intriguing observation with proneural expression of *out* raises some interesting possibilities as to how germ cells mismigrate to ectoderm. Though it is not yet known what the cellular substrates of *out* are, monocarboxylate transporters are known to move butyrate and pyruvate across membranes (Cuff et al., 2002). They can act as inhibitors of histone deacetylases (HDACs) (Thangaraju et al., 2006). In Drosophila, RNAi of HDAC3 in cell culture induces upregulation of many genes involved in lipid metabolism (Foglietti et al., 2006). HDAC1 RNAi and treatment with HDAC inhibitor trichostatin A both lead to upregulation of *wun*. Importantly, *wun* not only is involved in germ cell PCD, but also germ cell migration (Zhang et al., 1996). In addition, Hedgehog (Hh) protein has been demonstrated to work as a gradient to guide germ cells (Deshpande et al., 2001). Lipid metabolism has been suggested to affect functions and transport of Hh (reviewed in (Wendler et al., 2006)). It is puzzling that the unusual germ cell migration patterns were only observed when *out* was expressed in proneural cells and not in other somatic cells. However, it is possible that proneural cells mediate some guidance signaling involving lipid metabolism, and ectopic expression of *out* may potentiate the signaling effect. Also, based on *wun*/*wun2* functions in germ cell development, it is conceivable that balance of *out* expressions between different tissues might affect germ cell movement and PCD. It would be interesting to assay combined expression of *out* in germ cells and various somatic tissues.

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References

Baker, N. E., Yu, S. and Han, D. (1996). Evolution of proneural atonal expression during distinct regulatory phases in the developing Drosophila eye. *Curr Biol* **6**, 1290-301.

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics* **167**, 761-81.

Bossing, T., Barros, C. S. and Brand, A. H. (2002). Rapid tissue-specific expression assay in living embryos. *Genesis* **34**, 123-6.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Burnett, C. and Howard, K. (2003). Fly and mammalian lipid phosphate phosphatase isoforms differ in activity both in vitro and in vivo. *EMBO Rep* **4**, 793-9.

Coffman, C. R., Strohm, R. C., Oakley, F. D., Yamada, Y., Przychodzin, D. and Boswell, R. E. (2002). Identification of X-linked genes required for migration and programmed cell death of Drosophila melanogaster germ cells. *Genetics* **162**, 273-84.

Cuff, M. A., Lambert, D. W. and Shirazi-Beechey, S. P. (2002). Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. *J Physiol* **539**, 361-71.

Deshpande, G., Swanhart, L., Chiang, P. and Schedl, P. (2001). Hedgehog signaling in germ cell migration. *Cell* **106**, 759-69.

Foglietti, C., Filocamo, G., Cundari, E., De Rinaldis, E., Lahm, A., Cortese, R. and Steinkuhler, C. (2006). Dissecting the biological functions of Drosophila histone deacetylases by RNA interference and transcriptional profiling. *J Biol Chem* **281**, 17968-76.

Hanyu-Nakamura, K., Kobayashi, S. and Nakamura, A. (2004). Germ cell-autonomous Wunen2 is required for germline development in Drosophila embryos. *Development* **131**, 4545-53.

Ingham, P. W. and Fietz, M. J. (1995). Quantitative effects of hedgehog and decapentaplegic activity on the patterning of the Drosophila wing. *Curr Biol* **5**, 432-40.

Karess, R. E. and Rubin, G. M. (1984). Analysis of P transposable element functions in Drosophila. *Cell* **38**, 135-46.

Renault, A. D., Sigal, Y. J., Morris, A. J. and Lehmann, R. (2004). Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science* **305**, 1963-6.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. *Science* **218**, 341-7.

Starz-Gaiano, M., Cho, N. K., Forbes, A. and Lehmann, R. (2001). Spatially restricted activity of a Drosophila lipid phosphatase guides migrating germ cells. *Development* **128**, 983-91.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-5.

Thangaraju, M., Gopal, E., Martin, P. M., Ananth, S., Smith, S. B., Prasad, P. D., Sterneck, E. and Ganapathy, V. (2006). SLC5A8 triggers tumor cell apoptosis through pyruvate-dependent inhibition of histone deacetylases. *Cancer Res* **66**, 11560-4.

Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol* **8**, 243-6.

Wendler, F., Franch-Marro, X. and Vincent, J. P. (2006). How does cholesterol affect the way Hedgehog works? *Development* **133**, 3055-61.

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* **82**, 67-76.

Yamada, Y., Andersen, D. K. and Coffman, C. R. (2007). Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monocarboxylate transporter. *Development*, Accepted for publication, minor revisions pending.

Zhang, N., Zhang, J., Cheng, Y. and Howard, K. (1996). Identification and genetic analysis of wunen, a gene guiding Drosophila melanogaster germ cell migration. *Genetics* **143**, 1231-41.

CHAPTER 4. PRIMORDIAL GERM CELL AND SOMATIC GONADAL PRECURSOR INTERACTIONS IN MUTANTS

Introduction

Excess PGCs in *out* mutants overcome the PCD that is in effect during migration. However, not all the surviving PGCs are incorporated into the gonads. Instead, only the wild-type number of PGCs gets incorporated into the embryonic gonads. This intriguing observation poses some important questions: What restricts the number of PGCs that can be incorporated into the gonads? When is this regulation applied to PGCs?

To seek answers for these questions, we carefully surveyed distribution of the PGCs in the developing embryo. The following stages were investigated: During stage 10, PGCs exit the gut primordium and emerge into mesoderm (Fig. 2.2, A). In the stage 11 wild-type embryo, they become organized into two elongated clusters on either side of the ventral midline (Fig. 2.2, B). At stage 12, the PGCs in the clusters gather closer and by stage 13 they align with somatic gonadal precursor cells (SGPs) to form a band of cells (Fig. 2.2, C, D). During stage 14, PGC and SGPs coalesce to form the embryonic gonads in the 5th abdominal segment (Fig. 2.2, E)(Jaglarz and Howard, 1995; Moore et al., 1998; Warrior, 1994).

Materials & Methods

Fly stocks

Flies were maintained on standard media at 25 °C. The following stocks were obtained from the Bloomington Stock Center: P{UAS-*rpr*} (Aplin and Kaufman, 1997), P{GAL4::VP16-*nos*.UTR} (Van Doren et al., 1998), P{UAS-*shg*}5&9 (Sanson et al., 1996).

Scoring of embryos

Embryos from the cross between *out* ^{2}/*out* ^{2}; *nos*-GAL4/*nos*-GAL4 and w^{1118}/Y; UAS-*rpr*/UAS-*rpr*, are sometimes completely depleted of PGCs by stage 14. To distinguish those stage 14 embryos lacking PGCs from unfertilized eggs, ß-Galactosidase activity negative embryos were carefully examined under a differential interference contrast microscopy. Distinctive midgut shape of the stage 14 embryo was used for staging of the embryos.

Germ cell counts

Germ cells were labeled using X-Gal staining. Germ cells were counted using differential interference contrast microscopy. Our criterion for a mutant phenotype in stage 14 embryos was more than three germ cells ectopic to the gonads.

Results

Bilateral Segregation of PGCs in *out* mutants

Since the defective germ cell PCD of *out* mutants are first observed in stage 11 embryos, we inspected where PGCs are situated at this stage. It has been shown that functions of *wunen*/*wunen2* expressed in CNS are necessary and sufficient for bilateral segregation of germ cells during stage 11(Sano et al., 2005). We questioned if the PGCs in *out*, including those fated to become ectopic PGCs, are able to respond to *wun*/*wun2* guidance cues for formation of bilateral clusters. To address this issue, we quantified PGCs that fail to respond to *wun*/*wun2* signaling and remain in the middle of the embryo (Table 2.2). Bilateral segregation of PGCs starting at stage 11 was assayed by counting the number of PGCs left in the midline and those that migrate laterally away from the midline. PGCs were scored as middle cells when the cells remained close to the middle and isolated from other PGCs that moved laterally forming bilateral clusters. In wild type, an average of 1.8 PGCs are left in the midline at stage 11. In *out* mutants, the average numbers of midline PGCs are as follows: 1.9 in *out* ^{1}, 4.6 in *out* ^{2}, 1.5 in *out* ^{4}, 1.3 in *out* ^{5}, and 1.7 in KG07784. Therefore, most PGCs are able to form bilateral clusters. The *out* ^{2} embryos have a greater average number of PGCs remaining at the

midline. This allele also has a greater number of ectopic PGCs at stage 14 than the other *out* alleles (Supplemental Table 2.2).

At stage 12, PGCs in the bilateral clusters begin to associate and condense with the somatic gonad cells. During this stage, the average number of PGCs in the midline declines to 0.4 in wild type. It appears that those left in the midline are able to undergo PCD in the wild-type embryo, perhaps due to Wun-dependent signaling (Sano et al., 2005). In *out* mutants, the numbers of PGCs in the midline are relatively constant reflecting a failure to undergo PCD. An interesting exception is the KG07784 allele in which midline cells are eliminated similar to wild type. The average numbers of PGCs left in the midline at stage 12 are; 2.9 in *out* ^{1}, 4.1 in *out* ^{2}, 1.1 in *out* ^{4}, 1.3 in *out* ^{5}, and 0.6 in KG07784. Similar numbers are observed in stage 13 embryos.

Some PGCs are separate from the PGC/SGP cluster at stage 13

PGCs in *out* mutants appear to partially respond to *wun*/*wun2* signaling to avoid the middle, separating into bilateral clusters at stage 11. No apparent differences could be observed to distinguish the prospective intragonadal PGCs from those destined to be ectopic to the gonads. At stage 12, association of PGCs within the bilateral clusters is indistinguishable from wild type. In stage 13 *out* mutant embryos, a subset of PGCs are seen in assembly migrating to form embryonic gonads while the rest are starting to become isolated from the PGC/SGP cluster. Double labeling of stage 13 embryos for PGCs and SGPs illustrates this phenomenon (Fig. 2.6, A, B). In stage 13 wild-type embryos, PGCs and SGPs co-localize. In *out* mutants, a similar alignment of PGCs and SGPs is seen. However, there is a subset of PGCs that is separated from the SGPs. (Fig. 2.6, C, D, arrows).

PGCs in *out* mutants partially respond to *rpr*

Since the isolation between PGC/SGP clusters and ectopic PGCs become apparent at stage 13, we wondered if the events that take place during stage 12 might be the key to understand the cause. During stage 12, PGCs and SGPs contact with each other and start to closely associate. We hypothesized that the SGPs maybe the limiting factor

for the number of PGCs that are incorporated into the gonads. Even though *out* mutant germ cells are able to overcome the PCD in stage 11and continue to survive, only a subset of those surviving germ cells is permitted to form the gonads. PGCs that are unable to closely interact with the limited number of SGPs may end up in the overlying ectoderm, which is a commonly observed feature of PGCs in *out* mutants (Fig. 2.2, O).

To explore this possibility, we asked whether reducing the number of PGCs in *out* by germ cell-specific expression of the potent pro-apoptotic gene *reaper* (*rpr*) would affect the incorporation of PGCs into the gonads (White et al., 1994). PGC-specific expression of *rpr* in the otherwise wild-type embryo induces massive PCD of PGCs and the embryo retains the total of 3-4 PGCs in the gonads (Fig.4.1, B) Thus, *rpr* can eliminate the majority of PGCs that normally get incorporated into the gonads. If the ectopic PGCs in *out* mutants are simply the result of excess PGCs that could not get enough contacts with SGPs, then reducing the number of PGCs by *rpr* expression would rescue the ectopic germ cell phenotype. We would expect to see most of the surviving PGCs to be in the gonads. Alternatively, if the ectopic PGCs in the *out* mutants are excluded from the gonads due to other factors such as genetic flaws and are marked for exclusion, then *rpr* expression would not rescue the ectopic germ cell phenotype. We would still expect to see 3-4 intragonadal PGCs and approximately the same number of errant PGCs outside the gonads.

We drove expression of UAS-*rpr* specifically in PGCs using *nos*-GAL4 in the out^2 mutant background (Aplin and Kaufman, 1997; Van Doren et al., 1998). Of 35 embryos examined from the cross, half of them contained the wild-type copy of *out*, thus they would not have left any ectopic PGCs. This is clearly observed where 18 of the embryos have zero ectopic PGCs while the average of 4.1 PGCs are found inside the gonads. The rest of the 17 embryos had the average of 5.8 PGCs in the gonads and 1.8 ectopic PGCs (Table 4.1, Fig.4.1, C). There were more intragonadal PGCs in *out* mutants compared to the wild type. It may be that PGCs in *out* were more resistant to the killing effect of *rpr*. The presence of ectopic PGCs despite the substantially declined number of intragonadal PGCs indicates that the number of PGCs is not what determines the destination of *out* PGCs.

E-cadherin expression in *out* mutants does not rescue misplacement of germ cells

Simply reducing the number of germ cells was not sufficient to rescue the mispositioning of *out* germ cells. Ectopic PGCs were still found in the posterior region. In *out* mutants, ectopic PGCs are mostly dispersed as single cells instead of clumping. This observation prompted us to examine potential involvement of *shotgun* (*shg*), which encodes E-cadherin that functions in homophilic cell adhesion (Yap et al., 1997). In Drosophila, PGCs and SGPs have been shown to express E-cadherin during migration, and the importance of E-cadherin expression in mouse PGC is also suggested (Di Carlo and De Felici, 2000; Jenkins et al., 2003). It is thought that *shg* expression is especially important for PGC-SGP association starting at stage 12 for these two cell types need to condense into forming embryonic gonads (Jenkins et al., 2003). From the observation that the ectopic PGCs in *out* are mostly singly dispersed, it is conceivable that E-cadherin expression in PGCs are somehow impaired in these errant germ cells and they are unable to associate with one another or SGPs.

To test this hypothesis, we expressed UAS-*shg (Sanson et al., 1996)* in PGCs of *out* mutants (Table 4.2). out^2/ out^2; *nos*-GAL4 females were crossed to UAS-*shg* males. Half of the offspring were hemizygous for out^2 and the other half were heterozygotes. We observed 56% mutant embryo frequency in offspring carrying a single copy of UAS-*shg* in either 2nd or 3rd chromosome. The mutant embryo frequency of 57% was observed in embryos carrying two copies of UAS-*shg* on both 2nd and 3rd chromosomes. As a control, out^2/ out^2; *nos*-GAL4 females were crossed to wild-type males. This resulted in 47% mutant embryo frequency. The expression of *shg* in germ cells did not rescue the singly misplaced ectopic germ cells.

Discussion

PGCs in *out* mutants were able to respond to *wun*/*wun2* cues for proper bilateral segregation at stages 11 and 12. Close examination of germ cell migration in *out* mutants revealed that isolation of some PGCs starts at around stage 13 while the rest of the PGCs

are able to stay connected to SGPs. This is when germ cells start the process of coalescence with SGPs. Specification and formation of SGPs in the mutants appeared to be normal. This was evidenced by EYA positive SGPs and their co-localization with PGCs. Since only the wild-type number of germ cells is incorporated into the gonads of the *out* mutants, we hypothesized that excess PGCs in *out* might not get enough contacts with SGPs to form the gonads. To test this hypothesis, we decreased the number of *out* PGCs by germ cell-specific expression of *rpr*. It did not rescue the ectopic germ cell phenotype of *out* mutants. It is possible that *out* PGCs were more resistant to the killing effects of *rpr* expression, and most of the PGCs might not have been eliminated until stage 12. Germ cell counts in stage 12 embryos would be the next step to examine this possibility. Also, it would be important to include *rpr* germ cell expression in the wild-type background as a control.

Alternatively, the ectopic PGCs in *out* may be defective for germ cell-SGP interactions. To address this issue, we tested one of the components of germ cell-SGP interactions. We speculated that homophilic cell adhesion molecule E-cadherin is down regulated in *out* germ cells. Murine E-cadherin has been shown to mediate formation of PGC aggregates in vitro (Di Carlo and De Felici, 2000). *shg* overexpression in germ cells of *out* mutants did not rescue the mutant germ cell phenotype. Ectopic germ cells were still singly dispersed. Therefore germ cell expression of E-cadherin in *out* mutants appears insufficient to incorporate excess PGCs into the gonads. However, it is important to note that the study was performed using out^{2}, which leaves the most cells at the midline (Yamada et al., 2007). It would be interesting to test the effect of shg expression in KG07784. This allele leaves fewer midline cells and more PGCs appear to stay close to SGPs until stage 13. In addition, it would be important to confirm effective expression of *shg* in germ cells by in situ.

Further investigation will help determine whether downregulation of E-cadherin is involved in ectopic migration of excess germ cells in *out*. It is possible that *out* functions may be required downstream of *shg* for E-cadherin cell surface localization or protein turnover. It would be interesting to examine E-cadherin distribution using anti-E-cadherin antibody in *out* germ cells and SGPs (Oda et al., 1994). Creating

transheterozygotes with of *out* and *shg* mutants could be used the test the hypothesis that they may genetically interact. Studies involving E-cadherin in *out* background may provide valuable insights into study of metastasis because cancer cells avoid PCD and disconnect from controls of surrounding tissues as they invade other tissues. Importantly, decrease or loss of E-cadherin has also been shown to correlate with cancer progression and invasive behavior (Wheelock et al., 2001). Ectopic germ cells in *out* mutants appear to have decreased cellular adhesion to each other or to SGPs, resulting in an increased cellular motility to end up in various locations through out the posterior half of the embryo. It would be interesting to decipher molecular mechanisms by which errant germ cells in *out* mutants detach from the PGC/SGP clusters to move to other parts of the embryo.

References

Aplin, A. C. and Kaufman, T. C. (1997). Homeotic transformation of legs to mouthparts by proboscipedia expression in Drosophila imaginal discs. *Mech Dev* **62**, 51-60.

Di Carlo, A. and De Felici, M. (2000). A role for E-cadherin in mouse primordial germ cell development. *Dev Biol* **226**, 209-19.

Jaglarz, M. K. and Howard, K. R. (1995). The active migration of Drosophila primordial germ cells. *Development* **121**, 3495-503.

Jenkins, A. B., McCaffery, J. M. and Van Doren, M. (2003). Drosophila E-cadherin is essential for proper germ cell-soma interaction during gonad morphogenesis. *Development* **130**, 4417-26.

Moore, L. A., Broihier, H. T., Van Doren, M., Lunsford, L. B. and Lehmann, R. (1998). Identification of genes controlling germ cell migration and embryonic gonad formation in Drosophila. *Development* **125**, 667-78.

Oda, H., Uemura, T., Harada, Y., Iwai, Y. and Takeichi, M. (1994). A Drosophila homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Dev Biol* **165**, 716-26.

Sano, H., Renault, A. D. and Lehmann, R. (2005). Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen 2. *J Cell Biol* **171**, 675-83.

Sanson, B., White, P. and Vincent, J. P. (1996). Uncoupling cadherin-based adhesion from wingless signalling in Drosophila. *Nature* **383**, 627-30.

Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol* **8**, 243-6.

Warrior, R. (1994). Primordial germ cell migration and the assembly of the Drosophila embryonic gonad. *Dev Biol* **166**, 180-94.

Wheelock, M. J., Soler, A. P. and Knudsen, K. A. (2001). Cadherin junctions in mammary tumors. *J Mammary Gland Biol Neoplasia* **6**, 275-85.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-83.

Yamada, Y., Andersen, D. K. and Coffman, C. R. (2007). Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monocarboxylate transporter. *Development*, Accepted for publication, minor revisions pending.

Yap, A. S., Brieher, W. M. and Gumbiner, B. M. (1997). Molecular and functional analysis of cadherin-based adherens junctions. *Annu Rev Cell Dev Biol* **13**, 119-46.

CHAPTER 5. ELUCIDATING THE COMPONENTS OF GERM CELL PCD MACHINERIES IN DROSOPHILA

Introduction

Depending on cellular and developmental contexts, cells take different paths for self-termination. Type I PCD, apoptosis, and type II PCD, autophagy, are two prominent ways of self-destruction. The differences are reflected by distinct cellular morphologies seen during the process of demise. Apoptosis is marked by formation of a protein complex known as apoptosome that dismantles cellular structures. During apoptosis, the nucleus breaks apart causing DNA fragmentation, and the cell eventually breaks into smaller apoptotic bodies. Autophagy begins with formation of autophagosomes, membranes within the cell, which engulf cellular organelles. The autophagosome then fuses with lysosomes forming an autolysosome for degradation of cell components. Interestingly, cross-talk between these two mechanisms has also been noted in these crucial life and death decisions of cells (Edinger and Thompson, 2004; Levine and Yuan, 2005). In fact, there are molecular components common to both forms of cell death. Whether PGC death involves apoptosis and/or autophagy is largely unknown. For this reason, we examined mutants for genes important for apoptosis and autophagy, seeking to elucidate molecular mechanisms regulating germ cell PCD.

The core molecular components of apoptosis include cysteine proteases called caspases that, upon activation, dismantle cells in an orderly manner (Thornberry and Lazebnik, 1998). To regulate caspase activity, cells express Inhibitors of Apoptosis Proteins (IAPs) to negatively regulate the caspases for their survival (Fig. 5.1). In Drosophila, much of the embryonic PCD is carried out through induction of proapoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*), and *grim (Abbott and Lengyel, 1991; Chen et al., 1996; White et al., 1994)*. These genes have been shown to function in targeting of IAPs for ubiquitin-mediated degradation. In the absence of IAPs, caspases are activated to execute PCD. In mammals, the pro-apoptotic gene Bax has been implicated as having an important role in germ cell PCD. For example, Bax is required

for PCD of PGCs during migration to the genital ridges. Bax deficient mouse embryos exhibit a substantially increased number of ectopic PGCs compared to the wild type (Stallock et al., 2003). However, the ectopic PGCs later abolish, indicating that Bax-independent death machineries also function to eliminate the errant germ cells.

Recent studies have provided mounting evidence for *mTOR* (mammalian target of rapamycin) protein kinase to be the central node that connects cell survival and autophagy. *mTOR* functions to exert a negative regulatory effect on autophagy. During autophagy, *mTOR* is inactivated and downstream autophagy effectors partially dephosphorylate *autophagy-specific-gene 13* (*ATG13*), causing tighter association with ATG1 (Fig. 5.1). This leads to stimulation of ATG1 kinase activity that is required for autophagosome formation. Subsequent formation of autolysosome through docking and fusion of the autophagosome with the lysosome has been shown to be accomplished by components of the SNARE machinery (Klionsky, 2005). Sequestered cellular components then go through lysosomal degradation, and degeneration of autophagic bodies results in the elimination of the cell. Many ATG genes have been identified and studied in yeast (Klionsky, 2005). The Drosophila genome contains 11 homologs of the ATG genes. In addition, there are other genes recently demonstrated to play roles in autophagy. For example, Sphingosine kinase 1 (SK1), a lipid phosphate phophatase substrate, is part of mTOR signaling that positively regulates autophagy (Lavieu et al., 2006). Recently, two Drosophila lipid phosphate phosphatases (LPP) homologs, *wunen* and *wunen-2* (*wun/wun2*), have been shown to play important roles in both migration and PCD of germ cells (Hanyu-Nakamura et al., 2004; Sano et al., 2005; Zhang et al., 1997). This suggests a possible connection between *wun/wun2* and autophagic cell death. Moreover, *p53* also has an effect on autophagy by inhibiting mTOR (Feng et al., 2005). Collectively, this evidence suggests a role for autophagy in germ cell elimination. Additionally, studies have implicated that germ cell PCD in normal development may be non-apoptotic (Renault et al., 2004; Sano et al., 2005).

Materials & Methods

Stocks

Stocks were maintained on standard media. The ATG alleles, EP362, KG03090, EY07351, and EP3697, were generated in the Berkeley Drosophila Genome Project and obtained from the Bloomington Stock Center. EP362 is homozygous viable, and the rest are lethal alleles. P{UAS-*rpr*}(Aplin and Kaufman, 1997) and P{GAL4::VP16-nos.UTR} (Van Doren et al., 1998) were also provided by the Bloomington Stock Center. The following fly stains were generously provided by Kristen White: *hid* and *grim* deletion Df(3L)X14, *hid* deletion Df(3L)X25, *rpr* deletion Df(3L)XR38 (Peterson et al., 2002). The Dronc^{51} was provided by John Abrams and is a null allele (Chew et al., 2004).

Fly crosses

Occasionally, mutant lethal mutations balanced over inversions give a germ cell phenotype. Thus, all of the lethal mutants were first crossed to wild-type flies (w^{1118}, P{w^{+}, *fat facets-lacZ*}). Virgin females and males were selected based on their lack of Dominant balancer markers. Subsequently, these flies were mated and F2 embryos were scored for a mutant germ cell phenotype.

X-gal Staining

For embryos laid by females containing w^{1118}, P{w^{+}, *fat facets-lacZ*}, X-gal staining was performed. Embryos were collected on apple juice agar caps and developed until the appropriate stage was reached. They were dechorionated in 50% bleach for 4 minutes. After washing with PBST, the embryos were fixed in 25% gluteraldehyde for 10 minutes. Several washes with PBST were followed for 4 hours. The embryos were then soaked in a standard 0.08% X-gal staining solution for 4 hours.

Results

Testing components of apoptosis

To test whether the core apoptosis execution machinery is present in germ cells, a potent apoptosis inducer, *rpr*, was expressed specifically in PGCs using the GAL4-UAS

system (Aplin and Kaufman, 1997; Brand and Perrimon, 1993). *rpr* expression driven by *nos*-GAL4 caused massive death of germ cells (Van Doren et al., 1998), and only 3-4 germ cells could be found in gonads at stage 14 (Fig. 5.2, B). It is a drastic effect since the wild-type embryos contain an average of 16.5 PGCs in the goands at stage 14 (Supplemental Table 2.2, Fig. 2.2, A). UAS-GFP expression in PGCs was used as a negative control (Yeh et al., 1995). Though it has not been shown that *rpr* is normally expressed in PGCs, this experiment demonstrated that the death machinery downstream of *rpr* can be activated to eliminate PGCs.

Since *rpr* expression in PGCs induced the downstream death machinery to carry out PGC elimination, it prompted us to test if *rpr* is indeed required for the PGC death that normally takes place during migration. Df(3L)H99, a deletion that removes the *rpr*, *grim*, and *hid* genes, was tested for a PGC death phenotype (Abbott and Lengyel, 1991). Individual loss-of-function mutants of *rpr*, *hid*, as well as a *grim hid* double mutant were also assayed (Peterson et al., 2002). None of these mutants showed noticeable defects in PGC development (Table 5.1). The mutants had 2-4 PGC outside the gonads, slightly more than the wild-type. However, the phenotypes were not as severe as *out* or *p53*.

Caspases are the core executioners of apoptosis. Studying if there is a requirement for caspases is another way of testing apoptotic death of germ cells. We tested involvement of Dronc, a functional homolog of Caspase 9 using a null allele, *Dronc* (*Dronc*^{51}) (Chew et al., 2004; Dorstyn et al., 1999). Similar to Df(3L)H99, Dronc mutant embryos had a slightly more germ cells outside the gonads, but no severe PGC death phenotype was observed (Table 5.1). To confirm the results, we took another approach to test if caspases are dispensable for germ cell PCD. This was tested by driving expression of Baculovirus *p35* caspase inhibitor in PGCs (Hay et al., 1994). Again, this did not result in any apparent phenotypic changes.

Testing components of autophagy

Since the studies testing the apoptotic genes implied a PGC death machinery independent of apoptotic signaling, it prompted us to test involvement of genes involved in autophagy in germ cell PCD. Of 11 ATG homologs in Drosophila, we examined

mutants in four different genes that were available from the Drosophila stock center. In order to test involvement of the ATG genes, the mutant alleles were tested for a defective germ cell PCD phenotype (Table 5.1).

ATG8a, a component of the pre-autophagosomal structure, was assayed. ATG8a is one of two homologs of the mammalian LC3 gene (Scott et al., 2004). The EP362, ATG8a mutant, showed some slight germ cell PCD defects (Fig. 5.2, C)(Bellen et al., 2004). The penetrance of the mutant phenotype was 43%. Though not as severe as *out* or *p53*, it was noticeable that there were more germ cells ectopic to the gonads than normal. ATG8 appears to be important for a small subset of germ cells fated to die. In order to further understand ATG8a-dependent germ cell death, germ cell counts were performed on the EP362 mutants (Fig. 5.3). At stage 11, the average number of germ cells in the embryo was 31.8 in EP362 mutants, similar to the wild-type average of 33.7. Therefore, PGC production is normal in EP362 mutants. At stage 14, an average of 18.1 germ cells were incorporated into the gonads. The wild-type average was 17.0. This indicates that the incorporation of PGCs into the gonads is normal in EP362 mutants. The average numbers of PGCs ectopic to the gonads were 3.6 in EP362 mutants and 0.4 in wild-type. The EP362 mutants had more ectopic PGCs than the wild-type. This was significantly different ($P < 0.0001$, Student's t-test). Importantly, ectopic germ cell range was 0-3 in wild type and 1-9 in EP362 mutants.

Additionally, the following ATG mutants were assayed (Table 5.1). The P-insertion KG03090 has been shown to localize in an exon of ATG18 and also been shown to cause defective autophagy in Drosophila fat body development (Bellen et al., 2004; Scott et al., 2004). ATG18 has been shown to be involved in recycling of ATG9, an integral membrane protein, required for autophagosome formation (Fig. 5.1)(Suzuki et al., 2001). In order to test the requirement of ATG18, KG03090 mutants were assayed for a germ cell phenotype. No noticeable germ cell death defect was observed in the KG03090 mutants. EY07351 is a P-insertion in an exon of ATG1 protein kinase that is part of the pre-autophagosomal structure (Bellen et al., 2004). The EY07351 mutants too did not show germ cell death defects. EP3697 is inserted in the 5' region of ATG2, an

important component of the pre-autophagosomal structure (Bellen et al., 2004). EP3697 showed normal germ cell development.

Discussion

Our observations on the components of apoptosis are consistent with what other research groups have reported (Sano et al., 2005). Additionally, germ cell death induced in *wun*/*wun2* genetic backgrounds was TUNEL negative and was not affected by germ cell expression of p35, a dominant negative form of the caspase Dronc, DIAP1, or DIAP2. The Kobayashi group investigated presence of an active form of the Caspase Drice in germ cells of wild-type embryos using Caspase 3 antibody (Hanyu-Nakamura et al., 2004). An average of 2 germ cells were Caspase 3 positive, suggesting that the majority of germ cells undergo Drice-independent PCD. Collectively, the reports from the other research groups as well as our unpublished data suggest normal germ cell PCD machinery that is independent of many of the hallmark components of apoptosis. However, it is important to note that PGCs are able to undergo apoptosis in certain contexts. Expression of *hid* in PGCs causes PGC to undergo apoptosis as these dying germ cells are TUNEL positive. Also, it has been shown that PGCs normally express *hid* mRNA and PGCs lacking *nos* undergo apoptosis in *hid*-dependent manner (Sato et al., 2007). Since *nos* is important for repression of somatic cell fate in germ cells (Hayashi et al., 2004), it is thought that apoptosis eliminates germ cells with defective *nos* expression to ensure the integrity of the germline.

Assays involving the ATG genes did not yield any drastic germ cell PCD phenotype. It would be informative to test the remaining ATG mutants, ATG4-7. Importantly, ATG8a showed weak germ cell phenotype. It would be interesting to investigate if the mutant phenotype can be enhanced by creating tranheterozygous embryos with the other ATG genes. Also, it is important to note that ATG8a embryos were from homozygous mothers. The other lines were lethal, and we were only able to test embryos from heterozygous mothers. In order to test maternal requirements for the ATG genes, we would need to create germline clones. Another approach would be to

perform RNAi of ATG genes by injecting dsRNA into germ plasm of embryos. It has been shown to be effective for blocking both maternally loaded and zygotically expressed genes (Fire et al., 1998; Misquitta and Paterson, 1999). It would also be interesting to examine whether the ATG genes have mRNA expression in germ cells.

Alternatively, these results may be explained by the possibility of cross-talk among different components of cell death machineries. For example, when apoptosis is blocked by caspase inhibition, neurons can still undergo PCD via autophagy (Lang-Rollin et al., 2003). In order to observe abnormal cell death, intermediaries such as *p53* must be knocked out. It is not yet unclear whether PGCs normally undergo autophagy, apoptosis, or some combination. Double mutant analyses for both pro-apoptotic and pro-autophagic genes would be worth attempting, such as ATG8 and caspase Dronc. Detection of molecular markers for autophagy would also be useful. For example, cells that are undergoing autophagy can be detected by PGC-specific expression of UAS-LC3-GFP (Rusten et al., 2004) driven by *nos*-GAL4. LC3 is a mammalian homolog of ATG8, and upon induction of autophagy, it is incorporated into the autophagosome. Additionally, ATG6 might be an interesting gene to examine for potential involvement in germ cell PCD. The mammalian homolog of ATG6, haploinsufficient tumor suppressor Beclin-1, has been shown to interact with Bcl-2 family of proteins and also important for autophagy (Liang et al., 1999; Liang et al., 1998; Pattingre et al., 2005; Qu et al., 2003).

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References

Abbott, M. K. and Lengyel, J. A. (1991). Embryonic head involution and rotation of male terminalia require the Drosophila locus head involution defective. *Genetics* **129**, 783-9.

Aplin, A. C. and Kaufman, T. C. (1997). Homeotic transformation of legs to mouthparts by proboscipedia expression in Drosophila imaginal discs. *Mech Dev* **62**, 51-60.

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics* **167**, 761-81.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Chen, P., Nordstrom, W., Gish, B. and Abrams, J. M. (1996). grim, a novel cell death gene in Drosophila. *Genes Dev* **10**, 1773-82.

Chew, S. K., Akdemir, F., Chen, P., Lu, W. J., Mills, K., Daish, T., Kumar, S., Rodriguez, A. and Abrams, J. M. (2004). The apical caspase dronc governs programmed and unprogrammed cell death in Drosophila. *Dev Cell* **7**, 897-907.

Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible Drosophila caspase. *Proc Natl Acad Sci U S A* **96**, 4307-12.

Edinger, A. L. and Thompson, C. B. (2004). Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* **16**, 663-9.

Feng, Z., Zhang, H., Levine, A. J. and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A* **102**, 8204-9.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-11.

Hanyu-Nakamura, K., Kobayashi, S. and Nakamura, A. (2004). Germ cell-autonomous Wunen2 is required for germline development in Drosophila embryos. *Development* **131**, 4545-53.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-9.

Hayashi, Y., Hayashi, M. and Kobayashi, S. (2004). Nanos suppresses somatic cell fate in Drosophila germ line. *Proc Natl Acad Sci U S A* **101**, 10338-42.

Klionsky, D. J. (2005). The molecular machinery of autophagy: unanswered questions. *J Cell Sci* **118**, 7-18.

Lang-Rollin, I. C., Rideout, H. J., Noticewala, M. and Stefanis, L. (2003). Mechanisms of caspase-independent neuronal death: energy depletion and free radical generation. *J Neurosci* **23**, 11015-25.

Lavieu, G., Scarlatti, F., Sala, G., Carpentier, S., Levade, T., Ghidoni, R., Botti, J. and Codogno, P. (2006). Regulation of autophagy by sphingosine kinase 1 and its role in cell survival during nutrient starvation. *J Biol Chem* **281**, 8518-27.

Levine, B. and Yuan, J. (2005). Autophagy in cell death: an innocent convict? *J Clin Invest* **115**, 2679-88.

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402**, 672-6.

Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B. and Levine, B. (1998). Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol* **72**, 8586-96.

Misquitta, L. and Paterson, B. M. (1999). Targeted disruption of gene function in Drosophila by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc Natl Acad Sci U S A* **96**, 1451-6.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D. and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927-39.

Peterson, C., Carney, G. E., Taylor, B. J. and White, K. (2002). reaper is required for neuroblast apoptosis during Drosophila development. *Development* **129**, 1467-76.

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E. L., Mizushima, N., Ohsumi, Y. et al. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* **112**, 1809-20.

Renault, A. D., Sigal, Y. J., Morris, A. J. and Lehmann, R. (2004). Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science* **305**, 1963-6.

Rusten, T. E., Lindmo, K., Juhasz, G., Sass, M., Seglen, P. O., Brech, A. and Stenmark, H. (2004). Programmed Autophagy in the Drosophila Fat Body Is Induced by Ecdysone through Regulation of the PI3K Pathway. *Developmental Cell* **7**, 179-192.

Sano, H., Renault, A. D. and Lehmann, R. (2005). Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen 2. *J Cell Biol* **171**, 675-83.

Sato, K., Hayashi, Y., Ninomiya, Y., Shigenobu, S., Arita, K., Mukai, M. and Kobayashi, S. (2007). Maternal Nanos represses hid/skl-dependent apoptosis to maintain the germ line in Drosophila embryos. *Proc Natl Acad Sci U S A* **104**, 7455-60.

Scott, R. C., Schuldiner, O. and Neufeld, T. P. (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* **7**, 167-78.

Stallock, J., Molyneaux, K., Schaible, K., Knudson, C. M. and Wylie, C. (2003). The pro-apoptotic gene Bax is required for the death of ectopic primordial germ cells during their migration in the mouse embryo. *Development* **130**, 6589-97.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y. (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *Embo J* **20**, 5971-81.

Thornberry, N. A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281**, 1312-1316.

Van Doren, M., Williamson, A. L. and Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. *Curr Biol* **8**, 243-6.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-83.

Yeh, E., Gustafson, K. and Boulianne, G. L. (1995). Green fluorescent protein as a vital marker and reporter of gene expression in Drosophila. *Proc Natl Acad Sci U S A* **92**, 7036-40.

Zhang, N., Zhang, J., Purcell, K. J., Cheng, Y. and Howard, K. (1997). The Drosophila protein Wunen repels migrating germ cells. *Nature* **385**, 64-7.

CHAPTER 6. DNA DAMAGE-INDUCED PROGRAMMED CELL DEATH: POTENTIAL ROLES IN GERM CELL DEVELOPMENT

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Abstract

The detection of DNA damage is necessary to protect against proliferation of potentially harmful cells. DNA damage often results in cell cycle arrest and programmed cell death. Key components of DNA damage signaling networks include ATM, CHK2, p53, and Bax. Mutations in these genes are linked to tumorigenesis and developmental abnormalities. Expression of some of these genes in primordial germ cells (PGCs) argues that PGCs may utilize DNA damage-induced signaling mechanisms to select against germ cells that are genetically defective, thus maintaining the integrity of the germline. This paper summarizes the roles of these DNA damage signaling molecules and addresses their potential involvement in germ cell development.

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Introduction

In animals, genetic information is passed from one generation to the next via the germline. The formation of the germline begins when primordial germ cells (PGCs) differentiate during embryogenesis. PGCs undergo a series of complex developmental processes giving rise to gametes in adults. Proper regulation of germ cell development is vital to integrated transmission of genetic material between generations.

Germ cell PCD has been shown to be essential in the prevention of germ cell tumor formation and control of germ cell numbers (Furuchi et al., 1996; Rodriguez et al., 1997; Schneider et al., 2001). Because of its importance in human medicine, the regulation of germ cell PCD has been the subject of numerous cancer and reproductive studies. Since the DNA molecule is highly reactive and susceptible to damage by cellular metabolites, most organisms appropriate DNA damage-induced PCD to eliminate genetically flawed cells. Molecular analyses using organisms from nematodes to mammals have revealed conserved molecular components of the genome surveillance machinery. While it seems reasonable that germ cells might utilize DNA damage-induced PCD, this possibility has not been explored.

This review highlights some of the key findings in DNA damage-induced PCD. Focusing mainly on *Drosophila* and mammalian systems, we note some interesting observations and unresolved questions in this rapidly advancing field. Additionally, we comment on implications these findings may have for germ cell development.

An overview of damage-induced PCD

Dissection of the intricate molecular networks of DNA damage signaling has identified gatekeeper molecules that prevent defective cells from potentially harmful proliferation. The first line of defense employs DNA damage sensor molecules that monitor the state of the genome. Cells incurring DNA damage respond by halting the cell cycle to allow repair or promoting PCD if the damage is beyond repair (Zhou and Elledge, 2000). Failure to respond properly to DNA damage can result in the loss of

genomic material and carcinogenesis (Elledge, 1996; Hoeijmakers, 2001; Olive, 1998; van Gent et al., 2001).

Gatekeeper molecules in the cellular response to DNA damage include ataxia telangiectasia mutated (ATM) and CHK2. In addition, p53 is a key regulator of DNA damage-induced PCD. ATM-mediated damage signaling pathways have been linked to p53 activation. Activated p53 acts primarily as a transcription factor, entering the nucleus to bind to target enhancers. Numerous downstream pro-PCD genes are regulated by p53, including mammalian *Bax* and *Drosophila reaper* (*rpr*)*, hid,* and *sickle* (*skl*) (Fig. 6.1).

ATM

Eukaryotic genome integrity is maintained in part by cell cycle checkpoint pathways. One of these involves the activation of a phosphatidylinositol-3 kinase-related kinase, ATM, when DNA double-strand breaks (DSBs) are recognized (Valerie and Povirk, 2003). ATM triggers a biochemical cascade that relays and amplifies the damage signal to downstream effectors (Shiloh, 2003). Following the induction of DSBs by ionizing radiation (IR), ATM dimers autophosphorylate to become potent protein kinase monomers that carry out the signaling function (Bakkenist and Kastan, 2003). Simultaneously, a fraction of ATM is also found adhered to the site of DNA damage (Andegeko et al., 2001). To date, the mechanisms by which ATM is recruited to the DSB sites and how DSBs trigger ATM autophoshprylation remain unclear.

In humans, *ATM* mutations are present in patients with ataxia-telangiectasia(A-T), leading to progressive neuromotor dysfunction, immunodeficiency, infertility, and higher cancer susceptibility (Harnden, 1994; Shiloh, 1995). Notably, the phenotypes of *ATM* knockout mice resemble those of the A-T patients (Kuljis et al., 1997; Xu et al., 1996). The importance of ATM in DNA damage-induced PCD signaling is exemplified by observations of *ATM*-deficient mice in response to IR. In wild-type mice, IR leads to PCD in many regions of the CNS, whereas IR fails to induce cell death in *ATM*-deficient mice (Herzog et al., 1998).

Drosophila lacking the ATM ortholog, *dATM*, die as pupae or manifest eye and wing malformations (Song et al., 2004). Flies carrying hypomorphic mutations in *dATM*

have small, rough eyes and bristle abnormalities. The wings display notches with occasional blister formation. The defects resulting from the absence of *dATM* suggest it has important developmental functions.

CHK

Propagation of the DNA damage signal initiated by activation of ATM often involves actions of CHK2, a serine/threonine kinase, which is a primary target of ATM in response to DNA DSBs. For example, CHK2 is not modified or activated in ATM-deficient cells exposed to IR (Matsuoka et al., 1998). However reintroduction of ATM into these cells leads to modification and activation of CHK2 in response to IR.

In humans, mutations in *CHK2* are present in a subset of patients with Li-Fraumeni syndrome who are predisposed to developing diverse types of cancers (Bell et al., 1999; Lee et al., 2001; Vahteristo et al., 2001). Li-Fraumeni syndrome is also linked to mutations in *p53*. *CHK2*-deficient mice show no obvious defects under normal conditions (Hirao et al., 2002). However, thymic tissues of *CHK2*-deficient mice exhibit resistance to IR-induced PCD, while wild-type tissues undergo PCD (Hirao et al., 2002). Furthermore, following irradiation, *CHK2* mutant mice form tumors earlier than wild-type animals, and they develop a greater number of tumors (Hirao et al., 2002). Collectively, these findings suggest an important role of CHK2 in DNA damage response signaling.

Drosophila CHK2 (MNK) has a crucial function in the DNA damage response pathway mediating cell cycle arrest and PCD (Masrouha et al., 2003; Peters et al., 2002; Xu et al., 2001). In wild-type wing imaginal discs, IR causes extensive PCD. In contrast, PCD is blocked in *mnk* mutants after irradiation. Reintroduction of an *mnk* wild-type gene restores irradiation-induced PCD. These results demonstrate that MNK is involved in regulating DNA damage-induced PCD. Intriguingly, the MNK protein is expressed in ovaries and germ cell nuclei of the embryo, suggesting that it may act as a safeguard against genetically crippled PGCs (Oishi et al., 1998).

p53

p53 has been referred to as the guardian of the genome, owing to its roles in various cellular processes necessary to maintain genome integrity and stability (Lane, 1992). The key function of p53 involves transcriptional regulation. In DNA damage signaling pathways, ATM and CHK2 are primary candidate molecules that aid in activation and stabilization of p53 (Sutcliffe and Brehm, 2004). Downstream targets of p53 are employed in diverse cellular activities, such as *p21* in G1 growth arrest, *GADD45* in DNA repair, and *Bax* in PCD (Vousden and Lu, 2002). This suggests the important roles for p53 in coordinating cellular responses to DNA damage. Modifications on the p53 protein are likely to regulate its function, however exactly how activation and stabilization of p53 are controlled remains unclear (Sutcliffe and Brehm, 2004).

Over 50% of human cancers carry mutations in *p53* (Greenblatt et al., 1994). Studies of families with germline *p53* mutations have revealed high incidences of breast cancers, sarcomas of bone and soft tissues, and brain cancers (Kleihues et al., 1997). In mice, loss of *p53* leads to an early onset of spontaneous tumor formations (Ghebranious and Donehower, 1998). Involvement of *p53* in DNA damage-induced PCD is demonstrated in the following examples from mice. Intestinal crypt cells undergo PCD in response to IR. However, cells deficient for *p53* are resistant to induction of PCD in response to IR (Clarke et al., 1994). In culture, *p53* null thymocytes are resistant to IR induced PCD, while wild-type thymocytes undergo PCD following IR (Clarke et al., 1993). Finally, wild-type postmitotic CNS neurons, but not *p53* mutant neurons, undergo PCD in response to IR (Enokido et al., 1996).

Drosophila p53 (*Dmp53*) has also been shown to mediate DNA damage-induced PCD (Brodsky et al., 2000; Brodsky et al., 2004; Ollmann et al., 2000). Overexpression of *Dmp53* in the developing eye results in PCD, giving a small eye phenotype. Also, it has been shown that radiation-induced PCD in the wing and eye imaginal discs is suppressed by dominant negative *Dmp53* transgene expression (Brodsky et al., 2000; Ollmann et al., 2000). Studies of *Dmp53* transcripts during embryogenesis reveal global RNA expression that becomes more restricted to PGCs and hindgut cells during

development (Ollmann et al., 2000). Given its central role in DNA damage-induced PCD, it is likely that *Dmp53* plays an important part in regulating germ cell development.

Bax

Bax, a pro-PCD gene belonging to the Bcl-2 family, appears to be an important component in PCD (Kirkin et al., 2004). The Bcl-2 family, defined by the presence of conserved BH domains, includes both pro- and anti-PCD genes. In mammals, interactions of Bcl-2-related proteins are central to death or life decisions of cells. Upon death stimuli, the Bax protein undergoes oligomerization followed by integration into the outer mitochondrial membrane. This relocalization appears important for propagation of death signaling.

Bax is often mutated in human colorectal and hematopoietic malignancies (Meijerink et al., 1998; Rampino et al., 1997). In mice, loss of *Bax* promotes tumor formation (Ionov et al., 2000). Bax has also been shown to have a role in DNA damage-induced PCD. For example, in *Bax* knockout mice, the dentate gyrus is resistant to IR-induced PCD, whereas IR triggers massive PCD in wild-type animals (Chong et al., 2000). *Bax* expression appears to be regulated by p53 (Miyashita and Reed, 1995). Gel retardation assays demonstrate that wild-type but not mutant p53 protein binds to the p53-binding sites of the *Bax* promoter. In *p53*-deficient tumor cells, a wild-type but not a mutant p53 transgene is capable of transactivating the *Bax* promoter to drive the expression of a reporter construct. Furthermore, *Bax* has been shown to be important for *p53*-dependent PCD and contributes to *p53*-dependent tumor suppression in some cells (Cregan et al., 1999; Xiang et al., 1998; Yin et al., 1997).

Though a link between DNA damage-induced PGC PCD and *Bax* has not been described, there is convincing evidence for a role of *Bax* in germ cell PCD (Perez et al., 1999; Rucker et al., 2000). Recent findings show that Bax is required for PCD of migratory PGCs in mice. *Bax*-deficient mouse embryos exhibit a substantially increased number of ectopic germ cells compared to the wild type (Stallock et al., 2003). However, the ectopic germ cells later die, indicating the involvement of other PCD pathways.

rpr, *skl*, and *hid*

In higher eukaryotes, antagonistic interplay of cell death regulators maintains the fine balance between death and survival of cells. Inhibitors of Apoptosis Proteins (IAPs) are present to block the proteolytic actions of caspases, key executors of PCD. In *Drosophila*, *rpr*, *grim*, *hid* and *skl* are IAP antagonists that play major roles in promoting PCD (Bergmann et al., 2003). Recently, it has been proposed that *rpr*, *hid*, and *skl* function in IR-induced PCD based on the observation that flies deficient for *rpr*, *skl*, and *hid* exhibit reduced IR-induced PCD in wing imaginal discs (Brodsky et al., 2004).

rpr has been shown to be involved in Dmp53-mediated responses to radiation (Brodsky et al., 2004; Sogame et al., 2003). Dmp53 binds to a radiation responsive cis-regulatory region of *rpr*. After IR, the *rpr* radiation-responsive element is activated in *Dmp53* wild-type embryos, but not in *Dmp53*-deficient embryos (Brodsky et al., 2000). Genome-wide analysis of IR-induced transcription shows that *rpr* is induced following IR (Brodsky et al., 2004). However, this is not the case in *Dmp53* or *mnk* (*CHK2*) mutant backgrounds.

Similarly, microarray profiles of IR-responsive genes reveal that *skl* and *hid* are highly induced by IR in wild type, but not *Dmp53* or *mnk* (*CHK2*) deficient backgrounds (Brodsky et al., 2004). Therefore, *skl* and *hid* are potential targets of MNK(CHK2)/Dmp53-mediated damage signaling.

Concluding remarks

The previous sections present evidence that *ATM*, *CHK2/mnk*, *p53*, *Bax*, *rpr*, *hid*, and *skl* all play important roles in DNA damage-induced PCD. Are these core components of DNA-damage signaling a part of PGC development helping to ensure elimination of genetically damaged germ cells? ATM expression has not been reported in PGCs. Involvement of *CHK2* and *p53* in mammalian PGC development are yet to be analyzed. However, MNK/CHK2 protein and *Dmp53* mRNA are expressed in *Drosophila* PGCs during embryogenesis making it likely that they have functions in this particular developmental context. Bax has been shown to be involved in PGC PCD in

mice. Whether Bax functions in DNA damage-induced PCD of PGCs to select against cells carrying defective DNA remains an open question. Intriguingly, there is evidence indicating *CHK2*- and *p53*-dependent activation of *Bax* in response to IR (Hirao et al., 2000; Takai et al., 2002). So far, no published studies show involvement of *rpr*, *skl*, and *hid* in germ cell development. However, *rpr*, *hid*, and *skl* transcript levels increase in *mnk*- and *Dmp53*-dependent manners following IR. Future studies will determine involvement of these pro-PCD molecules and their interactions with other components of the networks in PGC PCD in developing embryos.

References

Andegeko, Y., Moyal, L., Mittelman, L., Tsarfaty, I., Shiloh, Y. and Rotman, G. (2001). Nuclear retention of ATM at sites of DNA double strand breaks. *J Biol Chem* **276**, 38224-30.

Bakkenist, C. J. and Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**, 499-506.

Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F. et al. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science* **286**, 2528-31.

Bergmann, A., Yang, A. Y. and Srivastava, M. (2003). Regulators of IAP function: coming to grips with the grim reaper. *Curr Opin Cell Biol* **15**, 717-24.

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M. and Abrams, J. M. (2000). Drosophila p53 binds a damage response element at the reaper locus. *Cell* **101**, 103-13.

Brodsky, M. H., Weinert, B. T., Tsang, G., Rong, Y. S., McGinnis, N. M., Golic, K. G., Rio, D. C. and Rubin, G. M. (2004). Drosophila melanogaster MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. *Mol Cell Biol* **24**, 1219-31.

Chong, M. J., Murray, M. R., Gosink, E. C., Russell, H. R., Srinivasan, A., Kapsetaki, M., Korsmeyer, S. J. and McKinnon, P. J. (2000). Atm and Bax cooperate in ionizing radiation-induced apoptosis in the central nervous system. *Proc Natl Acad Sci U S A* **97**, 889-94.

Clarke, A. R., Gledhill, S., Hooper, M. L., Bird, C. C. and Wyllie, A. H. (1994). p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. *Oncogene* **9**, 1767-73.

Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. and Wyllie, A. H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362**, 849-52.

Cregan, S. P., MacLaurin, J. G., Craig, C. G., Robertson, G. S., Nicholson, D. W., Park, D. S. and Slack, R. S. (1999). Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. *J Neurosci* **19**, 7860-9.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science* **274**, 1664-72.

Enokido, Y., Araki, T., Tanaka, K., Aizawa, S. and Hatanaka, H. (1996). Involvement of p53 in DNA strand break-induced apoptosis in postmitotic CNS neurons. *Eur J Neurosci* **8**, 1812-21.

Furuchi, T., Masuko, K., Nishimune, Y., Obinata, M. and Matsui, Y. (1996). Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development* **122**, 1703-9.

Ghebranious, N. and Donehower, L. A. (1998). Mouse models in tumor suppression. *Oncogene* **17**, 3385-400.

Greenblatt, M. S., Bennett, W. P., Hollstein, M. and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* **54**, 4855-78.

Harnden, D. G. (1994). The nature of ataxia-telangiectasia: problems and perspectives. *Int J Radiat Biol* **66**, S13-9.

Herzog, K. H., Chong, M. J., Kapsetaki, M., Morgan, J. I. and McKinnon, P. J. (1998). Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system. *Science* **280**, 1089-91.

Hirao, A., Cheung, A., Duncan, G., Girard, P. M., Elia, A. J., Wakeham, A., Okada, H., Sarkissian, T., Wong, J. A., Sakai, T. et al. (2002). Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol Cell Biol* **22**, 6521-32.

Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J. and Mak, T. W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287**, 1824-7.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366-74.

Ionov, Y., Yamamoto, H., Krajewski, S., Reed, J. C. and Perucho, M. (2000). Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. *Proc Natl Acad Sci U S A* **97**, 10872-7.

Kirkin, V., Joos, S. and Zornig, M. (2004). The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Acta* **1644**, 229-49.

Kleihues, P., Schauble, B., zur Hausen, A., Esteve, J. and Ohgaki, H. (1997). Tumors associated with p53 germline mutations: a synopsis of 91 families. *Am J Pathol* **150**, 1-13.

Kuljis, R. O., Xu, Y., Aguila, M. C. and Baltimore, D. (1997). Degeneration of neurons, synapses, and neuropil and glial activation in a murine Atm knockout model of ataxia-telangiectasia. *Proc Natl Acad Sci U S A* **94**, 12688-93.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. *Nature* **358**, 15-6.

Lee, S. B., Kim, S. H., Bell, D. W., Wahrer, D. C., Schiripo, T. A., Jorczak, M. M., Sgroi, D. C., Garber, J. E., Li, F. P., Nichols, K. E. et al. (2001). Destabilization of CHK2 by a missense mutation associated with Li-Fraumeni Syndrome. *Cancer Res* **61**, 8062-7.

Masrouha, N., Yang, L., Hijal, S., Larochelle, S. and Suter, B. (2003). The Drosophila chk2 gene loki is essential for embryonic DNA double-strand-break checkpoints induced in S phase or G2. *Genetics* **163**, 973-82.

Matsuoka, S., Huang, M. and Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* **282**, 1893-7.

Meijerink, J. P., Mensink, E. J., Wang, K., Sedlak, T. W., Sloetjes, A. W., de Witte, T., Waksman, G. and Korsmeyer, S. J. (1998). Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. *Blood* **91**, 2991-7.

Miyashita, T. and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293-9.

Oishi, I., Sugiyama, S., Otani, H., Yamamura, H., Nishida, Y. and Minami, Y. (1998). A novel Drosophila nuclear protein serine/threonine kinase expressed in the germline during its establishment. *Mech Dev* **71**, 49-63.

Olive, P. L. (1998). The role of DNA single- and double-strand breaks in cell killing by ionizing radiation. *Radiat Res* **150**, S42-51.

Ollmann, M., Young, L. M., Di Como, C. J., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W. W., Buchman, A. et al. (2000). Drosophila p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* **101**, 91-101.

Perez, G. I., Robles, R., Knudson, C. M., Flaws, J. A., Korsmeyer, S. J. and Tilly, J. L. (1999). Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. *Nat Genet* **21**, 200-3.

Peters, M., DeLuca, C., Hirao, A., Stambolic, V., Potter, J., Zhou, L., Liepa, J., Snow, B., Arya, S., Wong, J. et al. (2002). Chk2 regulates irradiation-induced, p53-mediated apoptosis in Drosophila. *Proc Natl Acad Sci U S A* **99**, 11305-10.

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J. C. and Perucho, M. (1997). Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* **275**, 967-9.

Rodriguez, I., Ody, C., Araki, K., Garcia, I. and Vassalli, P. (1997). An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *Embo J* **16**, 2262-70.

Rucker, E. B., 3rd, Dierisseau, P., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., Flaws, J. A. and Hennighausen, L. (2000). Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. *Mol Endocrinol* **14**, 1038-52.

Schneider, D. T., Schuster, A. E., Fritsch, M. K., Hu, J., Olson, T., Lauer, S., Gobel, U. and Perlman, E. J. (2001). Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. *Cancer Res* **61**, 7268-76.

Shiloh, Y. (1995). Ataxia-telangiectasia: closer to unraveling the mystery. *Eur J Hum Genet* **3**, 116-38.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* **3**, 155-68.

Sogame, N., Kim, M. and Abrams, J. M. (2003). Drosophila p53 preserves genomic stability by regulating cell death. *Proc Natl Acad Sci U S A* **100**, 4696-701.

Song, Y. H., Mirey, G., Betson, M., Haber, D. A. and Settleman, J. (2004). The Drosophila ATM ortholog, dATM, mediates the response to ionizing radiation and to spontaneous DNA damage during development. *Curr Biol* **14**, 1354-9.

Stallock, J., Molyneaux, K., Schaible, K., Knudson, C. M. and Wylie, C. (2003). The pro-apoptotic gene Bax is required for the death of ectopic primordial germ cells during their migration in the mouse embryo. *Development* **130**, 6589-97.

Sutcliffe, J. E. and Brehm, A. (2004). Of flies and men; p53, a tumour suppressor. *FEBS Letters* **567**, 86-91.

Takai, H., Naka, K., Okada, Y., Watanabe, M., Harada, N., Saito, S., Anderson, C. W., Appella, E., Nakanishi, M., Suzuki, H. et al. (2002). Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. *Embo J* **21**, 5195-205.

Vahteristo, P., Tamminen, A., Karvinen, P., Eerola, H., Eklund, C., Aaltonen, L. A., Blomqvist, C., Aittomaki, K. and Nevanlinna, H. (2001). p53, CHK2, and CHK1 genes in Finnish families with Li-Fraumeni syndrome: further evidence of CHK2 in inherited cancer predisposition. *Cancer Res* **61**, 5718-22.

Valerie, K. and Povirk, L. F. (2003). Regulation and mechanisms of mammalian double-strand break repair. *Oncogene* **22**, 5792-812.

van Gent, D. C., Hoeijmakers, J. H. and Kanaar, R. (2001). Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* **2**, 196-206.

Vousden, K. H. and Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604.

Xiang, H., Kinoshita, Y., Knudson, C. M., Korsmeyer, S. J., Schwartzkroin, P. A. and Morrison, R. S. (1998). Bax involvement in p53-mediated neuronal cell death. *J Neurosci* **18**, 1363-73.

Xu, J., Xin, S. and Du, W. (2001). Drosophila Chk2 is required for DNA damage-mediated cell cycle arrest and apoptosis. *FEBS Lett* **508**, 394-8.

Xu, Y., Ashley, T., Brainerd, E. E., Bronson, R. T., Meyn, M. S. and Baltimore, D. (1996). Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. *Genes Dev* **10**, 2411-22.

Yin, C., Knudson, C. M., Korsmeyer, S. J. and Van Dyke, T. (1997). Bax suppresses tumorigenesis and stimulates apoptosis in vivo. *Nature* **385**, 637-40.

Zhou, B. B. and Elledge, S. J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-9.

CHAPTER 7. GENERAL CONCLUSIONS

The previous chapters have provided insights into PCD machineries using the germ cells as a model system. Here are some concluding remarks.

Drosophila PGC death is regulated by *p53* and *out*

Careful and extensive germ cell counts showed PCD of germ cells takes place between stages 10-12 of embryogenesis. This is when germ cells exit the midgut primordium and sort bilaterally to move to the mesoderm. We have demonstrated that *p53* and *outsiders* (*out*) are required for PCD of excess germ cells between stages 10-12. In both mutants, the wild-type numbers of germ cells successfully reach the gonads while errant germ cells persist outside the gonads. Neither *p53* or *out* is haplo-insufficient for the mutant germ cell phenotype. However, interallelic non-complementation is observed when these two mutations are combined *in trans*. Some additional controls using p53 alleles generated from different genetic backgrounds remain to be examined. However, overexpression of *p53* in germ cells partially restores the germ cell PCD in *out* mutants. These observations argue that *p53* functions downstream of or parallel to *out* in germ cell PCD.

p53 mediates various cell death machineries

p53 plays significant roles during germ cell development in other model organisms such as *C. elegans* and mice (Beumer et al., 1998; Derry et al., 2001; Yin et al., 1998). Importantly, *p53* mediates multiple modes of cell death. Therefore, different possibilities can be speculated as to how germ cell elimination might be achieved. Reports show that *p53* has roles in caspase-independent modes of programmed cell death (Feng et al., 2005; Coureuil et al., 2006; Crighton et al., 2006). Death of terminally differentiating murine germ cells induced by *p53* overexpression is excecuted by calpains rather than caspases (Coureuil et al., 2006). *p53* activation can inhibit mTor activity and promote autophagy in mouse embryonic fibroblasts (Feng et al., 2005).

out has PCD functions in germ cells

out is predicted to encode a monocarboxylate transporter (MCT) (Crosby et al., 2007). MCTs are known to localize at the plasma membrane and/or mitochondrial membranes, and their substrates include butyrate and pyruvate (Halestrap and Price, 1999). We demonstrated that expression of *out* in germ cells partially restores germ cell PCD in mutants. Though *in situ* hybridization studies did not show *out* RNA expression in germ cells, *out* expression is detected using germ cell-specific microarray analyses, and it is present in a germ cell cDNA library (Kobayashi and Shigenobu personal communication, Yamada and Grantham unpublished data). Investigation of the *out* expression profile revealed that *out* has ubiquitous maternal expression and somatic expression starting at stage 11. Between stages 11-12, *out* is expressed in the primordium of the ventral epidermis. During stages 13-16, *out* expression is seen in midgut and hypopharynx. We did not observe *out* expression between stages 4-10, possibly due to low expression levels. Further investigation will use anti-Out antibody or epitope-tagged reporter constructs to determine spatial and temporal expression patterns.

Expression of *out* in somatic tissues did not rescue the mutant phenotype. Expression in mesoderm, CNS, and midgut showed no effects on germ cell development. Forced expression in proneural cells disrupted germ cell migration. Ubiquitous expression of *out* in somatic cells showed 100% mutant germ cell phenotype similar to *out* mutants and 100% larval lethality. Significance of these observations is yet to be investigated. It would be important to confirm the negative results obtained from *out* expression in mesoderm, CNS, and midgut. The next step would be to examine effectiveness of the GAL4 drivers by expressing UAS-LacZ. Also, it would be informative to use whole mount in situ hybridization to examine UAS-*out* expressions. In addition, using different GAL4 drivers that reflect Out protein expression patterns would be informative. *out* may be required in both germ cells and somatic tissues. Thus, combined expression in both germ cells and somatic tissues might also be interesting.

In addition, our work has shown that *p53* functions downstream of or parallel to *out* in germ cell PCD. To expand this model to somatic cells, it would be interesting to test whether *p53* mutations can suppress the larval lethality associated with *out*

overexpression in the entire somatic cells. Global *p53* overexpression results in male lethality. And this is not rescued by *out* mutations (Yamada and Andersen unpublished data). This is still consistent with our model.

Germ cells are able to undergo both apoptotic and non-apoptotic death

Testing loss of function alleles of genes involved in apoptosis revealed that *grim*, *rpr*, *hid*, and the caspase *Dronc* were not required for proper germ cell PCD. Also, germ cell expression of caspase *p35* did not affect germ cell PCD. This is consistent with the reports from other groups (Sano et al., 2005). Assays involving *wun*/*wun2*, DIAP1, DIAP2, the caspase Drice, and TUNEL staining show germ cells in normal development do not undergo PCD via apoptosis to any significant extent (Hanyu-Nakamura et al., 2004; Renault et al., 2004). However, cell death is extremely context dependent. We demonstrated that *rpr* expression in PGCs induced massive PCD. Similar observations showed that extensive apoptosis was induced by germ cell expression of *hid* (Renault et al., 2004). It has also been shown that zygotic *hid* mRNA expression is observed in PGCs, and PGCs lacking *nos* undergo apoptosis in a *hid*-dependent manner (Sato et al., 2007).

In addition, testing several loss-of-function mutations in components of autophagy for germ cell PCD did not yield a severe germ cell PCD phenotype. ATG8a mutants showed a subtle germ cell phenotype. Since there is another homolog of ATG8, ATG8b, in Drosophila, it is possible that they have redundant functions. Currently, there are no available mutants for ATG8b. It would be interesting to assay double mutants for both ATG8a and ATG8b. It may result in more severe germ cell PCD defects. Whether germ cells utilize mechanisms of autophagy remains to be investigated further. The next step would be to test the remaining ATG mutants, ATG4-7. Also, it would be interesting to test whether the ATG8a mutant phenotype can be enhanced by creating transheterozygotes with other ATG genes that have been shown to interact with ATG8a in other systems. Importantly, ATG1, 2, and 18 mutations were lethal. Therefore, maternal requirements of these genes for germ cell PCD were not tested in this study. In order to test maternal requirements, germline clones and RNAi approaches may be taken

(Fire et al., 1998; Misquitta and Paterson, 1999). Additionally, use of a molecular marker for autophagy would be useful. LC3-GFP has been shown to become incorporated into autophagosomes to allow detection of autophagy (Rusten et al., 2004). UAS-LC3-GFP can be expressed in germ cells by *nos*-GAL4 (Rusten et al., 2004) (Yamada unpublished data).

Germ cells may utilize multiple PCD machineries

Lack of drastic effects assaying loss of function alleles of apoptotic and autophagy genes implies germ cells may utilize multiple cell death machineries, and blocking one may not be sufficient to stop germ cells from dying. For instance, when apoptosis is blocked by caspase inhibition, neurons can still undergo PCD via autophagy (Lang-Rollin et al., 2003). Therefore, double mutant analyses for both pro-apoptotic and pro-autophagic genes, such as Dronc and ATG8a, would be interesting. Additionally, like *p53*, the mammalian homolog of ATG6, Beclin-1 has been shown to interact with Bcl-2 family of proteins and also promote autophagy. It would be interesting to investigate whether ATG6 has functions in germ cell PCD (Liang et al., 1999; Liang et al., 1998; Pattingre et al., 2005; Qu et al., 2003).

Molecular models of PCD involving MCT and p53

How MCT and *p53* regulate germ cell death remains an open question. Some potential avenues to investigate include mTor-Akt pathway and histone deacetylation. The mTor-Akt pathway links nutrient levels to autophagy (Kamada et al., 2004; Lum et al., 2005). *p53* has been shown to genetically interact with *Akt* (Quevedo et al., 2007). It would be interesting to examine components of mTor-Atk pathway for their potential involvements in germ cell PCD. For example, germ cell expression of the negative regulator of PCD, *Akt*, using UAS-*Akt*, may disrupt germ cell PCD in wild-type embryos. Studies involving PTEN may also be interesting. PTEN negatively regulates the pro-survival Akt signaling (Cantley and Neel, 1999). PTEN is a tumor suppressor gene, and mutations in PTEN are associated with various types of cancers (Dahia et al., 1997; Li et al., 1997; Okami et al., 1998; Risinger et al., 1997; Steck et al., 1997). Importantly,

PTEN is normally expressed in murine PGCs and PGC-specific loss of PTEN leads to testicular teratomas in male newborn mice (Kimura et al., 2003). Loss of functions of alleles are available to test the hypothesis that PTEN is required for Drosophila germ cell PCD (Huang et al., 1999; Oldham et al., 2002). UAS-PTEN would also be useful to examine if it can induce germ cell PCD (Gao et al., 2000). Using UAS-PTEN dsRNA for RNAi in germ cells would also be interesting (Exelixis, Inc).

In addition, involvement of histone deacetylase (HDAC) would be interesting to explore. Inhibition of HDAC has been shown to result in apoptosis (Glaser, 2007). MCTs are known to regulate pyruvate and butyrate that act as HDAC inhibitors. The Drosophila genome contains five HDACs; Rpd3, HDAC3, HDAC4, HDAC6, and Sir2 (Barlow et al., 2001; Zeremski et al., 2003). Microarray analyses involving these HDACs have revealed genes that are regulated by HDAC expression. Studies of HDAC target genes that are involved in cell death would be very interesting. For example, *comm3* has been shown to be upregulated during autophagy induction (Gorski et al., 2003). Sir2 overexpression leads to downregulation of *comm3* (Cho et al., 2005). It would be interesting to examine whether loss of *comm3* affects germ cell PCD.

DNA damage-induced PCD may have roles in germ cell development

Also, studying components of DNA damage-induced PCD may provide some insights. Proper regulation of germ cell development is crucial for successful transmission of genetic material between generations. It is conceivable that germ cells may utilize DNA damage-induced PCD to ensure only germ cells with intact genomes reach the gonads. *p53* is one of the core components of DNA-damaged induced PCD (Clarke et al., 1994). For example, *p53* deficient intestinal crypt cells, thymocytes, and postmitotic CNS neurons have been shown to be unresponsive to ionizing radiation (IR), while wild-type cells respond to IR and undergo PCD. *mnk* is an upstream regulator of *p53* in DNA damage signaling. Mnk protein has been shown to be expressed in Drosophila PGCs (Oishi et al., 1998). It would be interesting to test whether *mnk* is required for germ cell PCD. Microarray studies have revealed that *skl* and *hid* are highly induced by IR in wild type, but not *p53* or *mnk* deficient animals (Brodsky et al., 2004).

hid and *skl* mediate apoptosis in germ cells lacking *nos* (Sato et al., 2007). It would be informative to investigate whether germ cells can be induced to undergo PCD upon IR and if it is dependent on *hid* and *skl*.

References

Barlow, A. L., van Drunen, C. M., Johnson, C. A., Tweedie, S., Bird, A. and Turner, B. M. (2001). dSIR2 and dHDAC6: two novel, inhibitor-resistant deacetylases in Drosophila melanogaster. *Exp Cell Res* **265**, 90-103.

Beumer, T. L., Roepers-Gajadien, H. L., Gademan, I. S., van Buul, P. P., Gil-Gomez, G., Rutgers, D. H. and de Rooij, D. G. (1998). The role of the tumor suppressor p53 in spermatogenesis. *Cell Death Differ* **5**, 669-77.

Brodsky, M. H., Weinert, B. T., Tsang, G., Rong, Y. S., McGinnis, N. M., Golic, K. G., Rio, D. C. and Rubin, G. M. (2004). Drosophila melanogaster MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. *Mol Cell Biol* **24**, 1219-31.

Cantley, L. C. and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* **96**, 4240-5.

Cho, Y., Griswold, A., Campbell, C. and Min, K. T. (2005). Individual histone deacetylases in Drosophila modulate transcription of distinct genes. *Genomics* **86**, 606-17.

Clarke, A. R., Gledhill, S., Hooper, M. L., Bird, C. C. and Wyllie, A. H. (1994). p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. *Oncogene* **9**, 1767-73.

Crosby, M. A., Goodman, J. L., Strelets, V. B., Zhang, P. and Gelbart, W. M. (2007). FlyBase: genomes by the dozen. *Nucleic Acids Res* **35**, D486-91.

Dahia, P. L., Marsh, D. J., Zheng, Z., Zedenius, J., Komminoth, P., Frisk, T., Wallin, G., Parsons, R., Longy, M., Larsson, C. et al. (1997). Somatic deletions and

mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. *Cancer Res* **57**, 4710-3.

Derry, W. B., Putzke, A. P. and Rothman, J. H. (2001). Caenorhabditis elegans p53: Role in Apoptosis, Meiosis, and Stress Resistance, vol. 294 (ed., pp. 591-595.

Feng, Z., Zhang, H., Levine, A. J. and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A* **102**, 8204-9.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-11.

Gao, X., Neufeld, T. P. and Pan, D. (2000). Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Dev Biol* **221**, 404-18.

Glaser, K. B. (2007). HDAC inhibitors: Clinical update and mechanism-based potential. *Biochem Pharmacol*.

Gorski, S. M., Chittaranjan, S., Pleasance, E. D., Freeman, J. D., Anderson, C. L., Varhol, R. J., Coughlin, S. M., Zuyderduyn, S. D., Jones, S. J. and Marra, M. A. (2003). A SAGE approach to discovery of genes involved in autophagic cell death. *Curr Biol* **13**, 358-63.

Halestrap, A. P. and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* **343 Pt 2**, 281-99.

Hanyu-Nakamura, K., Kobayashi, S. and Nakamura, A. (2004). Germ cell-autonomous Wunen2 is required for germline development in Drosophila embryos. *Development* **131**, 4545-53.

Huang, H., Potter, C. J., Tao, W., Li, D. M., Brogiolo, W., Hafen, E., Sun, H. and Xu, T. (1999). PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. *Development* **126**, 5365-72.

Kamada, Y., Sekito, T. and Ohsumi, Y. (2004). Autophagy in yeast: a TOR-mediated response to nutrient starvation. *Curr Top Microbiol Immunol* **279**, 73-84.

Kimura, T., Suzuki, A., Fujita, Y., Yomogida, K., Lomeli, H., Asada, N., Ikeuchi, M., Nagy, A., Mak, T. W. and Nakano, T. (2003). Conditional loss of PTEN leads to

testicular teratoma and enhances embryonic germ cell production. *Development* **130**, 1691-700.

Lang-Rollin, I. C., Rideout, H. J., Noticewala, M. and Stefanis, L. (2003). Mechanisms of caspase-independent neuronal death: energy depletion and free radical generation. *J Neurosci* **23**, 11015-25.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R. et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-7.

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. and Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402**, 672-6.

Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B. and Levine, B. (1998). Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol* **72**, 8586-96.

Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T. and Thompson, C. B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* **120**, 237-48.

Misquitta, L. and Paterson, B. M. (1999). Targeted disruption of gene function in Drosophila by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc Natl Acad Sci U S A* **96**, 1451-6.

Oishi, I., Sugiyama, S., Otani, H., Yamamura, H., Nishida, Y. and Minami, Y. (1998). A novel Drosophila nuclear protein serine/threonine kinase expressed in the germline during its establishment. *Mech Dev* **71**, 49-63.

Okami, K., Wu, L., Riggins, G., Cairns, P., Goggins, M., Evron, E., Halachmi, N., Ahrendt, S. A., Reed, A. L., Hilgers, W. et al. (1998). Analysis of PTEN/MMAC1 alterations in aerodigestive tract tumors. *Cancer Res* **58**, 509-11.

Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M. and Hafen, E. (2002). The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. *Development* **129**, 4103-9.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D. and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122**, 927-39.

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E. L., Mizushima, N., Ohsumi, Y. et al. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* **112**, 1809-20.

Quevedo, C., Kaplan, D. R. and Derry, W. B. (2007). AKT-1 regulates DNA-damage-induced germline apoptosis in C. elegans. *Curr Biol* **17**, 286-92.

Renault, A. D., Sigal, Y. J., Morris, A. J. and Lehmann, R. (2004). Soma-germ line competition for lipid phosphate uptake regulates germ cell migration and survival. *Science* **305**, 1963-6.

Risinger, J. I., Hayes, A. K., Berchuck, A. and Barrett, J. C. (1997). PTEN/MMAC1 mutations in endometrial cancers. *Cancer Res* **57**, 4736-8.

Rusten, T. E., Lindmo, K., Juhasz, G., Sass, M., Seglen, P. O., Brech, A. and Stenmark, H. (2004). Programmed Autophagy in the Drosophila Fat Body Is Induced by Ecdysone through Regulation of the PI3K Pathway. *Developmental Cell* **7**, 179-192.

Sano, H., Renault, A. D. and Lehmann, R. (2005). Control of lateral migration and germ cell elimination by the Drosophila melanogaster lipid phosphate phosphatases Wunen and Wunen 2. *J Cell Biol* **171**, 675-83.

Sato, K., Hayashi, Y., Ninomiya, Y., Shigenobu, S., Arita, K., Mukai, M. and Kobayashi, S. (2007). Maternal Nanos represses hid/skl-dependent apoptosis to maintain the germ line in Drosophila embryos. *Proc Natl Acad Sci U S A* **104**, 7455-60.

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T. et al. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15**, 356-62.

Yin, Y., Stahl, B. C., DeWolf, W. C. and Morgentaler, A. (1998). p53-mediated germ cell quality control in spermatogenesis. *Dev Biol* **204**, 165-71.

Zeremski, M., Stricker, J. R., Fischer, D., Zusman, S. B. and Cohen, D. (2003). Histone deacetylase dHDAC4 is involved in segmentation of the Drosophila embryo and is regulated by gap and pair-rule genes. *Genesis* **35**, 31-8.

APPENDIX

Tables

Table 2.1 *p53* mutant embryos are defective in germ cell PCD.

Female	Male	N	% mutant
$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	166	93
$pP53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	$p53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	158	96
$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	+/+	91	30
$pP53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	+/+	204	35
wt/$p53^{5A\text{-}1\text{-}4}$	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	165	42
wt/$p53^{11\text{-}1B\text{-}1}$	$p53^{11\text{-}1B\text{-}1}/p53^{11\text{-}1B\text{-}1}$	216	42
out^1/out^1	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	135	77
out^2/out^2	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	445	84
+/+	$p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	174	3
out^1/FM7Z; $p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	FM7Z/Y; $p53^{5A\text{-}1\text{-}4}/p53^{5A\text{-}1\text{-}4}$	95	96

Table 2.2. Germ cell counts for quantification of PGC bilateral segregation at stages 11 through 13.

	Stage 11			Stage 12			Stage 13		
Genotype	N	Bilateral ± s.d.	Middle ± s.d.	N	Bilateral ± s.d.	Middle ± s.d.	N	Bilateral ± s.d.	Middle ± s.d.
wild type	19	20.8 ± 2.8	1.8 ± 0.4	19	16.3 ± 2.7	0.4 ± 0.8	31	15.7 ± 2.5	0.5 ± 0.9
p53 $^{5A-1-4}$	21	31.0 ± 2.7	1.9 ± 0.3	18	30.1 ± 2.8	1.0 ± 1.2	16	29.1 ± 6.2	1.8 ± 2.9
p53 $^{11-1B-1}$	17	32.7 ± 2.8	2.5 ± 0.5	21	28.5 ± 3.7	3.4 ± 1.9	21	27.8 ± 3.5	2.5 ± 2.3
out^{1}	17	30.3 ± 5.0	1.9 ± 0.4	21	28.7 ± 4.1	2.9 ± 3.0	15	26.7 ± 2.3	1.7 ± 1.9
out^{2}	18	29.1 ± 3.1	4.5 ± 0.9	31	27.6 ± 4.4	4.1 ± 3.5	19	25.9 ± 4.3	4.6 ± 2.6
out^{4}	21	31.4 ± 3.5	1.5 ± 0.3	19	29.0 ± 3.5	1.1 ± 1.4	17	27.3 ± 3.0	1.8 ± 1.5
out^{5}	18	32.4 ± 3.7	1.3 ± 0.5	16	31.6 ± 5.0	1.3 ± 2.0	15	30.7 ± 2.1	1.1 ± 0.8
KG07784	17	31.3 ± 4.1	1.7 ± 0.4	17	28.5 ± 3.1	0.6 ± 1.2	15	28.8 ± 4.8	0.5 ± 0.8

Table 2.3. The P element insertion KG07784 uncovers the *out* germ cell phenotype.

Female	Male	N	% mutant
KG07784/ KG07784	KG07784	200	99
KG07784/ KG07784	*out*^{1}	273	97
KG07784/ KG07784	*out*^{2}	272	95
KG07784/ KG07784	*out*^{4}	271	90
KG07784/ KG07784	*out*^{5}	173	98
KG07784/ KG07784	*faf*	546	56
out^{1}/*out*^{1}	KG07784	165	92
out^{1}/*out*^{1}	*out*^{1}	257	96
out^{1}/*out*^{1}	*out*^{2}	234	95
out^{1}/*out*^{1}	*out*^{4}	154	92
out^{1}/*out*^{1}	*out*^{5}	190	90
out^{1}/*out*^{1}	*faf*	154	66
out^{2}/*out*^{2}	KG07784	411	95
out^{2}/*out*^{2}	*out*^{1}	251	98
out^{2}/*out*^{2}	*out*^{2}	180	96
out^{2}/*out*^{2}	*out*^{4}	497	95
out^{2}/*out*^{2}	*out*^{5}	542	96
out^{2}/*out*^{2}	*faf*	473	67
out^{4}/*out*^{4}	KG07784	401	95
out^{4}/*out*^{4}	*out*^{1}	200	96
out^{4}/*out*^{4}	*out*^{2}	354	90
out^{4}/*out*^{4}	*out*^{4}	1365	97
out^{4}/*out*^{4}	*out*^{5}	509	98
out^{4}/*out*^{4}	*faf*	196	56
out^{5}/*out*^{5}	KG07784	223	98
out^{5}/*out*^{5}	*out*^{1}	267	99
out^{5}/*out*^{5}	*out*^{2}	158	94
out^{5}/*out*^{5}	*out*^{4}	235	96
out^{5}/*out*^{5}	*out*^{5}	388	98
out^{5}/*out*^{5}	*faf*	243	59
faf/*faf*	KG07784	264	9
faf/*faf*	*out*^{1}	284	5
faf/*faf*	*out*^{2}	275	5
faf/*faf*	*out*^{4}	189	7
faf/*faf*	*out*^{5}	406	7
faf/*faf*	*faf*	241	4

KG07784 line, P{SUPor-P} BcDNA:LD28120KG07784, has a P-element insertion in *out*. The *out* alleles fail to complement each other. The mutant phenotype is restored by wild-type copy of the gene. X-gal staining was used to label the PGCs. Actual genotype of KG07784 females was KG07784/KG07784; Cyo, P{w^{+}, fat-facets-β-gal}/+.

Table 2.4. Partial Rescue of *out* mutant phenotype through PGC expression of *p53*

Female	Male	N	% mutant
+/+; *nos*-GAL4/*nos*-GAL4*	W^{1118}/Y	219	8
+/+; *nos*-GAL4/*nos*-GAL4	UAS-*p53*/UAS-*p53*	69	3
out^2/out^2; *nos*-GAL4/*nos*-GAL4	W^{1118}/Y	133	47[#]
out^2/out^2; *nos*-GAL4/*nos*-GAL4	UAS-*p53*/UAS-*p53*	90	32[#]

* *nos*-GAL4:VP16

[#]*out* is X-linked – 50% of the offspring are out/+ and 50% are out/Y

Supplemental Table 2.1 Total number of PGCs in embryos between St. 10 and St. 14 Embryos

	St. 10		St. 11		St. 12		St. 13		St. 14	
Genotype	N	PGC # ± SD	N	PGC # ± SD	N	PGC # ± SD	N	PGC # ± SD	N	PGC # ± SD
Wild type	46	33.7 ± 3.3	19	22.6 ± 3.3	19	16.6 ± 2.7	31	16.2 ± 2.9	79	17.0 ± 2.3
P53 $^{5A-1-4}$	50	35.0 ± 3.7	21	32.9 ± 3.3	18	31.1 ± 2.9	16	30.8 ± 8.0	56	27.8 ± 4.2
P53 $^{11-1B-1}$	50	36.0 ± 3.6	17	35.2 ± 3.4	21	31.9 ± 3.8	21	30.3 ± 3.1	65	28.0 ± 4.8
out^{1}	50	36.4 ± 4.4	17	32.2 ± 5.8	21	31.6 ± 3.6	15	28.4 ± 3.2	55	28.2 ± 5.0
out^{2}	52	34.0 ± 2.9	18	33.6 ± 3.5	31	31.7 ± 4.8	19	30.5 ± 4.7	61	27.8 ± 5.2
out^{4}	51	35.8 ± 4.0	21	32.9 ± 3.4	19	30.1 ± 3.7	17	29.1 ± 3.4	57	27.8 ± 5.0
out^{5}	50	35.6 ± 3.2	18	33.7 ± 3.3	16	32.9 ± 5.5	15	31.8 ± 2.2	57	28.4 ± 4.8
KG07784	53	36.2 ± 4.6	17	33.0 ± 4.2	17	29.1 ± 3.2	15	29.3 ± 4.8	81	28.1 ± 4.8

Supplemental Table 2.2. PGCs within the gonads and ectopic to the gonads at stage 14

		PGCs inside gonads	PGCs outside gonads
Genotype	N	PGC# ± SD	PG # ± SD
Wild type	79	16.5 ± 2.1	0.4 ± 0.7
P53 $^{5A-1-4}$	56	18.7 ± 2.7	9.1 ± 3.7
P53 $^{11-1B-1}$	65	18.7 ± 2.7	9.4 ± 3.6
out^{1}	55	14.8 ± 2.6	13.4 ± 4.0
out^{2}	61	13.7 ± 2.3	14.1 ± 4.5
out^{4}	57	16.0 ± 2.8	11.8 ± 3.3
out^{5}	57	15.3 ± 2.8	13.1 ± 3.5
KG07784	81	15.3 ± 2.5	12.8 ± 4.0

Table 3.1. Germline and soma expression of *out*.

	Female	Male	N	%mutant
A	*out*^{1}/FM7Z; *nos*-GAL4	w^{1118}/Y	103	44
B	*out*^{1}/FM7Z; *nos*-GAL4	w^{1118}/Y; P{UAS-*out*}2	189	30
C	*out*^{2}/FM7Z; P{UAS-*out*}3	*out*^{2}/Y; {P{UAS-*out*}3	40	87
D	*out*^{2}/FM7Z; *nos*-GAL4/ P{UAS-*out*}3	*out*^{2}/Y; P{UAS-*out*}3	180	74
E	KG07784/KG07784; *nos*-GAL4	KG07784/Y; *nos*-GAL4	22	100
F	KG07784/KG07784; *nos*-GAL4	w^{1118}/Y; P{UAS-*out*}2	44	36
G	KG07784/KG07784; *nos*-GAL4	w^{1118}/Y; P{UAS-*out*}3	86	35
H	*out*^{1}/ FM7Z; P{GawB}how[24B]	*out*^{1}/Y; P{GawB}how[24B]	23	96
I	*out*^{1}/ FM7Z; P{GawB}how[24B]	w^{1118}/Y	213	47
J	*out*^{1}/FM7Z; P{GawB}how[24B]	w^{1118}/Y; P{UAS-*out*}3	98	52
K	KG07784/KG07784; P{UAS-CG8062}3	w^{1118}/Y; P{GawB}how[24B]	24	58
L	KG07784/KG07784; P{UAS-*out*}3	w^{1118}/Y; P{GawB}34B	8	63
M	KG07784/KG07784; P{UAS-*out*}3	w^{1118}/Y; P{GawB}sca$^{109-68}$	55	91
N	KG07784/KG07784; P{UAS-*out*}3	w^{1118}/Y; P{GawB}227	6	67
O	KG07784/KG07784; P{UAS-*out*}3	w^{1118}/Y; *da*-GAL4	150	100

Offspring from females carrying a copy of *P{fat-facets-lacZ}* was stained using x-gal. The rest were stained using anti-Vasa antibody to label PGCs. P{UAS-*out*}2is insertion on the 2nd chromosome and P{UAS-*out*}3is on the 3rd chromosome. *nos*-GAL4 used here is P{GAL4::VP16-nos.UTR}.

Table 4.1. Germ cell counts in st. 14 embryos.

Genotype	N	Intragonadal Germ Cell # ± SD	SEM	Ectopic Germ Cell # ± SD	SEM
out^{2}*; UAS-rpr; nos-GAL4*	17	5.8 ± 2.0	0.48	1.8 ± 1.1	0.28

Table 4.2. Germline and soma expression of *out*.

Female	Male	N	% mutant
out^{2}/ *out*^{2}; *nos*-GAL4	P{UAS-*shg*}5	124	56
out^{2}/ *out*^{2}; *nos*-GAL4	P{UAS-*shg*}5&9	187	57
out^{2}/ *out*^{2}; *nos*-GAL4	P{UAS-*shg*}9	137	56
out^{2}/ *out*^{2}; *nos*-GAL4	+	133	47

Embryos were stained using X-Gal. *nos*-GAL4 used here is P{GAL4::VP16-nos.UTR}. P{UAS-*shg*}5 in on the 2nd chromosome and P{UAS-*shg*}9 are on the 3rd chromosome. P{UAS-*shg*}5&9 is the combination of the two. The wild-type males used were w^{1118}/Y.

Table 5.1. Testing other known cell death regulators for germ cell PCD.

Female	Male	N	% mutant
H99/+	H99/+	81	9
rpr/+	*rpr*/+	408	5
hid/+	*hid*/+	193	8
grim, hid/+	*grim, hid*/+	147	1
Dronc/+	*Dronc*/+	180	6
Atg8a	*Atg8a*	44	43
Atg1/+	*Atg1*/+	161	6
Atg2/+	*Atg2*/+	213	8
Atg18/+	*Atg18*/+	141	4

H99 is a deletion that removes *rpr*, *hid*, and *grim*. Atg genes are important for autophagy. The + indicates wild-type chromosomes.

Figures

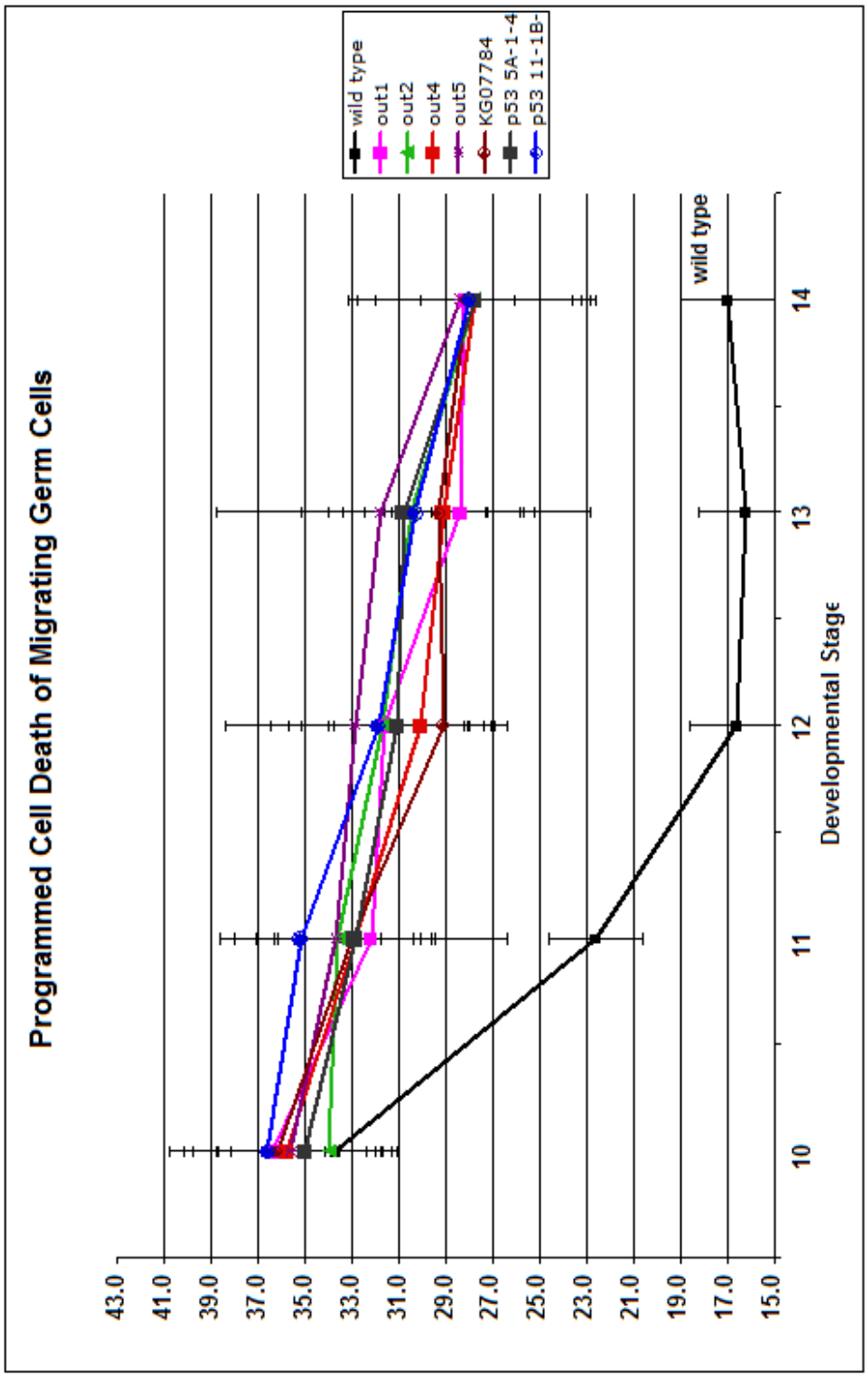

Figure 2.1. Programmed cell death of migrating germ cells in wild type, *p53*, and *out* mutants. Total numbers of PGCs in embryos between stages 10-14 were determined. In wild-type embryos, germ cell death was essentially complete by stage 12. In *p53* and *out* embryos, germ cell death was disrupted and similar numbers of PGCs were observed in both mutants with a gradual loss of germ cells between stages 10 and 14. Error bars represent s.d. See Supplemental Table 1 for additional data.

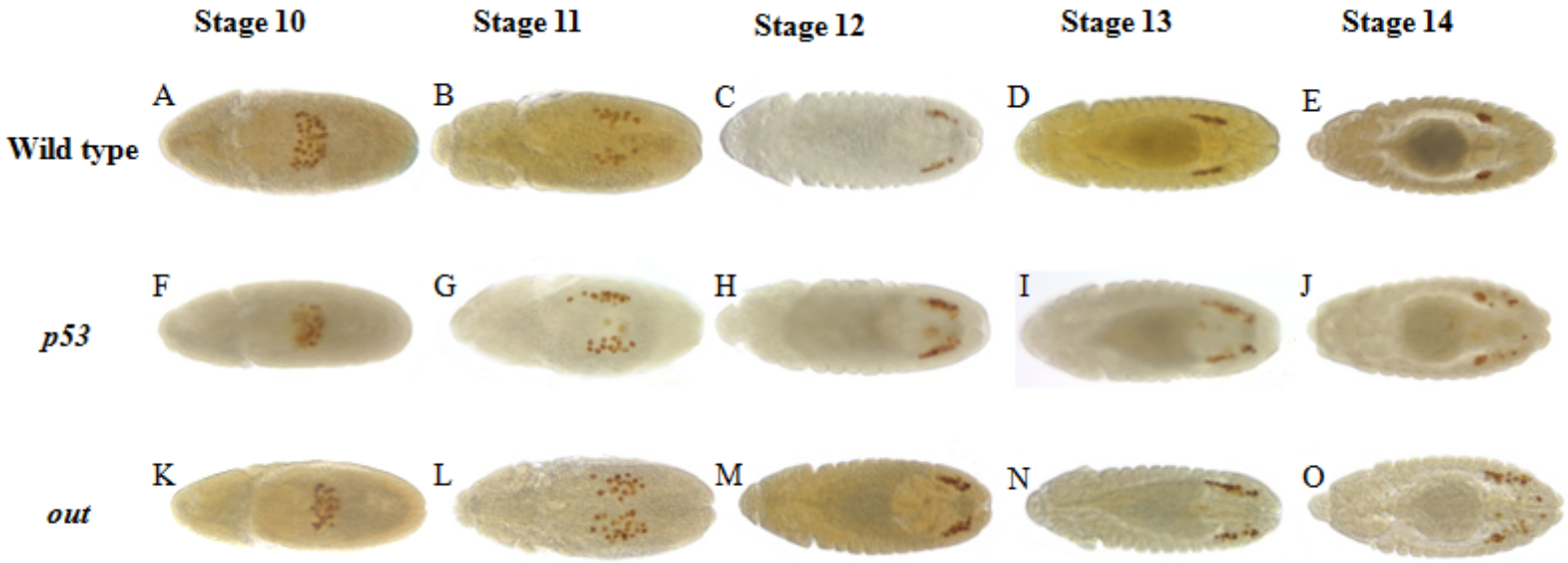

Figure 2 2. Dorsal views of *p53* and *outsiders* PGC development showed that mutants initiated migration and formed bilateral clusters similar to wild type embryos.
Anterior is left in all figures. PGCs are labeled with anti-Vasa antibody. **(A)** At stage 10, the wild-type PGCs moved out of the the posterior midgut toward mesoderm. **(F, K)** The PGCs movement in both *p53* and *out* appeared normal. **(B)** At stage 11, bilateral segregation of PGCs took place in the wild-type embryos. **(G)** PGCs in *p53* mutants were able to form bilateral clusters. However, there were a few PGCs left in the midline. **(L)** Formation of bilateral clusters was also observed in the *out* mutants. **(C)** During stage 12, PGCs formed clusters. **(H, M)** This was also seen in *p53* and *out* mutants. **(H)** Note that a few PGCs still remained in the midline of *p53* embryos. **(D)** At stage 13, the wild-type PGCs formed a tightly associated line of cells. **(I, N)** In both *p53* and *out* embryos, most PGCs were still in tight clusters, however, several isoloated PGCs were often observed. **(E)** At stage 14, PGCs in wild-type embryo coalesced with SGPs to from the gonads. Very few ectopic PGCs were observed. (**J**, **O**) *p53* and *out* mutant embryos exhibited very similar phenotypes. PGCs are able to migrate to the gonads, however many errant PGCs still persist ectopic to the gonads.

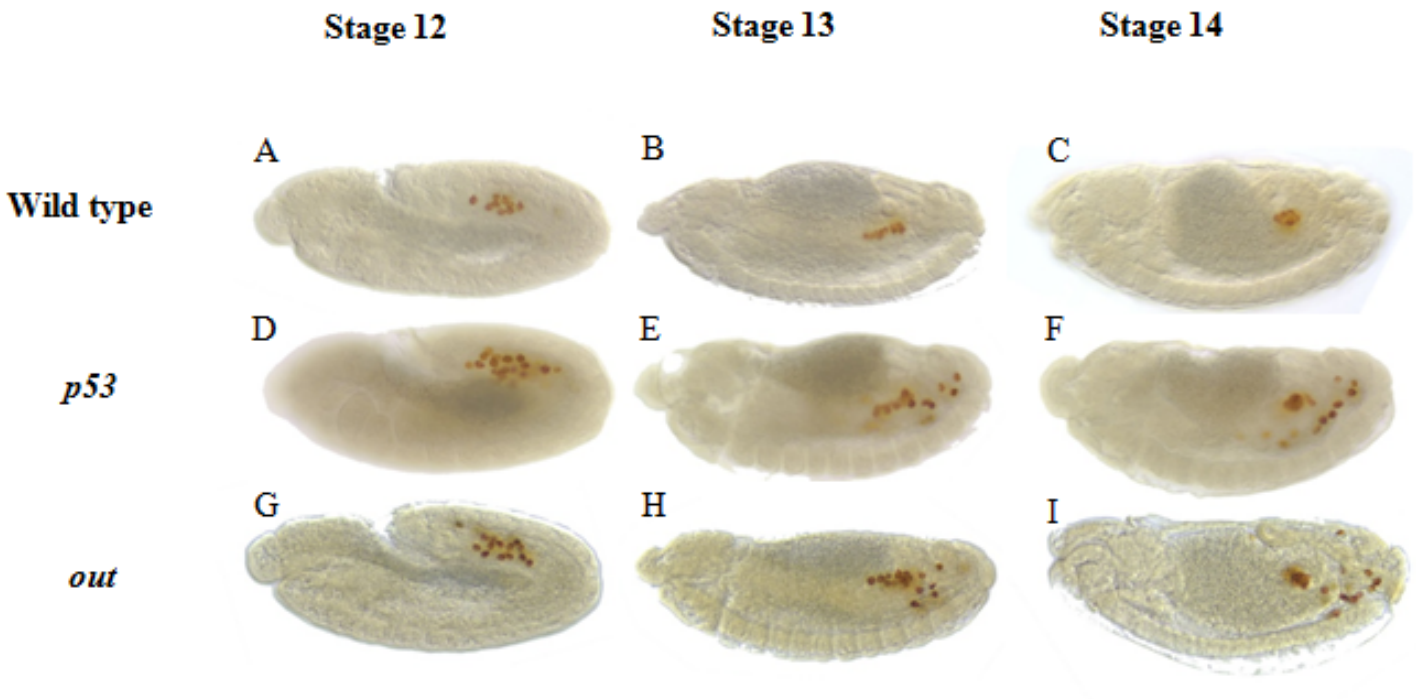

Figure 2.3. Lateral views of *p53* and *outsiders* PGC development showed that germ cells initiated migration, but germ cells ectopic to the gonads failed to die.
Anterior is left and dorsal up in all figures. PGCs are labeled with anti-Vasa antibody. **(A, D, G)** PGCs in stage 12 embryos were found in the mesoderm in wild type, *p53* and *out* embryos. **(B)** At stage 13, PGCs formed a band of cells and were associated with one another in wild-type embryos. **(E, H)** As some PGCs continued to form clusters, isolation of several PGCs became apparent in *p53* and *out* embryos. **(C)** At stage 14, wild-type PGCs reached the gonads, forming a colony. **(F, I)** In the mutants, normal numbers of PGCs successfully migrated to the gonads while errant PGCs were found ectopic to the gonads in mutants.

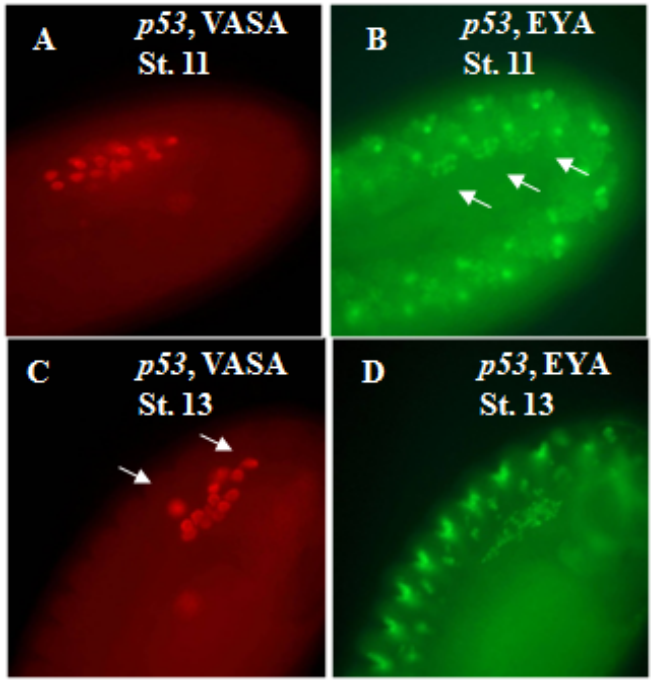

Figure 2.4. Double labeling for PGCs and SGPs showed that SGPs formed normally, but that some germ cells failed to associate with the SGPs.

Anterior is left in A and B and downwards in C and D. (**A**) Stage 11 *p53* embryo. PGCs were stained with an anti-VASA antibody. PGCs are seen migrating toward the SGPs. (**B**) Stage 11 *p53* embryo. SGPs were stained with an anti-EYA antibody. SGPs were specified in 3 clusters in parasegments 10-12. (arrows). (**C**) Stage 13 *p53* embryo. PGCs were stained with an anti-VASA antibody. The arrows indicate errant germ cells that failed to align with SGPs. (**D**) Stage 13 *p53* embryo. SGPs are stained with anti-EYA.

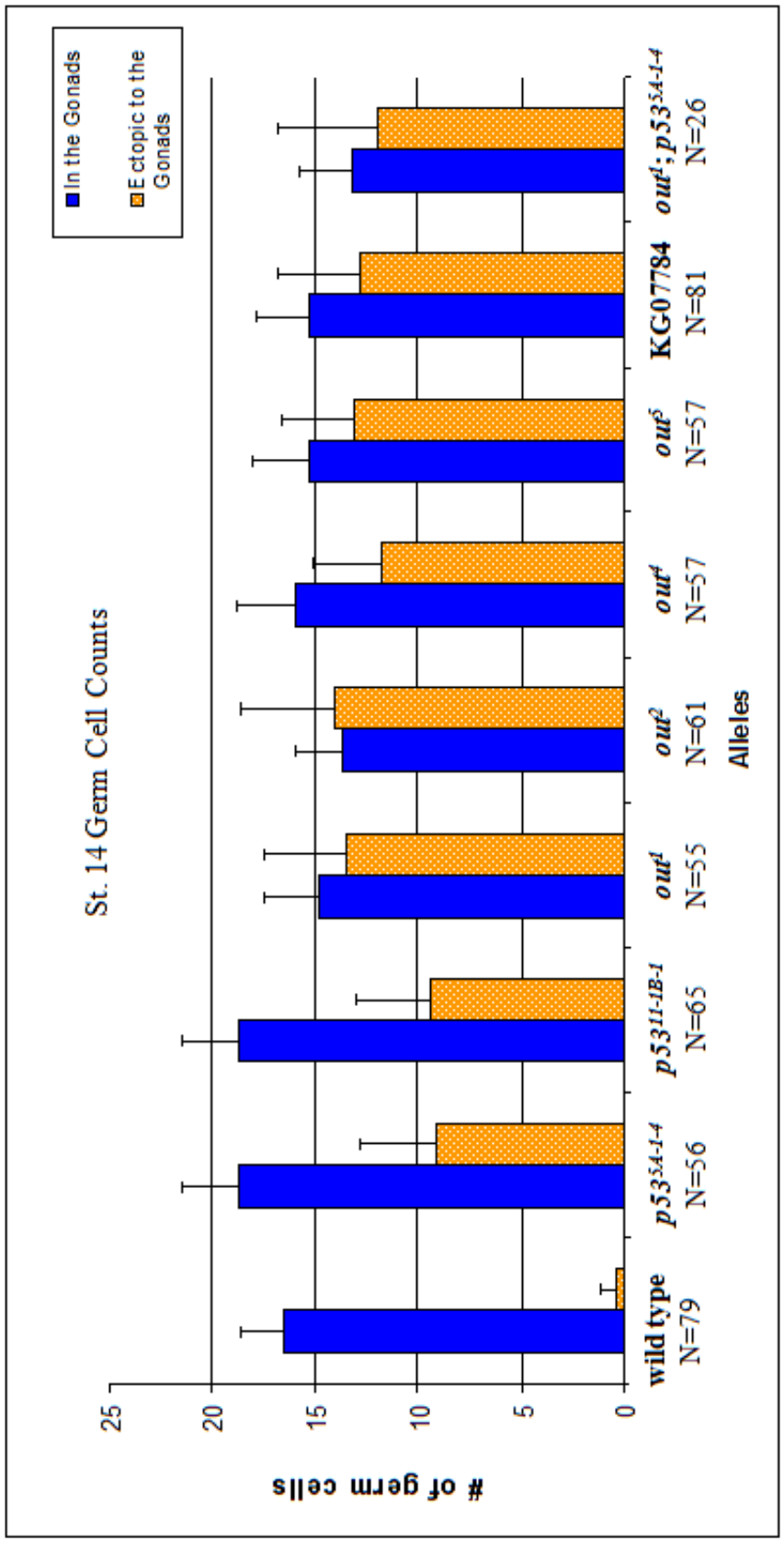

Figure 2.5. Germ cells ectopic to the gonads persist in *p53* and *out* mutants. Analyses of PGC distribution inside or outside of the gonads at stage 14 are shown. For each embryo examined, PGCs in the gonads (blue bars) and PGCs ectopic to the gonads (orange bars) were determined. PGC incorporation into the gonads was similar in wild type, *p53*, and *out*. 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 respresent s.d. See Supplemental Table 2 for additional data.

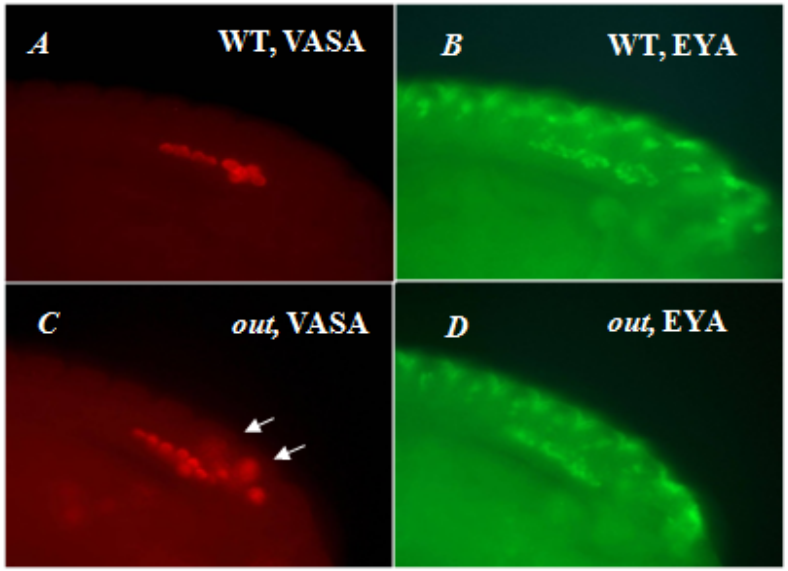

Figure 2.6. Double labeling for PGCs and SGPs reveals *out* germ cells failing to associate with somatic gonad precursor cells.

(**A**) Stage 13 wild-type embryo. PGCs were stained with an anti-VASA antibody and formed elongated clusters. (**B**) Stage 13 wild-type embryo. SGPs are stained with an anti-EYA antibody. SGPs aligned with the PGC staining. (**C**) A Stage 13 *out* embryo is shown. PGCs were stained with an anti-VASA antibody. The arrows indicate errant germ cells that failed to align with SGPs. (**D**) Stage 13 *out* embryo. SGPs are stained with an anti-EYA antibody. The formation of SGPs appeared normal.

Figure 2.7. 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**) *p53* mutants displayed germ cells ectopic to the gonads. (**C**) The *out*^{1} ; *p53* double mutant appears very similar to the *p53* mutants. The germ cells in the double mutant are labeled by using an anti-β-GAL antibody to label germ cells expressing the *fat facets-lacZ* transgene.

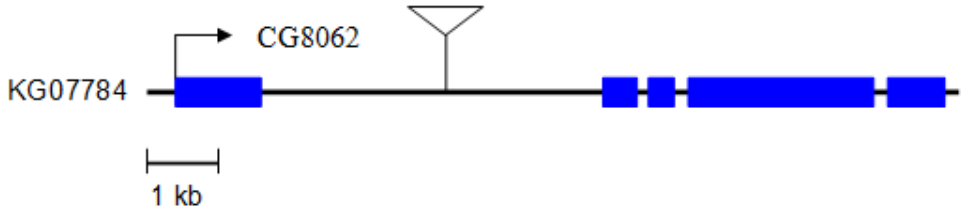

Figure 2.8. A P-element insertion in CG8062 disrupts germ cell PCD and this allele fails to complement *out*.
CG8062 is predicted to contain five exons and four introns. The P-element lies between exons 1 and 2.

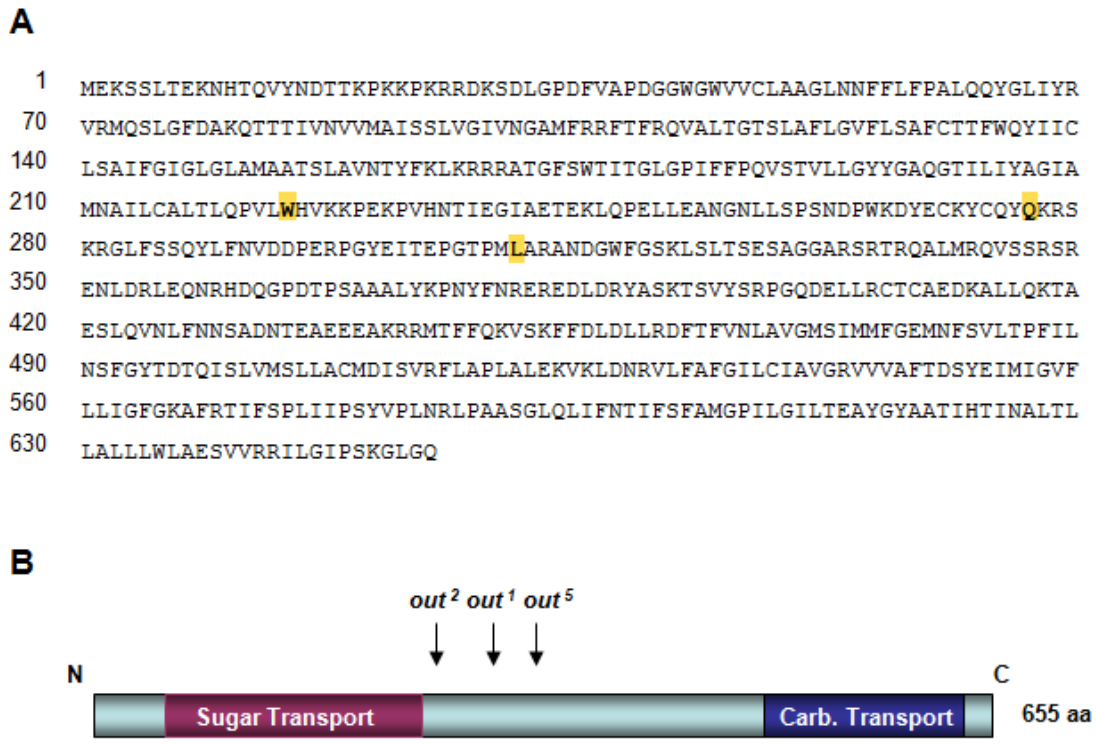

Figure 2.9. Three out alleles introduce premature stop codons in the CG8062 transcript.

(**A**) Amino acid sequence of OUT. The wild-type OUT peptide is comprised of 655 amino acids. The positions of the stop codons introduced by the alkylating agent ethyl methanesulfonate (EMS) are indicated by the bold letters and yellow boxes. (**B**) 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 *out*^{2}, *out*^{1}, and *out*^{5}, respectively.

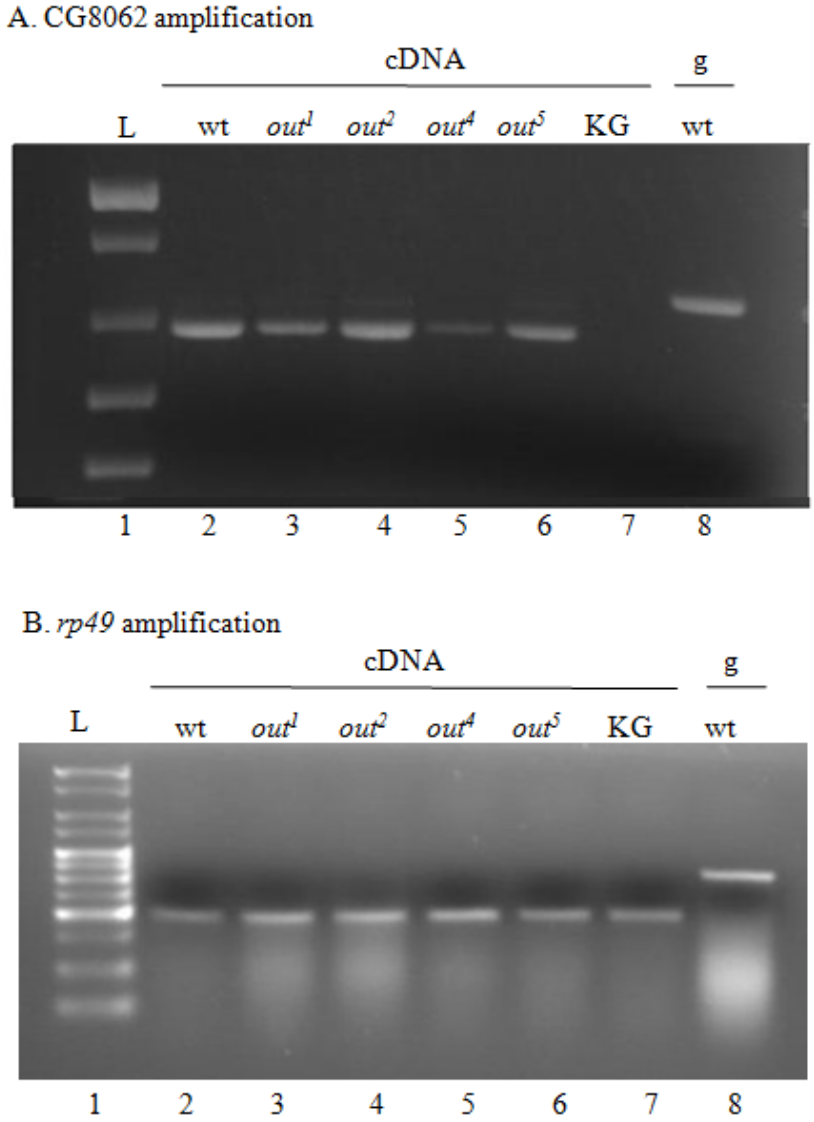

Figure 2.10. RT-PCR analysis of CG8062 expression. (**A**) Primers specific for CG8062 were designed to amplify a 634-bp cDNA, spanning exons 3 and 4. RNA was extracted from 0-15 hr old embryos. cDNAs derived from *out*^{1}, *out*^{2}, *out*^{4}, *out*^{5} and wild type amplified a 634-bp cDNA fragment of CG8062 (lanes 2-6). The CG8062 transcript was not detected in KG07784 (lane 7). The 716-bp fragment amplified from the genomic DNA containing intron 3 is shown as a control (lane 8). (**B**) Primers specific for *ribosomal protein 49* were used as a positive control. All samples amplified a 95bp cDNA fragment. The 157-bp genomic DNA fragment is also shown. (lane 8).

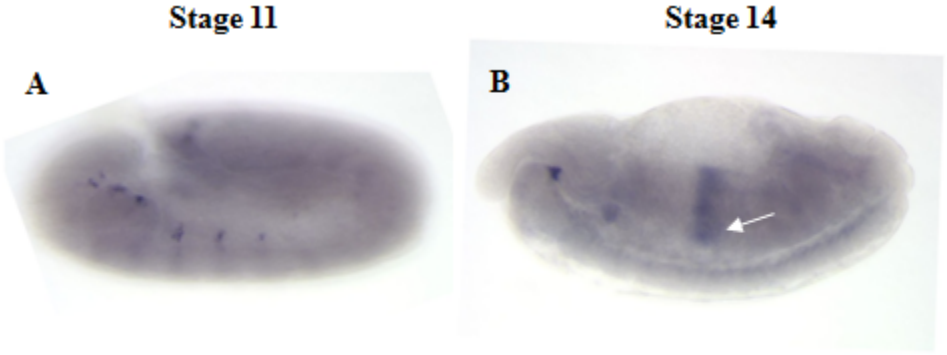

Figure 3.1. *out* RNA expression in soma.

Anterior is leftward. Lateral views are shown. (A) At stage 11, ventral epidermis primordium and atrium primordiam express *out* in anterior regions. Low levels of expression is also seen in the mesoderm. (B, arrow) At stage 14, midgut expression of *out* is observed. Also, hypopharynx expresses *out*.

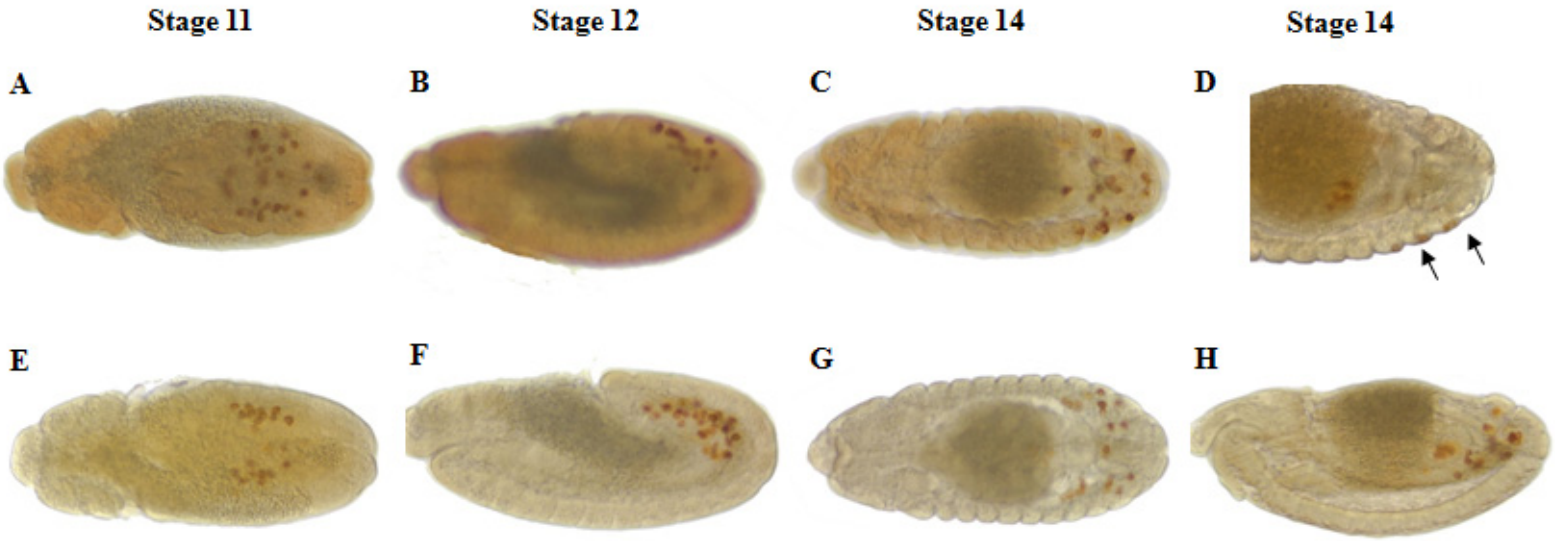

Figure 3.2. Phenotypes associated with *out* expression in somatic tissues.

Anterior is leftward. (A) At stage 11, KG07784; *sca*-GAL4; UAS-*out* embryo is defective for bilateral segregation of PGCs. Several PGCs remain in the midline. (E)The wild-type embryo has no midline PGCs. (B) At stage 12, PGCs in KG07784; *sca*-GAL4; UAS-*out* embryos are found in the ectoderm instead of mesoderm. (C) At stage 14, very few germ cells reach the gonads, leaving a scattered PGC phenotype. (D, arrows) Magnified view of the embryo reveals localization of PGCs in the epidermis. (F) KG07784; *da*-GAL4; UAS-*out* embryo shows that PGCs successfully migrate to the mesoderm at stage 12. (G) and (H) At stage 14, KG07784; *da*-GAL4; UAS-*out* embryos display germ cell phenotype very similar to *out*. Some germ cells successfully reach the gonads while errant PGCs persist ectopic to the gonads.

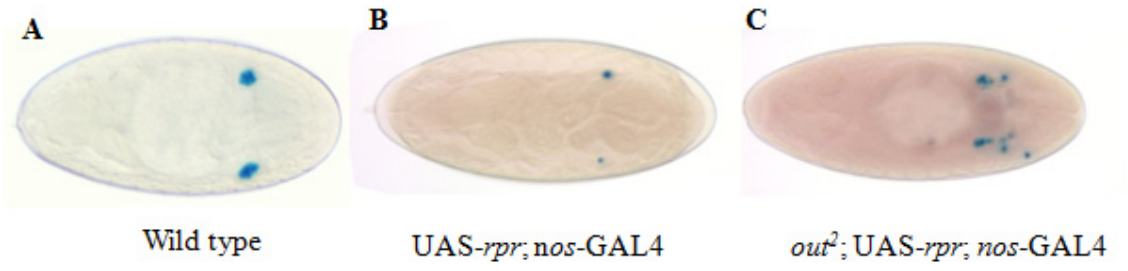

Figure 4.1. *reaper* expression in germ cells of wild-type and *out* mutants.
Anterior is leftwards. (A) The wild embryo has ~8 germ cells per gonad. No ectopic germ cells are observed. (B) UAS-*rpr*; nos-GAL4 embryo has 3-4 germ cells in the gonads. (C) *out*^{2}; UAS-*rpr*; *nos*-GAL4 shows reduced number of germ cells in the gonads. Errant germ cells still persist outside the gonads.

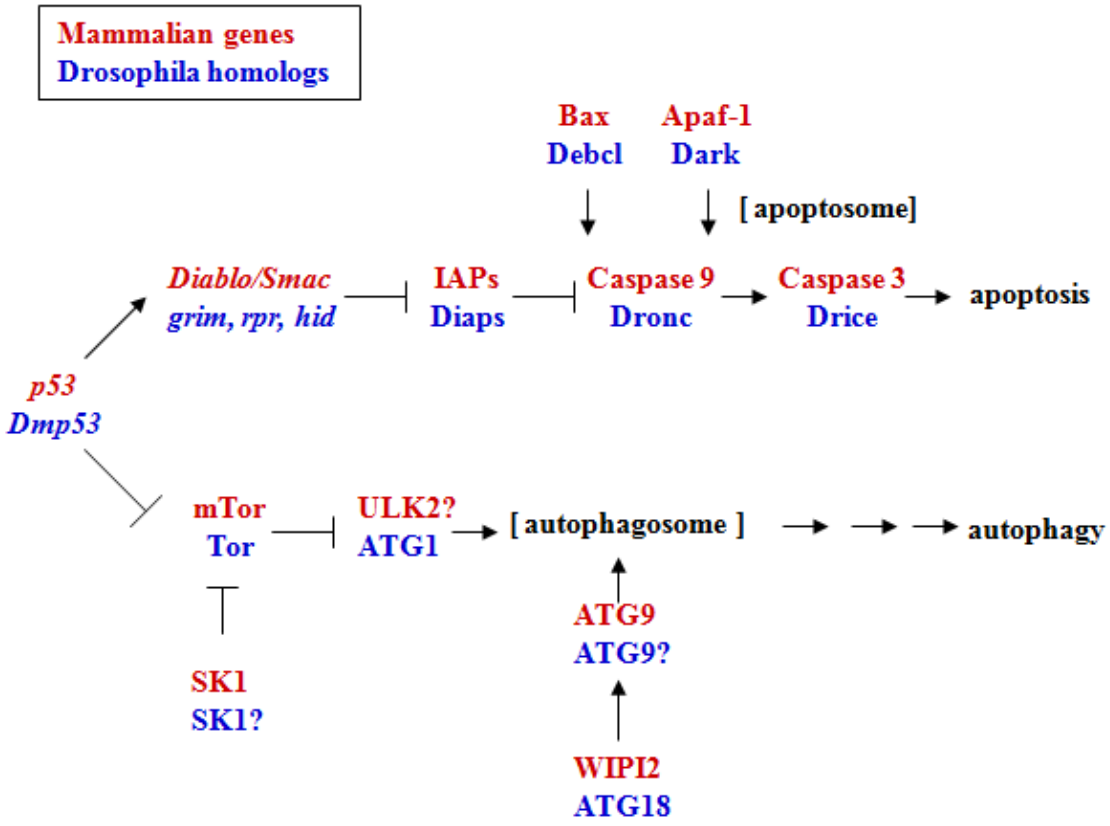

Figure 5.1. Molecular model for PCD pathways mediated by p53.

Mammalian genes are in red and Drosophila homologs are in blue. *p53* has been shown to be involved in both apoptosis and autophagy. *grim*, *rpr*, and *hid* are functional homologs of Diablo/Smac. Unlike *rpr* and *hid*, Diablo/Smac has not been shown to be activated by *p53*. SK1 in Drosophila and ULK2 show sequence similarities to their homologs. However, their role in autophagy have not been investigated. ATG9 has not been shown to be required for autophagy. However, its expression in upregulated upon autophagy.

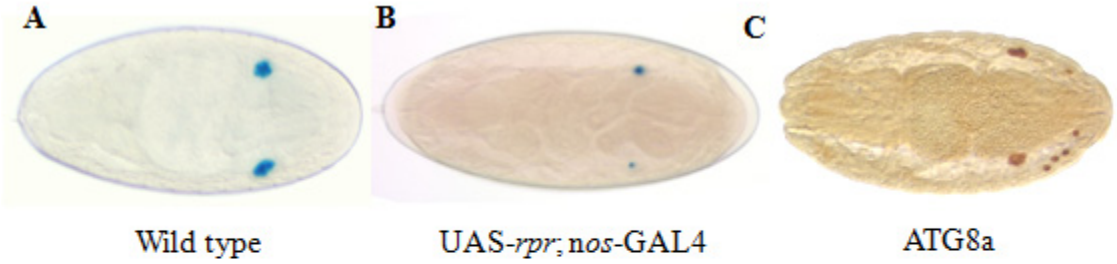

Figure 5.2. Cell death mutants.

Dorsal view of the embryos at stage 14. Anterior is leftwards. (A) Wild type embryo contains no ectopic PGCs are found. (B) PGC specific-*rpr*-expression was driven in wild-type embryo. Very few PGCs are left in the gonads. (C) ATG8a mutant embryo has several PGCs ectopic to the gonads.

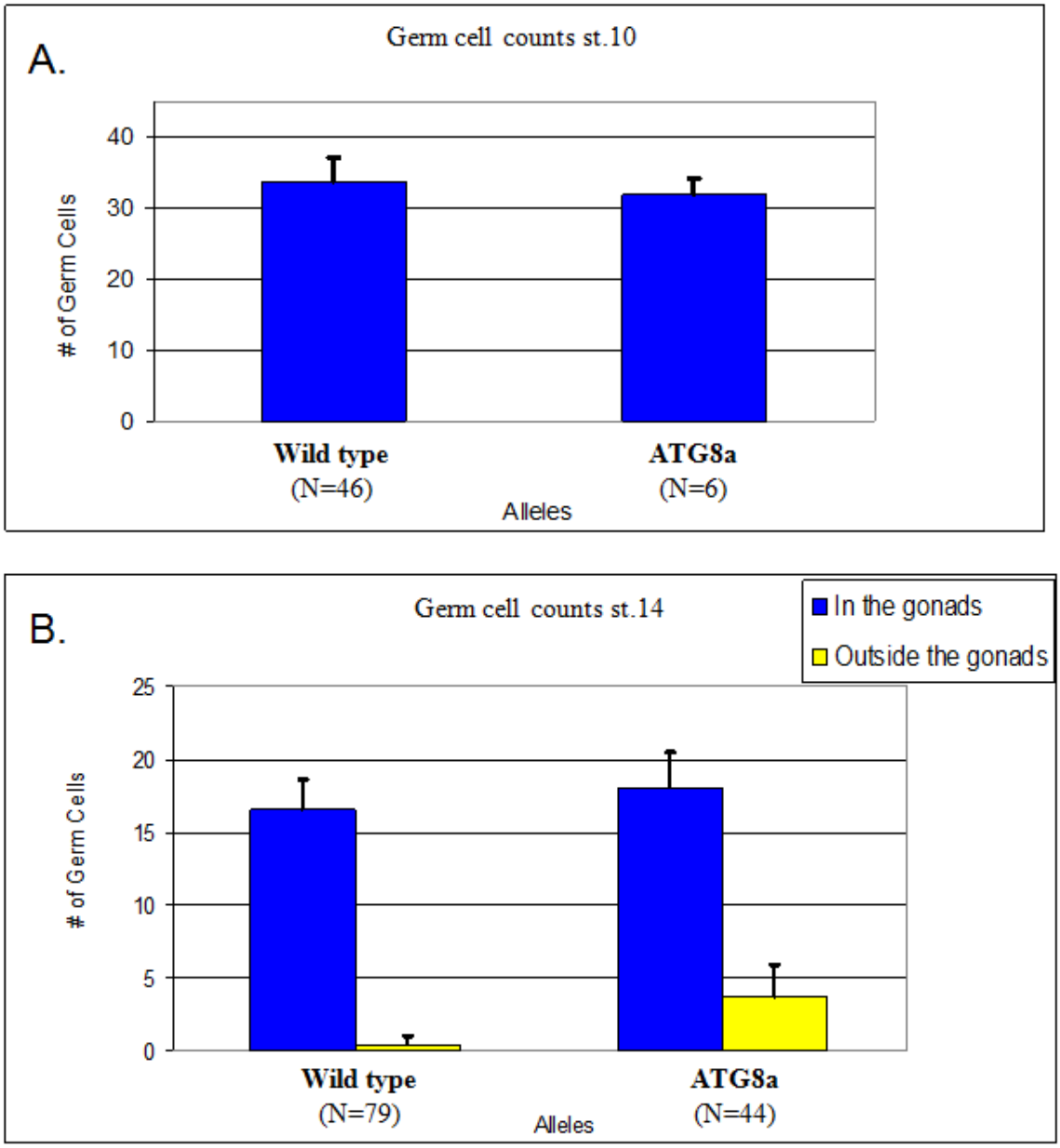

Figure 5.3. Germ cell counts of ATG8a mutants.

Error bars represent standard deviations. Graph A shows stage 11 PGC counts. The wild-type number of PGCs is produced in ATG8a mutants. Graph B shows stage 14 PGC counts. The wild-type number of PGCs are incorporated into the gonads. However, more PGCs remain ectopic to the gonads in ATG8a mutants. Ectopic germ cell counts in ATG8a showed the range from 1 to 9 germ cells. On contrast, ectopic germ cells counts in germ cells showed the range from 0 to 3 germ cells.

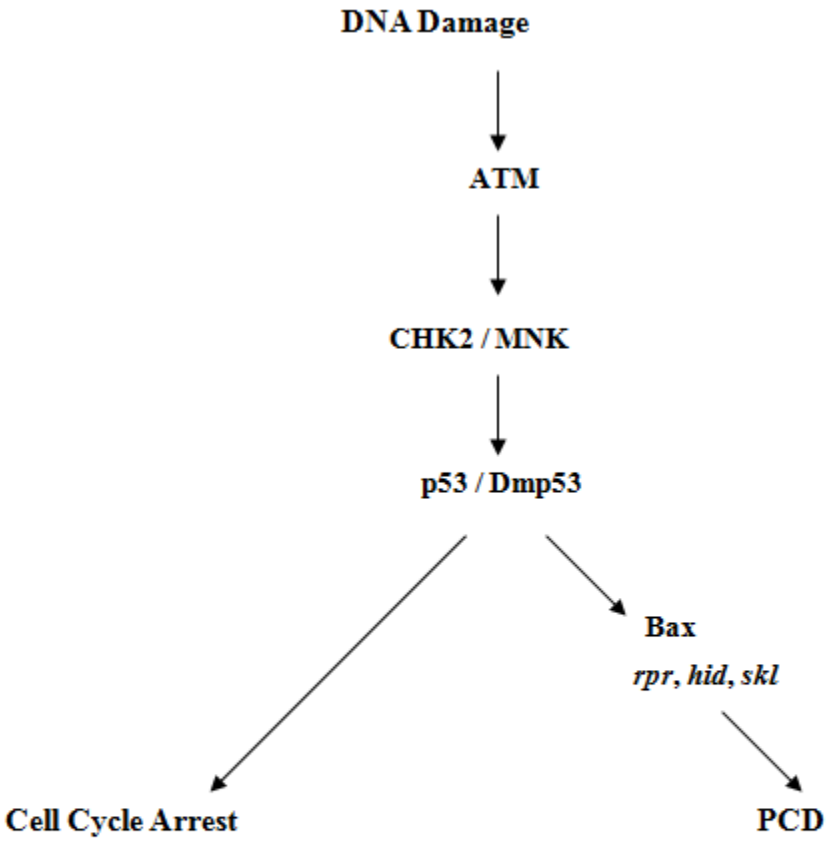

Figure 6.1. A simplified model of DNA damage-induced cellular responses. Core components of the DNA damage signaling in mammals and Drosophila. The DNA damage is sensed by ATM. The damage signaling is relayed and amplified by CHK2/MNK kinase. The downstream effector p53 is involved in PCD and cell cycle arrest responses in this signaling cascade. p53 regulates *Bax*, *rpr*, *hid*, and *skl* pro-PCD genes.

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