Identification of genes involved in germ cell programmed cell death in *Drosophila melanogaster*

by

Keri D. Davis

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Clark R. Coffman, Major Professor
Jeffrey J. Essner
Jo Anne Powell-Coffman

Iowa State University

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CHAPTER 1: General Introduction

Overview

Two central abilities of a metastatic cell are the ability to become migratory and the ability to overcome cell death signals. The knowledge gained from being able to study these two processes *in vivo* is essential to being able to develop new cancer therapies. Germ cell development in Drosophila is a wonderful model system to study. In normal development, germ cells must travel across tissues and overcome cell death signals in order to reach their ultimate location. Here I will describe the basics of germ cell development in Drosophila, explaining the basic migratory movements and cell death components of this system. One of these components, Outsiders, is a monocarboxylate transporter involved in germ cell death. I will briefly review how monocarboxylate transporters could be involved in this cell death process. An additional component involved in germ cell death is P53. This gene is especially intriguing as P53 is mutated in over 50% of human tumors (Greenblatt et al., 1994). I will review how P53 is involved cell death and also how it is regulated by posttranslational modifications specifically by acetylation.

Germ Cell Development In Drosophila

In Drosophila, germ cell development starts soon after fertilization. It begins with 8-10 nuclei moving into the germ plasm at the posterior pole and mitotically dividing 1-2 times. The germ cells undergo cellularization and may divide 1-2 times again until 30-40 pole cells are formed at the posterior pole (Sonnenblick, 1941; Underwood et al., 1980; Williamson and Lehmann, 1996). After these mitotic divisions the germ cells arrest in G2 where they do not proliferate again until late embryogenesis (Deshpande et al., 1999; Williamson and Lehmann, 1996). Two genes that repress mitotic division in the germline

are *nanos* and *pumilio*. Nanos and Pumilio were found to bind *cyclin B* mRNA. Cyclin B promotes the cell to enter mitosis (Asaoka-Taguchi et al., 1999; Kadyrova et al., 2007). Drosophila germ cells are unique from somatic cells in that they are one of the first cell types to cellularize yet do not transcribe their own mRNAs until later stages of development (Kobayashi et al., 1996). It is hypothesized that repressing transcription and isolating the cells outside of the embryo protects them from transdifferentiating into some other somatic cell type. Because they are transcriptionally repressed, germ cells are formed from maternally contributed germ plasm. The germ plasm is electron dense and contains the mRNA and protein that are needed to form pole cells such as Vasa, Oskar, Tudor, Nanos (Williamson and Lehmann, 1996). Studies using transplanted germ plasm have demonstrated that all required components to establish the germline are contained in the germ plasm as pole cells will form at the site of the transplanted germ plasm (Illmensee and Mahowald, 1974).

During the early stages of gastrulation the germ cells (associated with the posterior pole) are passively swept over the dorsal side of the embryo and into the posterior midgut primordium. It is here, around stage 8 where the germ cells start transcribing zygotic mRNA (Van Doren et al., 1998b; Zalokar, 1976).

In order to become migratory the germ cell changes its morphology from a smooth spherical cell to an amoeboid shape with protrusions. This happens at stage 10 (Callaini et al., 1995; Kunwar et al., 2008). The germ cells can then move through the midgut epithelial layer and into the mesoderm. A major player in transepithelial migration is the G protein-coupled receptor, Tre1. Tre1 has been identified as having a role in establishing germ cell polarity and down regulation of the adhesion protein E-cadherin. Tre1 was shown to be essential for the migration out of the midgut (Kunwar et al., 2008; Kunwar et al., 2003). Once in contact with the mesoderm, the germ cells bilaterally

segregate into two populations and migrate to the somatic gonadal precursor cells, which will ultimately form the presumptive gonad (Kunwar et al., 2006; Williamson and Lehmann, 1996). This process involves the coordination of many genes. For example, Wunen and Wunen 2 provide repulsive signals, which signal the germ cells to move towards the lateral mesoderm and away from the midline, segregating into two bilateral populations (Sano et al., 2005; Zhang et al., 1997). There are also attractive signals to guide the germ cells to the mesoderm and to the somatic gonadal precursor cells. These signals require lipid synthesis and Hedgehog signaling. One protein necessary for generating an attractive signal is Columbus, a HMG CoA reductase. Germ cells in embryos mutant for Columbus have migratory defects and fail to reach the lateral mesoderm (Renault and Lehmann, 2006; Van Doren et al., 1998a). Mutant alleles in other genes of the HMG CoA reductase pathway, for example Farnesyl-diphosphate synthase, have also been identified in having germ cell migration defects indicating a migratory signal maybe being produced via this pathway (Deshpande and Schedl, 2005). Once the germ cells have migrated and have met up with the somatic gonadal precursor cells (SGPs) multiple genes, *fear of intimacy* and *shotgun* being two examples, are employed to allow the SGPs to coalesce with the germ cells to form a compact gonad (Jenkins et al., 2003; Van Doren et al., 2003).

Germ Cell Death in Drosophila

Migration of primordial germ cells has been extensively studied and some genetic components of this process have been elucidated. However not much is known about the other part of germ cell development, the programmed cell death (PCD) of germ cells. Studies have shown that approximately half of the germ cells that are initially formed do not reach the gonad and are eliminated (Coffman et al., 2002; Technau, 1987; Underwood et al., 1980). Extensive germ cell counts showed that in wild-type embryos

the vast majority of germ cells programmed to die do so in stages 10-12 of embryogenesis (Yamada et al., 2008). These lost germ cells are predicted to be eliminated by PCD as it has been shown that germ cells do not seem to transdifferentiate during development (Technau, 1987; Underwood et al., 1980). The mechanism and the genetic components of germ cell death are still elusive. Here I will review some of what is currently known about germ cell death in Drosophila.

In Drosophila it has been shown that apoptosis is the main form of cell death in early embryogenesis and it is mediated by three proapoptotic genes *reaper, grim, and hid* (Abbott and Lengyel, 1991; Chen et al., 1996; Steller et al., 1994; White et al., 1994). Because of the massive apoptosis in early embryogenesis, it was unexpected to discover that embryos lacking these three important apoptotic genes zygotically were still able to initiate a PCD program in the germ cells (Sano et al., 2005; Yamada, unpublished). It has not been tested whether these genes have a maternal effect on germ cell PCD. In addition over expression of inhibitors of apoptosis such as DIAP1, DIAP2, and p35 were also unable to interfere with germ cell PCD (Hanyu-Nakamura et al., 2004). Studies with lipid phosphate phosphatase Wunen and Wunen 2 have also given some insight on germ cell death. Germ cells deficient for Wunen 2 undergo cell death. In addition, over expression of Wunen and Wunen 2 in the somatic cells result in germ cell death. This cell death is independent of the zygotic expression of *reaper, hid,* and *grim*. The germ cells that die are also unaffected by overexpressing a dominant negative from of caspase Dronc and they are TUNEL negative (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Sano et al., 2005). This evidence has led us to conclude that the germ cells most likely do not partake in a traditional caspase mediated form of cell death or have multiple means of dying.

Contrary to the evidence described above it has been shown that germ cells are able

to initiate apoptotic machinery and induce cell death under certain conditions. Germ cells in embryos mutant for *nanos* will die during development as they are migrating to the gonads (Forbes and Lehmann, 1998; Hayashi et al., 2004; Kobayashi et al., 1996). This cell death was shown to be apoptotic and it was shown to be *hid*, *grim*, and *sickle* dependent as removing these three genes represses *nanos* stimulated cell death. Nanos is also required for the establishment of the germ line. Germ cells in *nanos* mutant embryos lose the germ cell specific marker Vasa and also start expressing somatic cell markers (Hayashi et al., 2004). It has also been demonstrated that overexpressing genes in the apoptotic pathway, such as *reaper*, induces massive cell death (Sato et al., 2007, Yamada, unpublished).

P53 and Outsiders mediate germ cell PCD

Recently, we characterized two genes involved in Drosophila germ cell death, *outsiders* (*out*), a putative monocarboxylate transporter, and *p53,* a transcription factor well known for its role in cell death in a multitude of organisms (Yamada et al., 2008). The *out* gene was initially discovered in an EMS mutagenesis screen identifying X-linked genes required in germ cell development. Recombination and deletion mapping mapped the *out* alleles to 18C on the X chromosome (Coffman et al., 2002). P-elements in this region were crossed to the *out* alleles and one P-element inserted in gene CG8062 failed to complement the *out* alleles. The gene CG8062, encodes for a putative monocarboxylate transporter. Inverse PCR was performed to confirm that the P-element was disrupting this gene. Finally sequencing of the CG8062 gene in the *out* alleles isolated from the EMS screen revealed mutations in this gene. Characterization of the *out* alleles revealed that this gene was involved in germ cell death but the migration of the germ cells to the gonads was normal. In *out* mutants an average of 10 germ cells remain ectopic to the gonads. In wild type embryos excess germ cells are efficiently eliminated,

with an average of only 0.4 germ cells remaining ectopic to the gonads (Coffman et al., 2002).

Along with Out, Drosophila P53 (Dmp53) was also shown to be involved in germ cell death in Drosophila. When visualized for a germ cell phenotype, *dmp53* mutant embryos have a similar phenotype to *outsiders* mutant embryos (Yamada et al., 2008). Because of the similarity, it was hypothesized that these two genes could be working together to mediate germ cell PCD. Indeed both transheterozygote and double mutant analysis revealed a disruption in germ cell PCD, indicating that Dmp53 and Out worked in the same or parallel pathways. In addition to this data, it was shown that over expressing *dmp53* in an *out* mutant background could restore the germ cell death. Because expression of Dmp53 is able to mediate the germ cell death program in Drosophila in an *out* mutant background, it suggests that Out could be acting upstream of Dmp53 (Yamada et al., 2008).

Monocarboxylate Transporters

Out has been identified as a moncarboxylate transporter (MCT) based on its sequence similarity to other known proteins with monocarboxylate transporter activity. Out is one of the 18 predicted MCTs predicted to be encoded in the Drosophila genome (Crosby et al., 2007). MCTs are multi-transmembrane proteins that can reside either at the mitochondrial membrane or the plasma membrane. As their name implies, a main function of MCTs is to transport monocarboxylates, for example pyruvate or lactate across membranes (Poole and Halestrap, 1993). However because they transport important metabolites, such as pyruvate and lactate, MCTs are involved in many metabolic pathways giving MCTs an essential role in cell metabolism. One major pathway that MCTs are utilized in is glycolysis. In order to keep intracellular levels of lactate (the end product of glycolysis) in the cell down, MCTs must continually shuttle lactate out of

the cell. This transport allows the cell to keep its intracellular pH stable, as well as allowing glycolysis to continue so the cell can produce ATP to function. Other metabolic pathways that MCTs are involved in are ketone-body metabolism, gluconeogenesis, and the cori cycle (Enerson and Drewes, 2003; Halestrap and Price, 1999). Along with transporting important monocarboxylates, this transport is usually coupled with H^+, K^+, or Na^+ ions making transport electrogenic (Halestrap and Price, 1999).

One important protein that has been shown to interact with MCTs is CD147. CD147 is an immunoglobin glycoprotein that has been shown to be an inducer of matrix metalloproteinases that decrease cell adhesion during metastasis. It has been shown that increased expression of CD147 leads to an increased expression of MCT1 as CD147 helps transport MCTs to the plasma membrane (Kirk et al., 2000). This protein has also been correlated with tumor growth and invasiveness (Yan et al., 2005). Basigin, is the Drosophila homolog of CD147, and currently it is unknown whether Basigin interacts with Outsiders as it does in mammals (Crosby et al., 2007).

SLC5A8

In 2003 Li et al. identified SLC5A8 as a tumor suppressor. SLC5A8 encodes for a Na^+ coupled monocarboxylate transporter. It was first described as having a tumor suppressive role because it was observed that SLC5A8 was down regulated in multiple cancers due to hypermethylation (Coady et al., 2004; Ganapathy et al., 2005; Li et al., 2003; Miyauchi et al., 2004). Hypermethylation of SLC5A8 was particularly noticed in colon and gastric cancers. Cell culture work demonstrated that SLC5A8 expression coupled with the transport of pyruvate induced apoptosis in cell lines. Other short-chained fatty acids had this effect but not lactate. This is interesting to note that lactate did not induce apoptosis because one property of cancerous cells is to upregulate glycolysis and rapidly turn over pyruvate into lactate (Thangaraju et al., 2006). Pyruvate as well as other

short-chained fatty acids (SCFA) are considered histone deacetylase (HDAC) inhibitors. HDAC inhibitors have been shown to help increase the acetylation of histones and help induce gene transcription. Studies looking at the transport of SCFA by SLC5A8 have shown that there is more histone acetylation as well as upregulation of proapoptotic genes, including *p53*. In P53 neuroblastoma cells, in the presence of HDAC inhibitors, was shown to be hyper-acetylated. and this was correlated with increased cell death (Condorelli et al., 2008).

Siloon and LKB1

LKB1 encodes for a serine/threonine kinase. It was identified as a tumor suppressor and was shown to be required for P53-dependent apoptosis (Karuman et al., 2001). In Drosophila, LKB1 has also been shown to be involved in JNK-mediated apoptosis. However Dmp53 does not seem to be involved (Lee et al., 2006). Recently another MCT, Silnoon, was identified in Drosophila as being involved in LKB1-mediated cell death. It was shown that LKB1 and Silnoon are expressed together and when introduced to different monocarboxylates, LKB1 and Silnoon expressing cells initiated cell death. It was concluded that LKB1 kinase activity was necessary for this cell death as well as Silnoon's ability to transport monocarboxylates but it was unclear the exact mechanism by which these two genes are working together to mediate cell death (Jang et al., 2008; Lee et al., 2006). Besides Outsiders, Silnoon is the only other MCT in Drosophila to be identified in cell death. It is possible that Outsiders and Silnoon could be using similar mechanisms to mediate cell death but this has yet to be investigated.

Overview of P53 in Drosophila

In 2000, the identification of the Drosophila homolog of P53 (Dmp53) was confirmed by three laboratories (Brodsky et al., 2000; Jin et al., 2000; Ollmann et al., 2000). At the time of the initial identification, it was not apparent Dmp53 had a role in normal development as

dmp53 mutants are healthy and viable. Even now the only apparent phenotype under normal development that *dmp53* mutants show is in germ cell PCD (Yamada et al., 2008). Consequently many of the studies done on Dmp53 have been on exposing its role in DNA damage stress response. Dmp53 was first shown to be involved in the *reaper* response to irradiation. After irradiation, *reaper* mRNA is immediately transcribed and this response results in cell death (Nordstrom et al., 1996). This response to irradiation was found to be *dmp53* dependent as apoptosis was not induced in *dmp53* embryos after irradation. It was shown that *reaper* contains a *dmp53* response element and that upon DNA damage Dmp53 binds to reaper to induce the response (Brodsky et al., 2000; Sogame et al., 2003).

Another protein that has been shown to be integral to Dmp53 signaling is the kinase CHK2 (Brodsky et al., 2004; Peters et al., 2002). It was shown that over expression of Dmp53 in the eye induces apoptosis and this cell death is dependent on CHK2 as loss of function CHK2 resulted in inhibition of cell death. The same study showed that the Dmp53 apoptotic response to irradiation was also found to be dependent on CHK2. It was shown that after irradiation CHK2 phosphorylated Dmp53 (Brodsky et al., 2004). Deleting either p53 or CHK2 inhibited apoptosis, as well as over expressing a dominant negative form of CHK2. CHK2 however didn't affect Dmp53 protein levels though indicating that phosphorylation of Dmp53 increases its apoptotic activity (Peters et al., 2002; Brodsky et al., 2004).

As seen here, Dmp53 has been shown to be involved in apoptosis; it also potentially is involved in autophagy. In an RNAi screen looking for genes involved in starvation induced autophagy, *dmp53* was shown to inhibit autophagy when it's RNA expression was reduced (Hou et al., 2008). The role of P53 in autophagy has been explored in other systems, and it has been shown to sometimes inhibit the induction of autophagy but also it has been shown

to promote the induction of autophagy depending on the context (Tasdemir et al., 2008). At this time however, Dmp53's role in autophagy in Drosophila remains elusive.

Conservation of P53 Structure and Function

The Drosophila homolog of P53 was identified in a sequence homology search using human P53 (Hp53) (Ollmann et al., 2000). Dmp53 was initially found to be a protein 385 amino acids long, however more recently a second promoter was identified which has been shown to translate a 495 amino acid long isoform (Bourdon et al., 2005). This identification of a second promoter is only one of the key similarities between *hp53* and *dmp53*. Functionally it has been shown that the various isoforms of Hp53 have different expression patterns. For example *hp53*β mRNA is not found in muscle tissue but can be found in other tissues such as colon tissue and breast tissue. Whereas *hp53*γ*,* can be found in muscle tissue but not other tissues such as the testes. This is indicates the P53 isoforms may have distinct regulation and different functions. Three *dmp53* mRNAs from Drosophila have also been isolated, however it is unknown whether these mRNAs are tissue specific (Bourdon et al., 2005).

When identified, Dmp53 was shown to have structural homology to Hp53. Dmp53 and Hp53 were shown to contain a N-terminus transactivation domain, a central DNA binding domain, and lastly a C-terminus oligomerization domain. The Dmp53 DNA binding domain has the most homology to Hp53 as its amino acid sequence is 44% similar and 24% identical to Hp53 (Jin et al., 2000). With similar domains and structural homology, it was speculated that Dmp53 could reveal a conserved function for P53. Experiments were performed that showed that Dmp53 could bind Hp53 DNA binding sites and was able to activate transcription from these target sites (Jin et al., 2000). The C-terminus of Hp53 has been shown to have multiple sites for post-translational modifications. These modifications have been shown to regulate Hp53 activity (Harms and Chen, 2005; Mauri

et al., 2008; Weinberg et al., 2004). In addition, it has been shown that sumolyation of Hp53 is linked to transactivation and stabilization of Hp53 activity (Feng et al., 2005; Gostissa et al., 1999). In addition, Drosophila P53 has also been shown to be post-translationally modified by sumoylation. This sumoylation positively regulates Dmp53 and enhances its pro-apoptotic activity (Mauri et al., 2008).

P53 Acetylation

Because P53 has such an important role in determining cell fate it must be tightly regulated. Some of the post-translational modifications that have been shown to regulate P53 function are neddylation, ubiquination, methylation, and acetylation (Reviewed in Bode and Dong, 2004; Brooks and Gu, 2003; Xu, 2003). It has been said that P53 regulation is similar to the histone code in that different combinations of posttranslational modifications may lead to different p53 responses. For example ubiquination of P53 leads to P53 degradation while phosphorylation of P53 prevents ubiquination and allows P53 to become stabilized to induce the transcription of proapoptotic genes (Knights et al., 2006).

One post-translational modification, which has been under review, has been the acetylation of P53 by histone acetyltransferases (HATs). Hp53 was the first non-histone protein to be shown to be a target of HATs (Gu and Roeder, 1997). It has been shown that acetylated P53 is involved in inducing programmed cell death pathways. Specifically the role of two HATs, MOF and Tip60, have been examined in Hp53 signaling. These two HATs have been shown to be involved in Hp53 mediated cell death. However, the mechanism by which they act on Hp53 signaling is unclear (Tang et al., 2006). An RNAi screen has shown that Tip60 is required for the induction of Hp53 while another study showed that Tip60 had a co-activator role to p53 to induce apoptosis as well as helping prevent Hp53 from being degraded (Berns et al., 2004; Legube et al., 2004; Tang et al., 2006).

A main focus of my study is the characterization of the gene, *enoki mushroom* (*enok*). Enok is part of the MYST family of histone acetyltransferases. The MYST family was named after the initial proteins found in this family, Moz, Ybf/Sas2, Sas3, and Tip60 (Avvakumov and Cote, 2007). Enok has the most homology to human MOZ and MORF because of the additional NEMM (N-terminal part of Enok, MOZ, or MORF) domain. Human MOZ and MORF, when mutated have been shown to be linked to cancers such as acute myeloid leukemia. However, there has not been a link found between these two MYST family HATs and P53 as there is with Tip60 (Yang and Ullah, 2007). Enok also contains a zinc finger domain, a MYST HAT domain, and a neurofilament like domain. Not many studies have been done on *enok.* Its only shown biological function has been cell proliferation of mushroom bodies in Drosophila (Scott et al., 2001). A genome wide RNAi screen has also shown that *enok* is potentially involved as a downstream target of JAK/STAT signaling (Muller et al., 2005).

Conclusions

Here I have taken you through a review of genes involved in Drosophila germ cell death, specifically Dmp53 and Outsiders as well as a review of the role MCTs and HATs have in cell death. The main goals of my study were to further elucidate genetic components involved in Dmp53 and Out- mediated PCD in germ cells. From the literature and previous work done in our lab, I set out to address some outstanding questions regarding germ cell death in Drosophila. These include: Are HATs involved in PCD of germ cells? If so, are they involved in Dmp53 and Outsiders PCD? What other genetic components besides HATs could be involved in germ cell PCD? My thesis project revolved around these broad questions. The following is an outline of the rest of my thesis, which explains how I set out to approach these questions.

Overview of Thesis

Chapter 2: A MYST family histone acetyltransferase, *enok*, is involved in *p53* mediated germ cell death in *Drosophila melanogaster.* This chapter will describe the initial characterization of *enok* and its role in germ cell death. Experimental design and crosses done by Keri Davis. Germ cell counts done by Keri Davis and Clark Coffman. Wild-type and p53 data and germ cell counts provided by Yukiko Yamada.

Chapter 3: Preliminary data regarding the Dmp53 and Outsiders project. This chapter will give background on other potential genes found from literature searches that may be involved in germ cell development. Specifically I will go over the Drosophila genes *pcaf*, *basigin*, and *DLP*. It will also give a brief overview of my project involving the production of antibodies specific to Tre1 and Outsiders. Experimental design and antibody stainings done by Keri Davis.

Chapter 4: Discussion and future directions. This chapter will summarize the work done on the *out* and *p53* project. It will also propose models for how these genes could be working *in vivo* and give insight to the potential directions of this project.

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CHAPTER 2: A MYST family histone acetyltransferase, *enok*, is involved in *p53* mediated germ cell death in *Drosophila melanogaster*

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

Abstract

It has been shown that in Drosophila germ cell development approximately 50% of the germ cells undergo programmed cell death during the early phases of their migration to the gonads, and Dmp53 is a key player in mediating this germ cell death. However, the mechanism by which the germ cells die is still unknown. In order to identify regulators in Drosophila germ cell death, we analyzed a histone acetyltransferase (HAT) of the MYST family. Here we identify that Enok, a MYST family HAT, has a potential role in Dmp53-mediated germ cell death. *enok* mutant embryos have a defect in eliminating germ cells ectopic to the gonads. The phenotype is also apparent in *enok/+; dmp53/+* transheterozygotes. We propose that Enok may be acting to acetylate Dmp53, in order to more efficiently induce PCD in the germ cells.

Introduction

Germ cell development in Drosophila is a dynamic system that involves the coordination of multiple cellular processes. In order to populate the gonads, germ cells must actively migrate from an extra embryonic site of origin to the site of gonad formation. During the journey, only half of the germ cells make it to the gonads and the other half undergo programmed cell death (PCD) (Coffman, 2003; Coffman et al., 2002; Technau, 1987; Underwood et al., 1980; Yamada et al., 2008). While many components of germ cell migration have been revealed, how and why the other half of germ cells die is still very much an unknown (Reviewed in (Kunwar et al., 2006; Molyneaux and Wylie, 2004).

P53 is a transcription factor that has been dubbed, “guardian of the genome” because of its role in sensing cellular stresses and determining whether a cell will to go into cell cycle arrest and DNA repair or to undergo PCD (Lane, 1992; (Vousden, 2000; Vousden, 2002). Because of its pivotal role in determining cell fate, P53 must be tightly regulated. Post-translational modifications such as phosphorylation, ubiquination and acetylation are the most common mechanisms that have been shown to regulate p53 (Bode and Dong, 2004; Brooks and Gu, 2003). P53 was the first non-histone substrate shown to be acetylated by HATs (Gu and Roeder, 1997). From this initial discovery, it has been shown that acetylated P53 is involved in P53 stabilization as well as recruitment of other protein complexes to help initiate the activation of transcription (Barlev et al., 2001).

One specific HAT that has been shown to acetylate P53 is Tip60 (Tat-Interacting Protein). Tip60 is part of the MYST family of HATs. The MYST family of HATs was named after the founding proteins in this family, Moz, Ybf/Sas2, Sas3, and Tip60. All proteins in this family contain a conserved MYST domain which contains a zinc finger domain and a binding site for acetyl-CoA (Avvakumov and Cote, 2007). After DNA damage in humans, Tip60 directly acetylates K120 of P53, and this is required for the induction of apoptosis (Legube et al., 2004). Not only has Tip60 been shown to directly modify P53, but it has also been shown to modify ATM, a kinase that is well conserved in the P53 mediated apoptotic pathway (Sapountzi et al., 2006; Sun et al., 2005). Chameau, another MYST domain HAT, has also been shown to be linked to programmed cell death in Drosophila (Legube et al., 2004). It was shown that Chameau activated JNK mediated apoptosis by being a coactivator of Drosophila Fos and Jun, components of JNK signaling (Miotto et al., 2006).

We have previously shown that Drosphila P53 (Dmp53) is involved in mediating germ cell PCD as well as Outsiders, a monocarboxylate transporter (MCT) (Yamada et al.,

2008). Another MCT, SLC5A8, has been identified as a tumor suppressor in humans (Li et al., 2003). Studies have shown that SLC5A8 transports a histone deacetylase (HDAC) inhibitor into cells. The transport of the HDAC inhibitor is correlated with the up regulation of proapoptotic genes, including *p53* (Thangaraju et al., 2006). Because of the prevalence of P53 being acetylated prior to inducing apoptosis as well as the evidence that MCTs can act as tumor suppressors, we hypothesized that HATs may be involved in P53 and Outsiders mediated germ cell death. We examined different HATs for a germ cell phenotype. We identified Enok, a MYST family HAT as having a potential role in germ cell death. *enok* mutants have a cell death phenotype similar to both *outsiders* and *p53* mutants. In addition to a common phenotype, germ cell death is disrupted in *enok/+; dmp53/+* transheterozygotes indicating they may be part of a common cell death pathway. This paper describes the initial characterization of the *enok* germ cell phenotype as well as genetic evidence that suggests it is involved in P53 mediated cell death.

Materials and Methods

Fly Stocks

Fly maintenance and crosses were performed according to standard procedures at 25°C. The following fly stocks were used in this study. $enok^{2}$ (Scott et al., 2001), $enok^{c376}$, KG07784 (Bellen et al., 2004), out^{2} (Coffman et al., 2002), $p53^{5A-1-4}$ (Rong et al., 2002). $p53^{-ns}$ (Sogame et al., 2003). *P{w+, fat facets-lacZ}* was used as wild type (Fischer-Vize et al., 1992).

Immunohistochemistry

Germ cells were visualized using standard whole mount embryo immunohistochemistry (Johansen and Johansen, 2004). Embryos were collected on apple juice agar media and allowed to develop for 12-15 hours at room temperature (22-

25°C). Embryos were dechorionated in 50% bleach and fixed for 40 minutes in 4% paraformaldehyde. Embryos were blocked in 1.0% normal goat serum in PBST for at least 30 minutes and incubated at 4°C overnight in 1/10,000 anti-vasa antibody (a gift from K. Howard). Embryos were washed with PBST and incubated in 1/5000 biotinylated chicken IgG secondary antibody (Vector Labs) for 2.5 hours at 4°C. Embryos were washed again with PBST and incubated in ABC Elite Kit solution (Vector Labs) for 30 minutes. Embryos were developed using diaminobenzidine as the horseradish peroxidase substrate. For double labeling, immunofluorescence staining was done using 1:25 mouse anti-Clift (Eya10H6 Developmental Studies Hybridoma Bank- University of Iowa, Bonini et al., 1993) and Anti-Vasa. Alexa Fluor 488 conjugated goat anti-mouse antibody (Invitrogen) and Alexa Fluor 568 conjugated goat anti-chicken (Invitrogen) were used for secondary antibodies at a concentration of 1:500.

Germ Cell Counts

Germ cell counts were performed using differential interference contrast microscopy. Embryos were staged according standard morphology (Campos-Ortega and Hartensein, 1997). To determine penetrance of the mutant phenotype embryos were scored mutant if there were four or more germ cells ectopic to the gonads. A compact sphere of coalesced germ cells in the correct region abdominal segment 5 was identified as a gonad. Proper bilateral segregation of the germ cells was a requirement for identification of the gonad, any clumps of germ cells near the midline were considered ectopic to the gonad. When visible, gonadal sheath cells were used to help establish the limiting edge of the gonads.

Single Embryo PCR and Sequencing

Twelve *enok*[1] embryos were chosen for single embryo PCR. Embryos were lysed in a buffer containing 10mM Tris-HCl, 1 mM EDTA, 25mM NaCl, and 10 mg/ml Proteinase K

(Gloor et al., 1993). The *enok*^{1} sequence was amplified using the following primers; 5'-AATGCGAGGACGACATTCC-3', 5'-GCTGAAGTCGATCAGGAAGC-3'. Phusion™ High-Fidelity DNA Polymerase was used to amplify the genomic sequence (New England Biolabs). The Applied Biosystems 3730xl DNA Analyzer was used to sequence the amplified region (Iowa State DNA Sequencing Facility).

Results

Mutant analysis reveals a potential role for Enok in germ cell PCD.

Because of the role HATs have in modifying P53, we hypothesized HATs could also have a role in the Dmp53-mediated killing of germ cells in Drosophila. One of the MYST family HATs that was chosen for this study was Enoki mushroom (Enok). We chose three *enok* alleles for this study *enok*^{1}, *enok*^{2} and *enok*^{C376} *(Bellen et al., 2004; Scott et al., 2001)*. The *enok*^{2} allele results in an amino acid substitution in the conserved zinc finger domain which changes a cysteine to a tyrosine. This lethal allele is predicted to disrupt the zinc finger domain structure. In the context of general development and mushroom body formation, the *enok*^{2} allele behaves the same as Df(2)gek^{D23}, which is a deletion that covers both the *enok* genomic region as well as the neighboring gene, *genghis khan*. Because of the indistinguishable phenotypes between these two alleles, it is assumed that *enok*^{2} behaves as a null allele (Scott et al., 2001). The *enok*^{1} allele is the result of a premature stop codon at the end of the zinc finger domain. This allele is also lethal, and in mushroom body formation behaves the same as *enok*^{2} and the Df(2)gek^{D23} deletion. Due to the lethality of both *enok*^{2} and *enok*^{1}, these alleles are maintained over a balancer, SM6b. The *enok*^{C376} allele is the result of a P-element insertion just upstream of the coding sequence and this allele is homozygous viable.

In order to analyze whether *enok* is involved in germ cell development, we used whole mount immunohistochemistry using a germ cell-specific marker, Vasa.

Visualization of the germ cells in these mutant embryos revealed a germ cell death defect (Figure 2.1 E,F). When scored for penetrance of the mutant phenotype, the heterozygous *enok*^{2} allele was 21.6% mutant. Embryos with ≥ 4 germ cells ectopic to the gonads are considered mutant. The *enok*^{2} balancer was also tested separately to ensure the balancer was not causing a phenotype. The balancer stained separately had a mutant penetrance of 1.5%. Therefore, the phenotype does not result from the balancer chromosome. The homozygous *enok*^{C376} allele revealed a similar germ cell development defect with 47.7% of the embryos having 4 or more germ cells ectopic to the gonads. The transheterozygous *enok*^{1} allele did not show a mutant phenotype, as only 5.0% of embryos stained were mutant (Table 4.1).

It should also be noted that the *enok* stocks had a higher than usual number of embryos with 3 germ cells ectopic to the gonads. When scored for germ cells ectopic to the gonads, wild-type embryos had 3.3% of the entire population with 3 germ cells ectopic to the gonads. The *enok*^{2} allele had 14.9% and *enok*^{C376} had 6.9% with three germ cells ectopic to the gonads. To characterize the mutant phenotype, germ cell counts were done on embryos from the *enok*^{2} and *enok*^{C376} stock. As figure 2.2B indicates, embryos from these stocks have both wild-type and mutant phenotypes. Germ cell counts were done on both of these populations. In the mutant embryos that were scored the average numbers of germ cells ectopic to the gonad were 4.8 and 6.2 for *enok*^{2} and *enok*^{C376} respectively (Figure 2.2 and Supplementary Table 2.1). Wild-type embryos had an average of 0.4 germ cells ectopic to the gonad.

Because the *enok*^{1} stock did not show a germ cell development defect, sequencing was performed on single embryos to ensure the molecular defect was still present in this stock. Genomic DNA from twelve embryos was extracted and the *enok*^{1} gene was amplified and sequenced. The premature stop codon was still present in 2 of the 12

embryos in this balanced stock.

Enok does not seem to have a maternal effect in germ cell development

Scott et al. reported that *enok* mRNA was expressed in the early embryo and they indicated that *enok* could be maternally contributed (Scott et al., 2001). To further characterize Enok and its role in germ cell development, we tested whether Enok had a maternal effect by crossing homozygous $enok^{C376}$ females to wild-type males. This cross-resulted in 7.6% mutant embryos. We concluded that $enok^{C376}$ is recessive and does not show a maternal effect in germ cell development. A cross between $enok^{2}$/*SM6b* females and wild-type males resulted in an 8.0% mutant penetrance, arguing $enok^{2}$ is recessive (Figure 2.2B and 2.5).

Migration of germ cells appears normal in *enok* mutants.

In order to investigate if germ cell migration was abnormal in *enok* mutants, germ cell counts were done on embryos. In both wild-type embryos and *enok* mutant embryos around 17 germ cells are incorporated into the gonads (Figure 2.2 and Supplementary Table 2.1). The gonads appear fully formed in *enok* mutant embryos. To ensure that the germ cells were also able to migrate and coalesce properly with the somatic gonadal precursor cells (SGP), we double labeled $enok^{C376}$ mutant embryos with SGP specific antibody anti-Eyes absent as well as germ cell specific anti-Vasa. As Figure 2.3 shows, germ cells were capable of migrating and coalescing with the SGPs in the *enok* mutants but not all germ cells are incorporated into the formed gonad.

Enok and P53 transheterozygotes are defective in germ cell PCD.

A major goal of this study was to elucidate other genes that are involved in Drosophila germ cell PCD. After observing that Enok was involved in germ cell death, we tested for possible genetic interactions with Dmp53 in mediating germ cell death. We hypothesized that if Enok and Dmp53 were working together to mediate cell death,

knocking out one copy of each gene could potentially give a germ cell phenotype. To do this, we crossed *enok*^{2} and *enok*^{C376} females to *p53*$^{5A-1-4}$ males. The *dmp53* males were used as Dmp53 has a maternal effect in germ cell development (Yamada et al., 2008). The F1 transheterozygote embryos from these crosses were collected and stained to visualize the germ cells. The mutant phenotype penetrance was 36.5% and 38.4% for the *enok*^{2} cross and the *enok*^{c376} cross respectively (Figure 2.2). This is considerably higher than the control crosses which were only 8.0% and 7.6% mutant for *enok*^{2} and *enok*^{C376} respectively (Figure 2.2 and 2.5). As observed with the mutant stocks alone, there were many embryos with 3 germ cells ectopic to the gonads compared to the wild type control (Figure 2.2). Germ cell counts on the transheterozygote crosses were carried out and on average 6.6 germ cells persisted ectopic to the gonads in the *enok*^{C376}/+; *p53*$^{5A-1-4}$/+ transheterozygote mutant embryos. From the *enok*^{2}/ *Sm6b X p53*$^{5A-1-4}$ cross, the mutant embryos averaged 5.4 germ cells ectopic to the gonads.

Enok could also be potentially involved in Outsiders mediated cell death

We were also very interested in looking at the Enok's relationship with Outsiders. If Enok was involved in Outsiders mediated cell death, then a germ cell mutant phenotype might occur in *out/+;enok/+* transheterozygote embryos. As *outsiders* also has a maternal effect, *enok* females were crossed to *outsiders* males and the F1 progeny were stained and observed for a defective germ cell phenotype. As Figure 2.4 shows, the transheterozygote cross resulted in a mutant penetrance of 12.2%, this is slightly higher than the control cross that has a penetrance of 7.6% mutant embryos (Figure 2.4). Because this phenotype is very subtle, it is unclear whether Enok is involved in the Outsiders mediated cell death pathway.

Characterization of *p53^{-ns}* in germ cell development

Most of our previous work with Dmp53 mediated PCD was done using the *p53^{5A-1-4}* allele (Rong et al., 2002). This allele has a highly penetrant germ cell mutant phenotype at 93.0% (Yamada et al., 2008). Another allele, *p53^{-ns}*, was also shown to have a defect in germ cell PCD, but its effect was not well characterized. We have since done a more careful characterization and have found that this allele is not as penetrant as *p53^{5A-1-4}*. Only 34.2% of this stock showed a mutant germ cell phenotype (Figure 2.5). Germ cell counts of these mutant embryos also revealed a less severe phenotype compared to *p53^{5A-1-4}* (Supplemental Table 2.1). As we previously reported *p53^{5A-1-4}* embryos averaged 9.1 germ cells ectopic to the gonads. *p53^{-ns}* embryos on the other hand only average around 6.2 ectopic germ cells to the gonads. We also repeated the *enok/+; dmp53/+* transheterozygote experiment with the *p53^{-ns}* stock. This cross resulted in 9.3% of the embryos being mutant As expected with a low mutant penetrance of the *p53^{-ns}* stock, we did not see a noticeable difference between *enok^{2}/+; +/+* and *enok^{2}/+; dmp53^{-ns}/+* transheterozygotes as the percentage of mutant embryos in each population was similar.

Discussion

Here we describe a novel function of the histone acetyltransferase Enok in Drosophila germ cell development. Previously Enok was described as having a role in cell proliferation in mushroom bodies in the brain (Scott et al., 2001). Now, we illustrate a possible role for Enok, in programmed cell death. Here we establish that *enok^{2}* and *enok^{C376}* mutant embryos are defective in the programmed cell death of germ cells. Germ cells in *enok* mutants migrate to and populate the gonads as in wild-type embryos. However, the initiation of cell death pathways is not as efficient as wild-type controls, and consequently ectopic germ cells persist. Of the three *enok* alleles tested, the *enok^{1}*

allele did not show a defect in germ cell development. It is possible that the phenotype displayed by the *enok*^{2} and *enok*^{C376} alleles are the result of extragenic modifiers. In order to examine this possibility further, these two alleles should be crossed to see if they fail to complement. The *enok*^{1} allele should also be placed in trans to *enok*^{2} and *enok*^{C376}, as it would be interesting to see if these alleles failed to complement. In addition to this *enok*^{1} and *enok*^{2} should be placed over a different balancer containing a dominant embryonic marker, such as GFP. This would allow us to know which embryos are homozygous mutant for *enok* and allow for a more accurate characterization of the mutant phenotype.

The phenotype of these mutant embryos is similar to the mutant phenotypes of *p53* and *outsiders* (Yamada et al., 2008). One goal of this study was to further characterize how P53 and Outsiders are involved in the PCD of germ cells. The prevalence of P53 being acetylated in response to DNA damage to induce cell death is established in the literature, and it prompted us to look at whether HATs are involved in normal embryogenesis (Legube et al., 2004; Sapountzi et al., 2006). We have shown here that HATs are involved in normal Drosophila germ cell development.

As *enok/+; dmp53/+* transheterozygotes revealed nonallelic noncomplementation resulting in inefficient germ cell PCD, we propose that Dmp53 and Enok work together in a pathway to initiate PCD of the germ cells. It is possible that Enok could be acetylating Dmp53 directly to activate it. It is also possible that this result is because enok and p53 are working in parallel to mediate germ cell death. For example Enok could be acetylating histones around P53 DNA target sites. This would allow the DNA to be more accessible for P53 to bind and initiate transcription of pro-cell death genes. Another possibility is that enok and p53 are in separate pathways that mediate cell death. To investigate this further, a homozygous double mutant of *enok* and *p53* could be

constructed. If the phenotype of the double mutant is more severe than *p53* mutants, it is possible that enok and p53 are acting independently to mediate cell death.

Despite the fact that Enok and Outsiders did not show a strong transheterozygote phenotype it is still possible that Outsiders could be providing a histone deacetylase inhibitor such as pyruvate or butyrate. This inhibitor would be able to help keep Dmp53 acetylated, allowing cell death to occur.

Previously, Enok was shown to be essential for cell proliferation in the brain. Here we show a different role for Enok, mediating cell death in germ cells. I propose that Enok might function by acetylating Dmp53. If Enok is acting on Dmp53 via acetylation, it illustrates the role of another important post-translational modification in regulating P53 activity. Different combinations of post-translational modifications on histones result in either gene activation or repression (Sims and Reinberg, 2008). Similarly, P53 has been compared to the histone code with the multiple ways it can be regulated post-translationally. The different effects, such as repression and activation, post-translational modifications have on P53 activity are also comparable to the effects of the histone code. This has been dubbed, the “protein code” (Knights et al., 2006; Sims and Reinberg, 2008). We show here a potential role of Enok acetylating Dmp53 *in vivo*, thereby helping Dmp53 mediate the PCD of germ cells. What regulates Dmp53 is still being revealed, but it is possible that Enok has a role in its protein code.

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CHAPTER 3: Preliminary data regarding the Dmp53 and Outsiders project

Chapter 2 focused on Enok, a MYST family HAT involved in the PCD of germ cells. The focus of the work presented in this chapter was to identify additional genes in germ cell development as well as to develop antibodies to the Out and Tre1 proteins already identified in germ cell development.

In my search for genes involved in germ cell development, I came across three more genes that also could be involved. This chapter will give background on three genes; *pcaf*, *DLP*, and *Basigin* as well as show some preliminary data about these genes regarding their potential roles in germ cell development. With the preliminary data seen here, I will propose future experiments that will more definitively show if these genes are involved in germ cell development. Along with this work, I will describe a polyclonal antibody project that was designed to produce antibodies to the Outsiders and Tre1.

Pcaf

In addition to the MYST family of HATs, another family of HATs, the Gcn5-related N-acetyltransferases (GNAT) family of HATs has also been shown to be able to acetylate P53. One member of this family, Pcaf (p300/CBP associated factor) has been identified as being involved with P53 signaling. Specifically it has been shown using antibodies that target acetylated P53 lysine residues, that after DNA damage, Pcaf acetylates P53 (Liu et al., 1999; Sakaguchi et al., 1998). The regulation of acetylation on P53 by PCAF is controlled by MDM2, a mammalian P53 suppressor. Binding of MDM2 to P53 targets P53 for ubiquination and degradation (Haupt et al., 1997; Jin et al., 2002; Jin et al., 2004). It was shown that MDM2 could inhibit P53 acetylation by p300 (a Pcaf co-activator and HAT). This inhibition is caused by p300 and MDM2 forming a complex that prevents P53 from being acetylated

(Kobet et al., 2000). Interestingly, MDM2 can also target PCAF for degradation, giving it another role in regulating P53 activity (Jin et al., 2002; Jin et al., 2004).

In Drosophila there is only one Gcn5/Pcaf homolog termed Pcaf (sometimes called Gcn5). Similar to the GNAT complexes formed in yeast and mammals, Drosophila Pcaf has also been shown to form a protein complex that includes Ada2, Ada3, Spt3, and Tra. Homologs to these proteins have also been found in mammals and yeast (Kusch et al., 2003). When looking to see if the functions of these complexes are conserved between humans and Drosophila, Kusch et al. performed coimmunoprecipitations to see if Ada2 and Pcaf could bind to Dmp53. Indeed, Dmp53 was pulled down with both Pcaf and Ada2, indicating that these proteins are part of a complex (Kusch et al., 2003). With this data, I hypothesized that Pcaf could be also working with Dmp53 in germ cell development.

DLP

In humans, a Fas binding protein was identified that activated JNK mediated apoptosis. This protein was named Daxx for death domain-associated factor (Yang et al., 1997). Similar to Hp53, Daxx was found to be involved the cellular response to UV damage. UV induced cell death was suppressed in *daxx* mutants. This study also identified that Daxx was upstream of JNK, and that Daxx was upregulated upon UV exposure. This indicated that Daxx promoted cell death, and initiated a JNK mediated cell death pathway (Khelifi et al., 2005). Not only is Daxx involved in the same processes as P53, such as response to stress, Daxx has also been shown to interact with P53 *in vivo*. This Daxx-P53 interaction was shown to downregulate the transcription of p21, a gene regulating cell cycle arrest (Gostissa et al., 2004). In contrast, Daxx has been shown in mice to have anti-apoptotic effects. *daxx* mutant embryos undergo massive apoptosis and these mutants are lethal (Michaelson et al., 1999). Zhao et al. demonstrated that Daxx binds P53 *in vivo*. They also

have shown anti-apoptotic effects, and that this binding inhibited P53 transcriptional activity (Zhao et al., 2004).

In the Drosophila genome there is one Daxx homolog named Daxx- like Protein (DLP). It seems to have a conserved function as DLP and Dmp53 were shown to interact via Dmp53's C-terminus in a yeast two hybrid screen, and in a GST pull down assay (Bodai et al., 2007). Just as Daxx has been shown to have a pro-apoptotic function, DLP was also shown to potentially function in this way as DLP was shown to positively regulate the transcription of pro-apoptotic *ark*. In *DLP* mutants, *ark* mRNA levels are lowered, while over expressing DLP was able to increase the expression of *ark*. This points towards a regulatory role for DLP in apoptosis. However, DLP does not seem to have a role in irradiation response as after irradiation DLP mutants can initiate apoptotic pathways properly (Bodai et al., 2007). Because DLP has been shown to interact with Dmp53 *in vitro* and its known role in mammalian systems, I hypothesized that DLP could be involved in germ cell development and set out to test this hypothesis by staining DLP mutant embryos to look for a germ cell phenotype.

Basigin/CD147

As mentioned in Chapter 1, CD147 encodes for a single transmembrane molecule which co-localizes with monocarboxylate transporters (MCTs). CD147 has been shown to aid in MCT expression and targeting to the membrane (Kirk et al., 2000). CD147 has not only been found to be involved in being a auxiliary protein to MCTs, but it has also been named a major player in metastasis. In a screen developed to look for upregulated mRNAs in micrometastatic cells, it has been shown that CD147 is upregulated (Klein et al., 2002). CD147 also functions as an inducer of matrix metalloproteases (Guo et al., 1998). Matrix mettalloproteases are able to degrade the extracellular matrix allowing a cell to become metastatic and migratory (Ellerbroek and Stack, 1999). The Drosophila homolog of CD147 is

named Basigin. It is highly conserved between vertebrates and Drosophila with the transmembrane domain being over 50% identical. It has been identified as having a role in dorsal closure and disintegration of the aminoserosa in the embryo (Reed et al., 2004).

Because of the evidence in other systems that CD147 co-localizes with MCTs, I hypothesized that Basigin could be co-localizing with Out. In addition, Basigin could be inducing the production of metalloproteases, which could be involved in germ cell migration. A metalloprotease, Invadolysin, has been already been identified as being involved in germ cell migration in Drosophila (McHugh et al., 2004).

Materials and Methods

Fly Stocks

Fly maintenance and crosses were performed according to standard procedures at 25°C. The following fly stocks were used in this study. *DLP*^{KG01694}, *Bsg*^{gel} (Bellen et al., 2004). *pcaf*^{C137T} (Carre et al., 2005). *P{w*$^{+}$*, fat facets-lacZ}* was used for a wild-type control (Fischer-Vize et al., 1992).

Immunohistochemistry

Germ cells were visualized using standard whole mount embryo immunohistochemistry as described in Chapter 2.

Determination of mutant penetranc

Data was collected using differential interference contrast microscopy. Embryos were staged according standard morphology (Campos-Ortega and Hartensein, 1997). Embryos were scored based on number of germ cells ectopic to the gonads. A cluster of coalesced germ cells in abdominal segment 5 with the correct lateral and dorsal-ventral position were identified as gonads.

Results and Future Work

pcaf, DLP, and *basigin* mutant stocks were all stained with anti-Vasa antibody and scored for germ cells ectopic to the gonads (Figure 3.1). Because of Pcaf's role interacting with P53 in other systems, as well as its role in mediating cell death, we hypothesized that Pcaf could have a role in germ cell development in Drosophila. The *pcaf* allele used in this study, $pcaf^{C137T}$, results from an amino acid change from a cysteine to a tyrosine. The allele is lethal and maintained over a balancer. If Pcaf were involved in germ cell death we would expect to see a quarter of the embryos showing a mutant germ cell phenotype. 22.2% of the embryos showed a mutant phenotype of 3 or more germ cells ectopic to the gonads.

Because this allele resides over a balancer, the germ cell phenotype seen could be the result of a balancer effect. To test for this the $pcaf^{C137T}/TM3$ females were crossed to wild-type males. Females and males from the F1 progeny showing the balancer markers were then crossed and the F2 embryos were stained. Embryos were then scored for mutant penetrance. 14.47% of the embryos stained from the balancer cross had 3 or more ectopic germ cells to the gonads. We concluded that the balancer in the $pcaf^{C137T}$ stock partially contributes to the germ cell phenotype seen in this stock. However, because the $pcaf^{C137T}$ stock shows a 22.6% mutant penetrance, it does not rule out the possibility that Pcaf has a role in germ cell development. In order to more definitively test this gene and its role in germ cell development, the allele needs to be placed over a different balancer that does not give rise to a germ cell phenotype, re-stained, and scored. Also, because this phenotype could be due to other modifiers on the chromosome(s), different alleles from different genetic backgrounds should be tested. Another experiment to make ensure other chromosomes are not contributing to the germ cell phenotype would be to outcross the $pcaf^{C137T}$ allele. Outcrossing the chromosomes

with wild-type chromosomes should remove any modifiers that maybe contributing to the mutant phenotype observed in the stock. If the germ cell phenotype persists after these experiments are done, further testing should continue to see if *pcaf* and *dmp53* genetically interact possibly by doing a transheterozygote experiment as we have done with other HATs. An additional set of experiments would be to examine Pcaf for a maternal effect in germ cell PCD. The lethality of the $pcaf^{C137T}$ chromosome would require the generation of germline clones.

The *DLP* allele, $DLP^{KG01694}$ was also tested to see if could potentially have a role in germ cell development. This allele is homozygous viable, and results from a P-element insertion in the 5' UTR. When scored for germ cells ectopic to the gonads, 18.1% of the embryos had 4 or more germ cells ectopic to the gonads, and 15.3% of the embryos had 3 germ cells ectopic to the gonads. This indicates that DLP is potentially involved in germ cell development. Nevertheless, more rigorous tests must be performed to conclude DLP's role in germ cell development. Because only one allele was tested, other alleles should also be tested to see if this phenotype could be repeated in different stocks. The $DLP^{KG01694}$ allele should also be outcrossed to remove extragenic modifiers on other chromosomes. These alleles could also be used to set up a complementation cross. If the two alleles fail to complement and show a mutant germ cell phenotype, this would be indicative of DLP being involved in germ cell death. As my hypothesis is that *DLP* and *dmp53* are functioning together to mediate PCD and *dmp53* has a maternal effect, the maternal effect of *DLP* should also be tested. Because this allele is homozygous viable, females from this stock would need to be crossed to wild-type males. If *DLP* has a maternal effect, embryos from the F1 of this cross would show a germ cell phenotype.

Finally a third gene, *Basigin*, was tested to see if it was involved in germ cell development. The P-element insertion allele Bsg^{gel} was used in this study. This allele also resides over a balancer. When the mutant stock was stained and visualized no apparent germ cell defects were seen. Preliminary staining of this allele showed a germ cell phenotype. However, no further characterization was done with this staining and subsequent results did not show a germ cell phenotype. It is possible the original stock has been contaminated, and a new stock should be re-ordered and examined for a germ cell phenotype.

Production of antibodies to Tre1 and Outsiders

An essential part of knowing a protein's function *in vivo* is knowing when and where a protein is expressed. Our laboratory's main focus is to identify the roles of two proteins, Outsiders (Out) and Tre1, in germ cell development. Part of my thesis project was to develop a strategy to produce antibodies specific to these two proteins. Generating antibodies specific to Tre1 and Out would allow for visualization of these proteins *in vivo* and also allow us to examine how these proteins interact with other proteins. Here I will describe the main strategy I used to develop polyclonal sera to these proteins. As of now, no positive results indicating a specific antibody have been noted. I will briefly describe testing conditions used, and what other conditions could be tried in the future to potentially reveal a specific antibody to Tre1 or Out.

Both *tre1* and *out* encode for transmembrane proteins that span the membrane multiple times (Figure 3.2). Due to the hydrophobic insoluble nature of these proteins, a recombinant protein would be very hard to produce. Because of this, an anti-peptide strategy was employed to produce antibodies specific to Tre1 and Out. For each protein four peptide sequences were chosen to be injected into mice. These peptides were chosen based on their hydrophilic nature and the antigenicity. Multiple resources were utilized to decide what

peptides would be antigenic. Specifically, Design of Useful Peptide Antigens, written for the Association of Biomolecular facilities, was useful in describing the properties of amino acids in relation to how they would react in an animal (Angeletti, 1999). It also described how to design a peptide that would be easily synthesized. For example it is best to avoid multiple cysteines as they are both hard to synthesize and *in vivo* form disulfide bonds. As the goal is to inject a peptide that mimics our protein of interest, having a sequence that normally forms a specific secondary structure would not be represented in a short peptide form. “Antibodies: A laboratory manual” by Harlow and Lane, was also helpful in developing a strategy to produce antibodies (Lane, 1988). In deciding which peptides to choose to inject into mice, advice was taken from technical representatives at Alphadiagnostic, a company that specializes in producing antibodies, as well as Paul Kapke, the manager of the ISU hybridoma facility. The peptide sequences were subjected to a BLAST search to look for conservation among other proteins in Drosophila, mouse, and rabbit genomes. Using a specific peptide would maximize the chances of getting a specific antibody to our proteins of interest. In summary peptides were chosen based on antigenicity, ease of synthesis, BLAST results, and sites of potential post-translational modifications. Prediction programs used were NCBI BLAST, Prosite, SOSUI, and TopPred (Claros and von Heijne, 1994; de Castro et al., 2006; Hirokawa et al., 1998; Hulo et al., 2008). The peptides chosen are depicted in Figure 3.2.

The ISU Hybridoma facility did all animal husbandry, peptide injections, ELISAs, and bleeds. After peptides were synthesized by the ISU Protein facility, they were conjugated to KLH (keyhole limpet hemocyanin) protein and injected into mice. Three mice were chosen for each peptide. Because peptides are so small, in order to increase the chances of inducing an immune response they needed to be conjugated to KLH. Test bleeds were taken and an ELISA was performed to make sure all mice were responding to the peptide

injected. For the ELISA, the peptides were conjugated to ova albumin (OVA). This allowed us to look at only the antibodies that were produced in response to the peptide, and not to KLH. In order to induce the production of ascites fluid, Mouse SP2/0 myeloma cells were injected into the mice. This produces a tumor in the intraperitoneal cavity and the mice will then start producing mass quantities of ascites fluid, which will contain the polyclonal antibodies. ELISA tests were done a second time and mice that responded to the antigen were sacrificed and the ascites fluid was collected from the abdomen. Spleens were also frozen down for potential future monoclonal antibody projects.

The ascites fluid was then tested on whole mount Drosophila embryos to test whether the mouse produced a specific antibody. The ascites fluid was diluted 1/200 and was tested on embryos fixed in Bouins fluid for twenty minutes. The secondary antibody used was an anti-mouse IgM (Invitrogen). This was used at a standard concentration of 1/5000. There was no visualization of specific staining on these embryos. Some, but not all, ascites fluid was tested using a paraformaldehyde fixative as well as using a IgG specific secondary. Again, no visualization of specific staining was visualized. As we do not know where specifically the Tre1 or Out protein is being translated, there could be a specific binding between the protein and antibody, but it is not being seen. A possible reason for this would be that the staining is hidden by background or is being mistaken as background staining. Every antibody reacts differently however from experience with the Anti-Vasa antibody used our laboratory, background initially appears in the midgut region. If the Tre1 and Out are being expressed in this region, say in the germ cells as the germ cells are migrating towards the lateral mesoderm, it is possible the specific staining wasn't detected.

Even though two different fixatives and two different secondary antibodies were tested with the ascites fluid, other conditions need to be tested in order to rule out the failure to produce a specific antibody. First, a more complete screening of using paraformaldehyde

fixative by changing the time of fixation, as well as the IgG secondary antibody should be tested. Second, the ascites fluid could be purified using affinity purification columns, or pre-absorbed with other embryos to reduce non-specific binding. Pre-absorbing other antibodies used in our laboratory has significantly decreased background staining. Third, these antibodies should be screened using western blots, as they may be specific to the denatured protein even though they may not be specific to the native protein in the whole embryo. Because the antibodies did respond in an ELISA test, it is possible that they will also bind to our proteins of interest on a western blot. This is because in a western blot the proteins are denatured and will look more like the linear peptide that was injected into the mouse.

These antibodies, should they yield positive results in the future, could be incorporated into many current projects in the laboratory. Specifically, when looking at potential genes that regulate Out or Tre1, we could look at protein expression in various mutant backgrounds. For example, they could also be used to co-localize Tre1 and Out with other proteins. For example, even though Basigin does not seem to be involved in germ cell development we could use antibody staining to see if Basigin co-localizes with Outsiders. This would show an evolutionary conserved role of Basigin. In summary, being able to visualize these proteins using antibodies would give the laboratory additional tools to elucidate how these proteins act *in vivo* and this would make it much easier to study the genetic pathways that they are in.

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CHAPTER 4: Discussion and future directions

From this study, I have identified an additional gene that may be involved in germ cell programmed cell death (PCD), *enok*, a MYST family histone acetyltransferase. Other experiments and controls need to be performed in order to further establish this model. This chapter will outline the main conclusions of chapter 2, as well as some other tests that should be done to further characterize Enok's involvement in germ cell death. I will also discuss three other genes that could be involved in my proposed model and explain other experiments that could be done to show if these genes are acting to regulate PCD.

enok mutants are defective in germ cell PCD

Chapter two described the characterization of two alleles of *enok, enok*^{2} *and enok*^{C376}*.* This gene was found to be involved in germ cell development as mutant embryos were defective in germ cell PCD. Along with the work done on these two alleles, one other allele *enok*^{1} was also tested in this study. This allele is reported to be a null as in embryonic and larval development as well as in mushroom body formation it behaves the same as the deletion Df(2)gek^{D23} (Scott et al., 2001). This deletion covers the *enok* genome region as well as its neighboring gene, *genghis khan.* The molecular characterization of the *enok*^{1} allele revealed a nonsense mutation at the end of the zinc finger domain. Compared to the *enok*^{2} allele, the *enok*^{1} mutation occurs 20 amino acids after the *enok*^{2} mutation. As with *enok*^{2}, *enok*^{1} is lethal and must be maintained over a balancer. When stained to look for a germ cell phenotype, *enok*^{1} did not show a germ cell phenotype. Crossing the *enok*^{1} allele to *dmp53* or to *outsiders* alleles, also did not reveal a mutant phenotype (Figure 4.1). Because this result is conflicting with the data presented in chapter 2, other experiments must be done to elucidate why the *enok* alleles are acting differently in our system.

The most direct test to perform to test the *enok*^{1} allele would be to sequence the *enok*^{1} stock and look to make sure the nonsense mutation is still there, and that it didn't revert back to wild-type. Because this stock is maintained over a balancer, single-embryo PCR should be done on multiple embryos in order to sequence a homozygous *enok*^{1} embryo. If the allele has not reverted back to wild type, further tests should be done. Complementation between the *enok*^{1} and the other two alleles could be done to see if *enok*^{1} fails to complement these two alleles. If the two alleles fail to complement it could mean in germ cell development the *enok*^{1} allele is just less severe and doesn't have an effect by itself. Complementation should also be done between *enok*^{2} and *enok*^{C376}. If the germ cell defect we are seeing is due to the mutations disrupting the *enok* gene, these two alleles should fail to complement each other. It is possible that *enok*^{C376}/*enok*^{1} or *enok*^{C376}/*enok*^{2} mutants could be viable, and this could result in a more severe germ cell phenotype compared to the homozygous viable line *enok*^{C376}.

Another test that should be done to further characterize the *enok* alleles used in this study would be to look at the expression of *enok*. Because *enok*^{C376} is homozygous viable mRNA expression could be quantified doing *in situ* hybridization (to look for expression in the embryo) as well as QPCR to quantify how much mRNA is being produced between the *enok*^{C376} and wild-type embryos.

Enok and Outsiders do not show a transheterozygote phenotype

I propose that Enok and Outsiders could be indirectly working together to mediate PCD of germ cells. As monocarboxylate transporters (MCTs) can transport short-chained fatty acids (SCFA), which function as histone deacetylase inhibitors (HDAC inhibitors), I propose that Outsiders could be helping promote Dmp53 acetylation by inhibiting histone deacetylases (Figure 4.2). If they are indeed working indirectly, this could be a reason why Outsiders and Enok transheterozygotes did not show an obvious germ cell phenotype.

Another reason could be that one copy of each gene produced enough gene product to allow for efficient PCD. In order to further test a potential interaction between Outsiders and Enok, the UAS-Gal4 system could be used to overexpress *enok* in an *out* mutant background or express *out* in an *enok* mutant background. Overexpressing *enok* in an *outsiders* mutant background maybe able to rescue PCD in germ cells if *outsiders* is providing an HDAC inhibitor.

Dmp53 and Enok transheterozygotes show germ cell PCD defects

enok^{2}/+ ;*dmp53*$^{5A\text{-}1\text{-}4}$/+, as well as *enok*^{C376}/+; *dmp53*$^{5A\text{-}1\text{-}4}$/+ transheterozygotes, show defects in germ cell PCD. This indicates that Enok and Dmp53 are working together in a similar pathway to promote PCD in germ cells. I propose that Enok could be directly acting on Dmp53 by post-translationally acetylating Dmp53.

In order to directly test the hypothesis that Enok is indeed acetylating Dmp53, specific antibodies would have to be created that would recognize the acetylated lysine(s) in Dmp53. To date, no specific antibodies exist. If Enok is acetylating Dmp53, western blots could be done between wild-type and *enok* mutant embryos to look for changes in acetylated Dmp53 levels. An antibody to Dmp53 is available that is not specific to Dmp53's acetylation state.

Another piece of the Enok/Dmp53 story that needs to be revisited in order to establish how Enok and Dmp53 are interacting is the results found with the *p53*$^{-ns}$ data. When originally establishing the role for Dmp53 in germ cell development the *p53*$^{-ns}$ allele was not fully characterized. Here we show that the allele only has a 34.7% mutant penetrance. Sogame et al. generated this line using ends in homologous recombination (Sogame et al., 2003). The construct used for recombination removed the parts of the DNA binding domain and transactivation domain, as well as the entire oligomerization domain. After a recombination event had occurred in this line, both genomic PCR and northern blots revealed that the 1.6 KB genomic region of Dmp53 was disrupted and instead two genomic

and corresponding RNA transcripts (of 0.6 KB and 5.0 KB) were visualized instead. This disruption of the *dmp53* gene was enough to fully block the Dmp53-dependent irradiation response up regulating the *reaper* gene (Sogame et al., 2003). In our context of germ cell development, we first should test the stock to see if it has been contaminated. PCR can be done on genomic template DNA to see if there is a wild-type contaminant. If there is a contaminant, three different size bands should be amplified. If the stock does not have a contaminant, it needs to be evaluated if the two transcripts that have been shown to be transcribed do are producing protein. Western blots could be done with anti-Dmp53 antibody to see if there is production of Dmp53 protein. If Dmp53 is being produced, it is possible that it still could function in germ cell development and this is enough to mediate the PCD of germ cells.

Future Directions

The current working model is that Enok is modifying Dmp53 to induce programmed cell death in the germ cells. With this model we also hypothesize that Outsiders could also transport histone deacetylase inhibitors into the cell thus helping Dmp53 stay acetylated and in an active conformation (Figure 4.2). As with any other scientific discovery these findings open up a whole new set of questions to test.

This hypothesis was based on other research groups that had shown that Dmp53 is acetylated as I have described in chapter 1 (Barlev et al., 2001; Gu et al., 2004; Zhao et al., 2006). Along with acetylation, phosphorylation is also a common theme in P53 activation; in particular it has been shown that P53 can be activated via a phosphorylation-acetylation cascade (Sakaguchi et al., 1998). It also has been shown in response to DNA damage that HATs will acetylate kinases and after acetylation the kinase will phosphorylate P53 (Lavin and Kozlov, 2007). Here I will talk about different kinases that could potentially be involved in Dmp53 mediated PCD of germ cells, either directly or indirectly.

ATM and CHK2

One pathway that has been established to activate P53 is through ATM activation (Lavin and Kozlov, 2007; Sun et al., 2005; Sun et al., 2007). Upon DNA damage, ATM (Ataxia-Telangiectasia Mutated), a protein kinase, can phosphorylate a serine/threonine kinase CHK2. CHK2 in turn phosphorylates P53. It has been shown that on the onset of DNA damage, the HAT Tip60 acetylates ATM. Blocking Tip60 was shown to inhibit ATM's ability to phosphorylate CHK2 and P53 (Sun et al., 2005). The lysine in ATM that Tip60 acetylates after DNA damage has been identified, it is lysine 3016 (Sun et al., 2007). A basic alignment search between the human and Drosophila homologs of ATM show that this lysine is conserved. However, it is unknown whether the conserved lysine is acetylated in Drosophila. It is possible that Enok (or another HAT) could be acting on ATM in germ cells to induce PCD. This acetylation event could set off the phosphorylation cascade from ATM to CHK2 to Dmp53 (Figure 4.3). In support of this model, CHK2 mRNA is highly expressed in the germ cells (Figure 4.4) (Tomancak et al., 2002). Its phosphorylation activity on Dmp53 has also been well established in the context of DNA damage (Brodsky et al., 2004) (Peters et al., 2002). In order to establish that ATM and CHK2 are working in germ cell PCD, mutants in these genes should be to looked at to see if there is a germ cell phenotype. If it does look like these genes are involved in germ cell development it would be interesting to look at to see if HATs are involved in initiating this PCD pathway. This could be done by transheterozygote experiments with the HAT of interest (For example Enok or Tip60) and ATM. Western blots using an acetylated ATM antibody could also be performed to look for changes in acetylation of ATM in a HAT mutant. An acetylated ATM antibody that reacts to Human ATM has been made. It is possible that it could react to Drosophila ATM as well.

Tao-1

I described in my general introduction that germ cells in *nanos* mutant embryos undergo apoptosis (Forbes and Lehmann, 1998; Hayashi et al., 2004; Kobayashi et al., 1996). Recently it was shown that Nanos represses Tao-1, a serine threonine kinase (Sato et al., 2007). In embryogenesis, Tao-1 seems to be expressed primarily in the germ cells (Figure 4.4) (Tomancak et al., 2002). Tao-1 was shown to mediate germ cell death in *nanos* mutant embryos by promoting the pro-apoptotic gene *sickle (skl)* (Sato et al., 2007). It is unknown whether Tao-1 directly or indirectly promotes the expression of *skl*.

Besides the zinc finger domain and HAT domain, Enok also has a neurofilament- like domain (Scott et al., 2001). This domain has a predicted serine/threonine kinase target site. I propose that Tao-1 could be indirectly promoting *skl* expression by phosphorylating Enok at this target site. This phosphorylation event could trigger Enok to acetylate other proteins such as Dmp53 to mediate cell death (Figure 4.3).

As figure 4.3 shows, HATs can be involved in multiple steps leading to PCD. The previous chapters have described two HATs, Pcaf and Enok, that potentially could acetylate proteins at any of these steps. It is unknown as of now how HATs function in the context of germ cell PCD in Drosophila.

Concluding Remarks

The major goal of this thesis project was to reveal other genetic determinants in germ cell PCD. Chapter two described work on Enok, a MYST histone acetyltransferase. *enok* was shown to not only be involved in germ cell PCD, but also appears to genetically interact with Dmp53 to mediate PCD. It is unclear whether *enok* and *outsiders* genetically interact. Chapter three showed preliminary data on three genes *Basigin*, *pcaf*, and *DLP*. There is much more characterization that needs to be done on these genes and their potential role in germ cell development. Chapter 3 briefly describes the strategy used to produce polyclonal

antibodies to the Outsiders and Tre1 proteins. Finally, I describe in this chapter the main conclusions of the Enok characterization, as well as background on three kinases, ATM, CHK2, and Tao-1 and have described various reasons why these three genes could possibly tied into germ cell development.

As this story unfolds, it's likely that it will reveal more roles of post-translational modifications in development. Post-translational modifications provide a way of fine-tuning regulation, by not just upregulating or downregulating gene expression, but allowing the protein to become more or less active or functional. This type of regulation is extremely important in the case of Dmp53, as it controls cell fate.

Throughout this thesis, I have compared Dmp53 to Hp53 and have described how the function of cell death in Dmp53 is evolutionary conserved. In order to understand Hp53 and how, when mutated, it contributes to cancer, it is important to know how P53 is regulated. My work shows how acetyltransferases could be contributing to Dmp53 regulation, which in turn could ultimately help figure out how Hp53 is acting in humans.

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APPENDIX: DATA

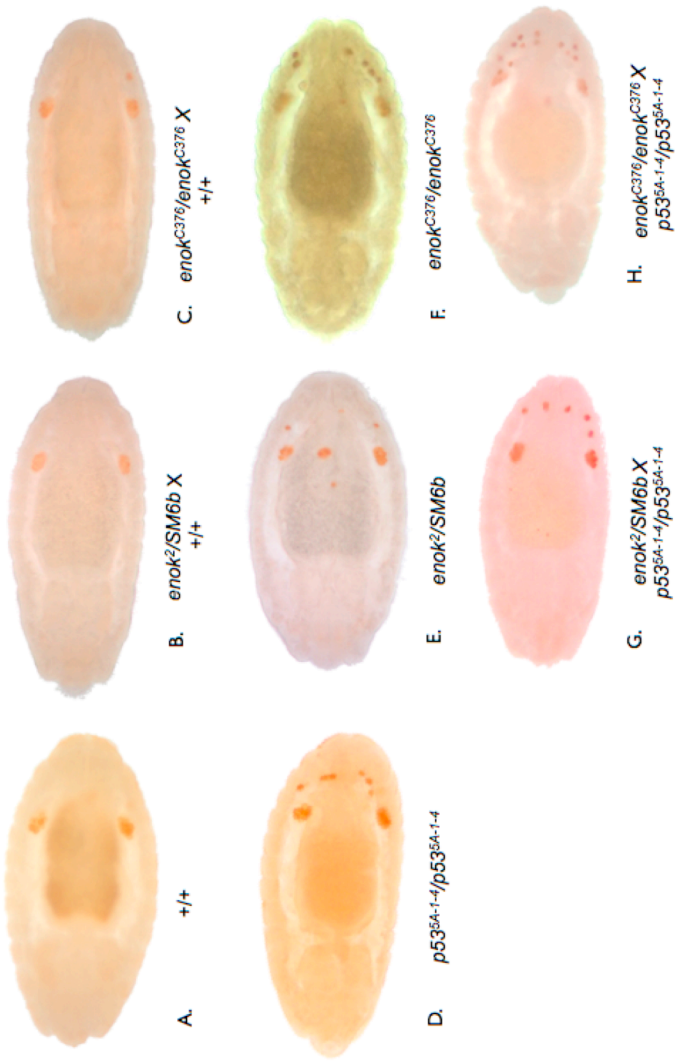


Figure 2.1 Germ cell phenotypes in various *enok* and *p53* mutant backgrounds
12-15 hour embryos were collected and stained with anti-Vasa antibody to visualize germ cells. Both *enok*^{2} and *enok*^{C376} alleles were defective in eliminating ectopic germ cells. This phenotype was also seen in *enok/+; p53/+* transheterozygote embryos indicating a role for *enok* in P53 mediated germ cell death. A) Wild-Type embryos eliminate ectopic germ cells efficiently. B and C) *enok* alleles were crossed to wild-type to test for maternal effect. A maternal effect was not seen. D) *p53*$^{5A-1-4}$ embryos show a germ cell PCD defect. E and F) *enok* alleles show a germ cell PCD defect. G and H) *enok/+; p53/+* transheterozygotes show a germ cell PCD defect.

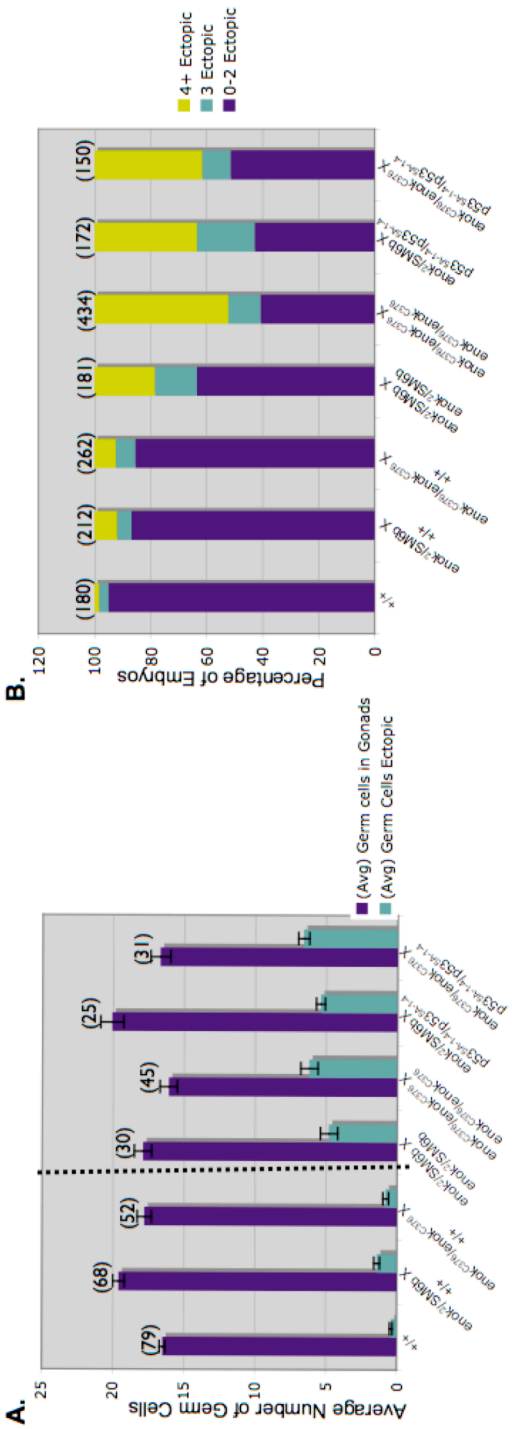


Figure 2.2 Enok and Dmp53 mediate germ cell PCD. Two alleles, *enok*^{C376} and *enok*^{2} show a germ cell PCD defect. To look for maternal effect *enok*^{C376}/*enok*^{C376} females were crossed to wild-type males. A maternal effect was not apparent in this cross. To ensure the nature of the allele is recessive, *enok*^{2}/*SM6b* females were crossed to wild-type males. After initial characterization of the *enok* alleles, the genetic interaction between *enok* and *dmp53* was tested. *enok* females were crossed to *p53* males and F1 embryos were collected and stained for a germ cell phenotype. A) Embryos were counted for total number of germ cells. Error bars represent S.E.M. Cumulative data are shown for +/+, *enok*^{2}/*SM6b* X +/+, *and enok*^{C376}/*enok*^{C376} X +/+ as very few embryos have ≥ 4 germ cells ectopic to the gonads. For the remaining crosses, only those embryos with ≥ 4 germ cells ectopic to the gonads are represented. B) Crosses were scored for penetrance of the mutant phenotype. Germ cell PCD is less efficient in the *enok*/+; *p53*/+ transheterozygote compared to *enok*/+ heterozygotes. Number of embryos in data set is denoted by parentheses.

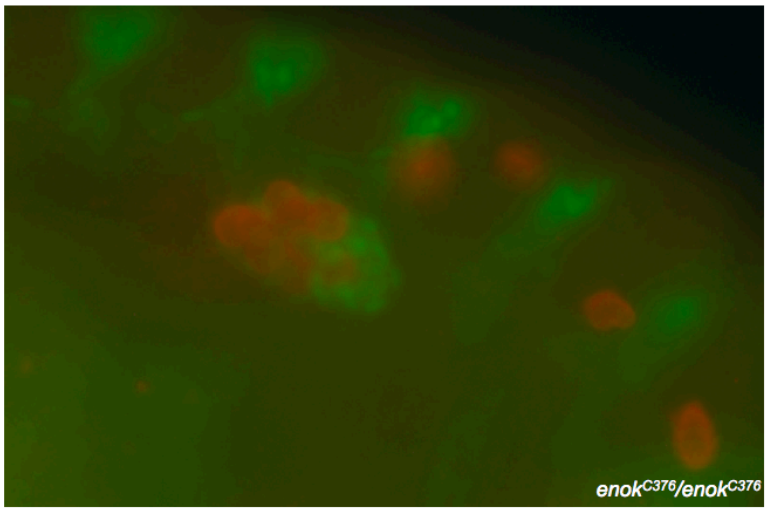


Figure 2.3 Gonad formation is normal in *enok* mutant embryos. *enok*^{C376} mutant embryos were double labeled with anti-Vasa (red) and anti- EYA (green) antibodies. Somatic gonadal precursors as well as germ cells were able to coalesce to form a gonad however not all germ cells migrated to the gonad.

A.

$enok^{C376}/enok^{C376}$ X +/+

$enok^{C376}/enok^{C376}$ X out^{2}/Y

$enok^{C376}/enok^{C376}$ X $out^{KG07784}/Y$

B.

Cross	Percentage of Embryos 0-2 Ectopic	3 Ectopic	4+ Ectopic	N
$enok^{C376}/enok^{C376}$ X +/+	85.5	6.9	7.6	262
$enok^{C376}/enok^{C376}$ X out^{2}/Y	80.3	7.4	12.2	188
$enok^{C376}/enok^{C376}$ X $out^{KG07784}/Y$	78	17.1	4.9	41

Figure 2.4. Outsiders may be involved in Enok mediated cell death. *enok* mutant females were crossed to *outsiders* mutant males and F1 embryos were collected and stained for a germ cell phenotype. Germ cells are labeled with anti-Vasa antibody. A. Phenotypes of $enok^{C376}/enok^{C376}$ X +/+ and *out/+;* $enok^{C376}$/+ transheterozygotes. B. Crosses were scored for penetrance of mutant phenotypes. Germ cell PCD is slightly less efficient in the *out/+;* $enok^{C376}$ /+ transheterozygote compared to $enok^{C376}$ /+ heterozygotes.

A.

p53^{-ns}/p53^{-ns}

enok^{2}/SM6b X p53^{-ns}/p53^{-ns}

enok^{c376}/enok^{c376} X p53^{-ns}/p53^{-ns}

B.

Female	Male	N	% Mutant
p53^{5A-1-4}/p53^{5A-1-4}	*p53^{5A-1-4}/p53^{5A-1-4}*	166	93.0%
p53^{-ns}/p53^{-ns}	*p53^{-ns}/p53^{-ns}*	234	34.2%
enok^{2} /SM6b	WT	212	8.0%
enok^{C376}/enok^{C376}	WT	262	7.6%
enok^{2} /SM6b	*p53^{5A-1-4}/p53^{5A-1-4}*	63	36.5%
enok^{C376}/enok^{C376}	*p53^{5A-1-4}/p53^{5A-1-4}*	268	38.4%
enok^{2} /SM6b	*p53^{-ns}/p53^{-ns}*	172	9.3%
enok^{C376}/enok^{C376}	*p53^{-ns}/p53^{-ns}*	150	4.7%

Figure 2.5. The *p53^{-ns}/p53^{-ns}* allele does not have a severe effect on germ cell PCD.
A) The *p53^{-ns}/p53^{-ns}* stock was stained with Anti-Vasa antibody to visualize germ cells and scored for germ cell mutant phenotypes. This stock was also crossed to the *enok^{2}* and *enok^{C376}* alleles to look for a phenotype in transheterozygous embryos. B) Embryos were scored for the penetrance of the mutant phenotype. The *p53^{-ns}* allele is only 34.2%. *enok/+; p53^{-ns}/+* transheterozygotes do not have severe germ cell PCD defects.

Female	Male	N		PGCs Inside Gonad	PGCs Outside Gonad
+/+	+/+	79		16.5 ±0.2	0.4 ± 0.1
$p53^{5A-1-4}/p53^{5A-1-4}$	$p53^{5A-1-4}/p53^{5A-1-4}$	56		18.7 ± 0.3	9.1 ± 0.5
$p53^{-ns}/p53^{-ns}$	$p53^{-ns}/p53^{-ns}$	48	≤ 3	18.8 ± 0.7	1.4 ± 0.5
		36	> 4	14.4 ± 0.7	6.2 ± 0.1
$enok^{2}/SM6b$	+/+	68		19.6 ± 0.4	1.4 ± 0.2
$enok^{2}/SM6b$	$enok^{2}/SM6b$	79	≤ 3	17.9 ± 0.4	1.5 ± 0.1
		30	> 4	17.6 ± 0.6	4.8 ± 0.2
$enok^{2}/SM6b$	$p53^{5A-1-4}/p53^{5A-1-4}$	37	≤ 3	18.6 ± 0.5	1.6 ± 0.2
		25	> 4	20.1 ± 0.8	5.4 ± 0.3
$enok^{2}/SM6b$	$p53^{-ns}/p53^{-ns}$	67	≤ 3	16.4 ± 0.9	0.4 ± 0.2
		18	> 4	18.4 ± 1.3	5.4 ± 0.3
$enok^{C376}/enok^{C376}$	+/+	52		17.8 ± 0.5	0.8 ± 0.2
$enok^{C376}/enok^{C376}$	$enok^{C376}/enok^{C376}$	28	≤ 3	14.9 ± 0.7	1.6 ± 0.2
		45	> 4	16.1 ± 0.6	6.2 ± 0.4
$enok^{C376}/enok^{C376}$	$p53^{5A-1-4}/p53^{5A-1-4}$	31	≤ 3	17.1 ± 0.6	1.8 ± 0.2
		31	> 4	16.7 ± 0.7	6.6 ± 0.4
$enok^{C376}/enok^{C376}$	$p53^{-ns}/p53^{-ns}$	23	≤ 3	16.4 ± 0.9	0.4 ± 0.2
		N.D.	> 4	N.D.	N.D.

Supplementary Table 2.1 The indicated crosses were set up and 12-15 hour embryos were collected and stained with anti-Vasa antibody. Cumulative data are shown for +/+, $enok^{2}/SM6b$ *X* +/+, *and* $enok^{C376}/enok^{C376}$ X +/+ as very few embryos have ≥ 4 germ cells ectopic to the gonads. Cumulative data is also shown for $p53^{5A-1-4}/p53^{5A-1-4}$. For the remaining crosses, embryos were divided into two populations,≤ 3 (wild-type) and ≥ 4 germ cells (mutant). Embryos were counted for total number of germ cells. Mean ± S.E.M. is displayed.

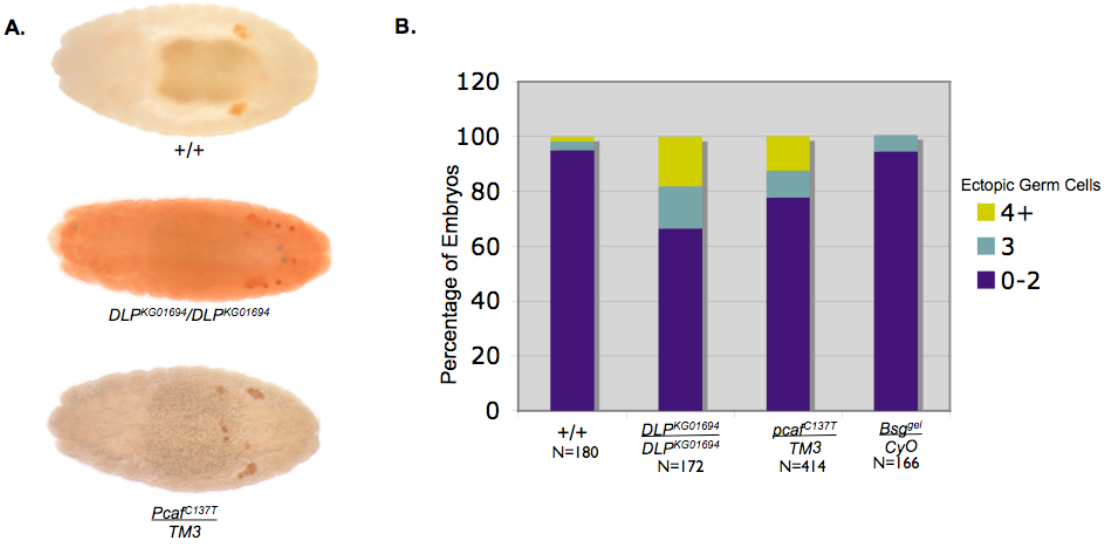
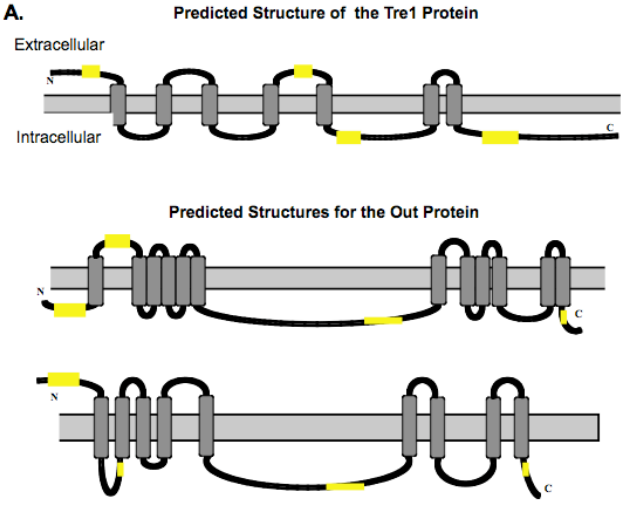


Figure 3.1. Mutants Screened For Germ Cell Development Phenotypes
A) 12-15 hour embryos were collected and stained with anti-Vasa antibody to visualize germ cells. Both *DLP* and *pcaf* showed defects in germ cell development. B) After mutant alleles were stained, embryos were scored based on the number of ectopic germ cells. Both *DLP* and *pcaf* alleles had an increase in ectopic germ cells compared to the wild-type control. *Basigin (Bsg)* did not show a mutant phenotype.

A.



B.

Protein	Peptide Name	Peptide Sequence	Different Sera Available
Tre1	3171-1	CTGYIQDADMQMDE	3
Tre1	3131-2A	CVLHQKKKIRNHDNFQ	3
Tre1	3171-3	CSRNYHQSKNSKELS	1
Tre1	3171-4	CTILKKEGRSSIKK	3
Out	8062-1	CTEKNHTQVYNDTT	2
Out	8062-2	CDNTEAEEEAKRRMTFFQK	1
Out	8062-3	CRVRMQSLGFDAKQTT	3
Out	8062-4	CDRLEQNRHDQGPDTPS	2

Figure 3.2 Schematic diagram of Tre1 and Outsiders Proteins
A) Protein structure prediction programs model both Tre1 and Out as membrane spanning proteins. Tre1 is shown as a 7 transmembrane protein. The Out protein is predicted as having either 9 or 12 membrane spanning domains. Yellow boxes indicate where peptides were chosen. Figures adapted from TopPred and SOSUI prediction programs.
B) Peptide sequences chosen for injection into mice. Different sera available refers to number of mice that responded when injected with the same peptide. These sera are available for further testing.

	Percentage of Embryos			
	0-2 Ectopic	3 Ectopic	4+ Ectopic	N
+/+	95.0	3.3	1.7	180
enok^{1}/*SM6b*	90.8	4.3	5.0	141
enok^{1}/*SM6b* x *p53*$^{-ns}$	76.5	17.6	5.9	17
enok^{1}/*SM6b* x *out*^{KG07784}/*Y*	96.3	1.1	2.6	268

Figure 4.1. *enok*^{1} embryos do not have a strong germ cell PCD defect.
12-15 hour embryos were collected and stained with anti-Vasa antibody to visualize germ cells. After mutant alleles were stained, embryos were scored based on number of ectopic germ cells.

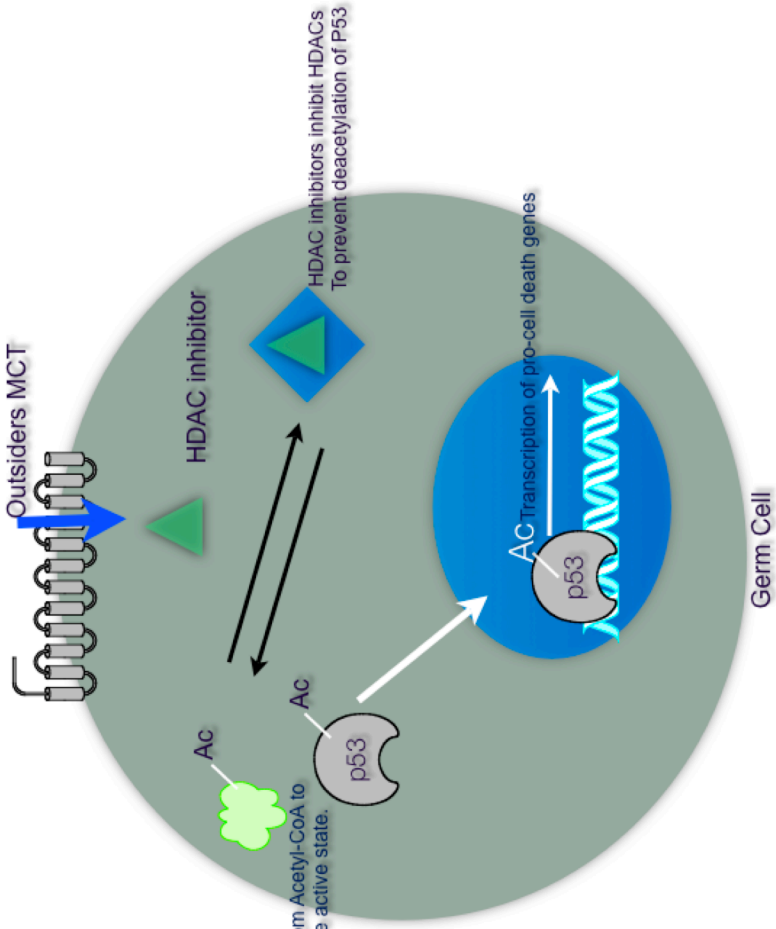


Figure 4.2. Proposed model of how HATs and MCTs may work together to mediate PCD in germ cells. Out could be functioning in germ cell PCD by transporting a HDAC inhibitor into the cell. At the same time, a HAT could be acetylating Dmp53. This acetylation event promotes Dmp53 to enhance the transcription of pro-cell death genes. HDAC and HATs maybe in a competition state and if either a HAT or HDAC inhibitor is mutated, Dmp53 will not be in an active state.

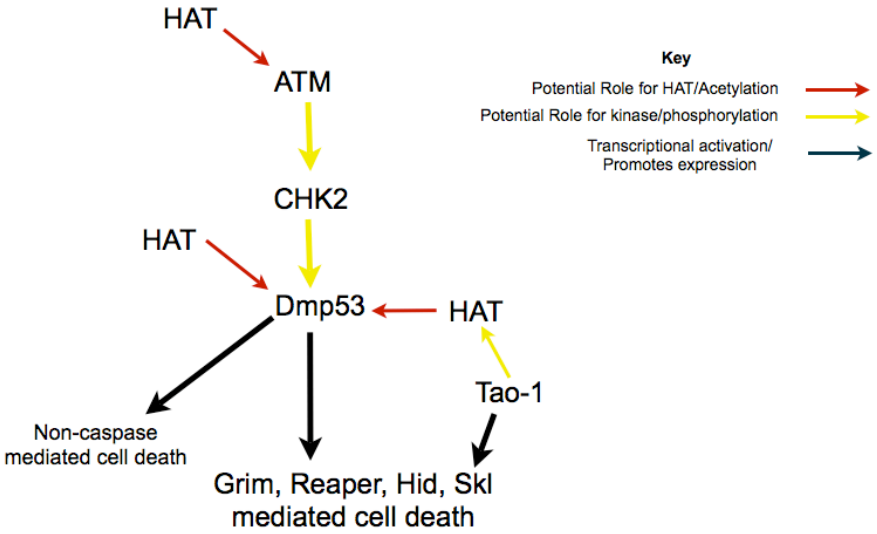


Figure 4.3 Potential entry points of HATs in germ cell PCD. HATs such as *enok* or *pcaf* may function in germ cell PCD via Dmp53 pathway activated by ATM. HATs may acetylate ATM or Dmp53 to mediate germ cell PCD. In addition, *t*ao-1 may be promoting pro-apoptotic *skl* indirectly by phosphorylating a HAT, such as *enok*. The HAT may then acetylate Dmp53.

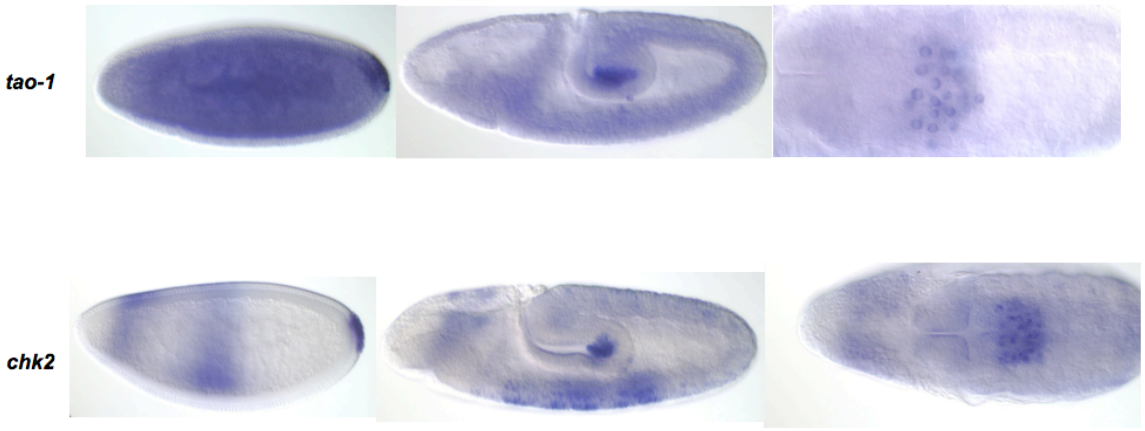


Figure 4.4. *tao*-1 and *chk2* mRNA is expressed in germ cells during embryogenesis. *In situ* probes specific to *tao-1* and *chk2* were used on embryos of various stages of development. Throughout embryogenesis both t*ao-1* and *chk2* mRNA is expressed primarily in the germ cells indicating a potential role in germ cell death. Images from the Berkeley Genome Drosophila Project (Tomancak et al., 2002).