p53 IN A GENETIC MODEL: ILLUMINATING ADAPTIVE RADIATION RESPONSES

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DEDICATION

To my parents, Ryoko & Mamoru Sogame, I dedicate this thesis.

I am grateful to my parents who fostered my respect for learning and who provided me all the resources one could ever wish for to accomplish my goals.

I also dedicate this thesis to my husband, Justin R. Swasey, for his never-ending encouragement.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor, John M. Abrams, for his guidance, enthusiasm, support, and patience.

I would also like to thank my thesis committee member, Helmut Kramer, Michael A. White, and Xiaodong Wang for providing a source of experience and knowledge, not to mention their support and encouragement.

Lastly, I would like to thank all members, past and present, of the Abrams' Lab, for their technical assistance as well as many laughs we shared.

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2005

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Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2005

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When cells are challenged by genotoxic stress, the tumor suppressor protein p53 promotes adaptive responses, including cell cycle arrest, DNA repair, or apoptosis. How these distinct fates are specified through an action of a single protein is not known. To study its functions *in vivo* we produced a targeted mutation at the *Drosophila* p53 (Dmp53) locus. I show that Dmp53 is required for damage-induced apoptosis but not for cell cycle arrest. Dmp53 function is also required for damage-induced transcription of two tightly linked cell death activators, *reaper* and *sickle*. When challenged by ionizing radiation, *Dmp53* mutants exhibit radiosensitivity and genomic instability, indicating in our model, apoptosis is important for maintenance of genomic stability in response to ionizing radiation. I also examined a global transcriptional change in response to ionizing radiation in the absence of Dmp53. Only 35

genes were constantly radiation responsive in wild type animals and Dmp53 was required for induction of a vast majority of the genes. The Radiation Induced p53 Dependent (RIPD) genes include genes implicated in apoptosis and DNA repair as well as genes with unknown functions. The functional significance of RIPD genes for the activation of apoptosis was tested using RNAi. Thus far, I uncovered *ribonucleotide reductase large subunit* (*RnrL*) as a novel Dmp53 target that is necessary for induction of caspase activation. Taken together, my study supports the notion that core ancestral functions of the p53 gene family are intimately coupled to cell death and possibly DNA repair as an adaptive response.

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PRIOR PUBLICATIONS

1. Christich*, A., Kauppila, S*., Chen, P*., Sogame, N*., Ho, S. I. & Abrams, J. M. The Damage-Responsive *Drosophila* Gene *sickle* Encodes a Novel IAP Binding Protein similar to but Distinct from *reaper*, *grim*, and *hid*. *Curr. Biol.* **12** 137-140 (2002)
2. Sogame, N., Kim, M., & Abrams, J. M. *Drosophila* p53 Preserves Genomic stability by Regulating Cell Death. *PNAS.* **100** 4696-4701 (2003)
3. Sogame, N. & Abrams, J. M. Radiation Responses in *Drosophila*. *Handbook of Cell Signaling,* Vol. **3**, Academic Press/Elsevier Science. 213-217 (2003)
4. Sogame, N., Christich, A., & Abrams, J. M. Genetic Determinants of Radiation Response in *Drosophila Melanogaster*. (Manuscript in preparation)

*Note: These authors contributed equally to this work.

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LIST OF DEFINITIONS

ATM – Ataxia telangiectasia mutated

ATR – Ataxia telangiectasia and Rad3 related

Caspase – Cysteinyl aspartate-specific protease

CDK – Cyclin dependent kinase

ChIP– Chromatin immuno precipitation

Ced – Cell death defective

Cep1 – Centrosomal protein 1

Dark – Drosophila apaf-1 related killer

Debcl – Drosophila executioner bcl

Dmchk2 – Drosophila melanogaster chk2

Dmp53 – Drosophila melanogaster p53

Dronc – Drosophila nedd2-like caspase

GFP – Green fluorescence protein

Hid – Head involution defective

IAP – Inhibitor of apoptosis protein

IR– Ionizing radiation

LOH – Loss of heterozygosity

Mei-41 – Meiotic-41

Mre11 – Meiotic recombination-11

Mwh – Multiple wing hair

RIPD – Radiation induced p53 dependent

RNAi – RNA interference

RnrL – Ribonucleoside diphosphate reductase large subunit

RnrS – Ribonucleoside diphosphate reductase small subunit

Rpr – Reaper

Skl – Sickle

TNF – Tumor necrosis family

CHAPTER ONE

GENERAL INTRODUCTION

Statement of objectives

Since its discovery, p53 has obtained a great deal of attention as a "guardian of the genome" due to its ability to halt propagation of damaged DNA by cell cycle arrest, DNA damage or apoptosis. After nearly three decades of studying this gene, a comprehensive understanding of the molecular mechanisms that cause p53 to activate each pathway is just beginning to emerge. Elucidating the molecular mechanism of apoptosis controlled by p53 is of particular interest in cancer research given many chemotherapeutic and radiation treatments work by inducing cell death in tumor cells, and in many cases resistance to these treatments indicates impaired apoptotic pathways (Malaguarnera, 2004).

Loss of genomic stability is one hallmark of cancer development and inactivation of p53 highly correlates with incidences of genomic instability (Attardi, 2005). However, it is not well understood which pathways (cell cycle arrest versus cell death) governed by p53 are indispensable to the safeguard mechanism imposed by p53 against cancer development. Much effort in the p53 field is focused on identifying p53 downstream targets for the p53-regulated pathways. However, the progress to identify true *in vivo* targets of p53 for each pathway has been slow due to the multifunctional roles played by p53 combined with the complexity of higher eukaryotic organism.

Studying p53 in *Drosophila* offers unique opportunities to circumvent this issue. Previous studies using dominant negative p53 transgenes suggest that Dmp53 promotes cell death but not cell cycle arrest (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000),

and the apoptotic response occurs in part by the p53-mediated transcription of *reaper*. Therefore, studying Dmp53 allows us to examine how p53 governs the specification of apoptotic fate without added complexities. The goal of my project was to understand the *in vivo* role of p53 in response to genotoxic stress using a genetically tractable organism, *Drosophila Melanogaster*. I had four specific aims: 1) Generation of a loss of function *Dmp53* mutant using a homologous recombination mediated targeting strategy. 2) Analyze the *in vivo* role of Dmp53 in response to IR. 3) Analyze an *in vivo* requirement of Dmp53 for the activation of *reaper* transcription. 4) Identify Dmp53 apoptotic targets by examining the genome-wide radiation response in *Drosophila*.

Mammalian p53

Tumor suppressor genes are a class of genes that prevent the transformation of normal cells to cancerous cells by inhibiting a transmission of harmful genetic materials. *p53* was discovered in 1979 by several labs (DeLeo et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979) and later classified as a tumor suppressor gene in 1989 (Eliyahu et al., 1989). Since its discovery, p53 has been under intense investigation due to its high mutation incidences exhibited in human cancer (50% of all human cancers contain a p53 mutation). The importance of p53 in tumor development was also confirmed by p53 knockout mice that showed a remarkable predisposition to lymphoma and other types of cancers (Donehower et al., 1992; Jacks et al., 1994).

p53 is said to play a role in senescence, differentiation, DNA repair and antiangiogenesis. However, the best characterized functions of p53 are initiation of cell cycle arrest and apoptosis in response to genotoxic damage. p53 regulates these pathways primarily as a sequence-specific transcription factor. Although transcription independent activities of p53 for induction of apoptosis have been documented (Bennett et al., 1998; Mihara et al., 2003), it remains unknown whether this represents a true *in vivo* event. The human p53 encodes 393 amino acids and is divided into several structurally and functionally discrete domains. At the N-terminus of p53, a transcription activation domain is located. Within the transcription activation domain, there is a MDM2 binding site. MDM2 is an ubiquitin ligase that controls the p53 stability via proteasome degradation (Kubbutat et al., 1997). Since the *mdm2* gene is a transcriptional target of p53, there is a negative feedback regulation loop involving p53 and MDM2 which keeps the level of p53 limited after exposure to genotoxic stress (Jones et al., 1995; Montes de Oca Luna et al., 1995). A sequence-specific DNA binding domain is located between amino acids 102-292 and binds to a tandem duplication of 10-mer DNA sequence 5'-PuPuPuC(A/T)-(T/A)GpyPyPy-3' (el-

Deiry et al., 1992). The majority of p53 mutations occur in this DNA binding region in the form of missense mutations, illustrating the importance of p53 as a sequence specific transcription factor (Sigal and Rotter, 2000). At its C-terminus, a tetramerization domain is located.

Various stimuli such as chemical, UV, IR and hypoxia activate p53. In normal cells, p53 has a relatively short half-life (Maki et al., 1996) because of the proteasome-mediated degradation of p53 via MDM2. However, upon stimulation by genotoxic stress, p53 is stabilized by several post-translational modifications. Kinases including ATM, ATR, Chk1 and Chk2 phosphorylate p53 at several N-terminal residues (Banin et al., 1998; Canman et al., 1994) in response to damage signals. The phosphorylation at the N-terminus inhibits the p53 interaction with MDM2, thereby, leading to an accumulation of p53. In addition to phosphorylation, acetylation appears to influence the p53 DNA binding activity (Gu and Roeder, 1997).

Once p53 is activated, it mediates various DNA damage responses such as cell cycle arrest and/or apoptosis. It is not clear how p53 chooses one biological outcome out of the many it regulates. Several models for how p53 determines a biological outcome have emerged (Vousden, 2000). One model suggests that p53 does not discriminate between the induction of cell cycle arrest versus apoptosis, in other words, p53 functions the same under all circumstances. In this model, activation of p53 always results in the induction of cell cycle arrest genes and apoptotic genes. What determines the cellular outcome in this case is a balance between survival signals that impedes cell death and apoptotic signals that augment cell death. Additional transcription factors may also be responsible for transcription of apoptotic genes in a context specific manner. The second model suggests that p53 is a determinant which is responsible for influencing cellular outcomes. In this scenario, the promoter binding ability of p53 seems to influence the decision between cell cycle arrest and apoptosis. For example, a cell cycle arrest gene, *p21,* has two p53 binding sites that are a

much better consensus to the known p53 binding sites compared with the binding sites of proapoptotic genes. It has been shown that a low abundance of p53 is able to activate cell cycle arrest genes, but fails to activate proapoptotic genes due to low affinity p53 binding sites in the vicinity of these genes (Ludwig et al., 1996). The corollary here is that persistent activation of p53 leads to apoptosis (Chen et al., 1996b). In this model, phosphorylations of p53 at different sites may also influence its DNA binding as well as the interaction with transcriptional coactivators (Shikama et al., 1999).

The consensus p53 binding site is highly degenerative and the estimated number of p53 binding sites in the human genome is as many as 200-400 (el-Deiry, 1998; el-Deiry et al., 1992). So far, a handful of genes have been identified as a direct p53 target. One of the most well characterized p53 targets is *p21*, a cyclin dependent kinase (CDK) inhibitor. Although as I show later, this does not occur in all models of p53 action. Using a $p21^{-/-}$ knockout mouse model, Deng *et al.* showed p21 is responsible for the induction of G1/S arrest in response to genotoxic stress (Deng et al., 1995). The promoter region of *p21* contains p53 binding sites and the expression of *p21* is p53 dependent (el-Deiry et al., 1993). p53 also plays a partial role in G2/M arrest, perhaps through regulation of 14-3-3, GADD45 and B99 (Hermeking et al., 1997; Utrera et al., 1998; Zhan et al., 1999), but we have an incomplete understanding of how these genes contribute to G2/M arrest and whether they represent a direct p53 target. The range of p53 dependent apoptotic target genes is broader than that of cell cycle target genes. *Bax,* a proapoptotic bcl2 member, and *fas*/*apo1*, a TNF receptor family member, are among the first p53 dependent proapoptotic genes identified (Miyashita and Reed, 1995; Munsch et al., 2000; Owen-Schaub et al., 1995). However, both genes seem to play only a partial role in the induction of apoptosis. First, *bax* or *fas* knockout thymocytes are capable of inducing apoptosis in response to IR, contrary to *p53* knockout thymocytes that fail to induce apoptosis (Fuchs et al., 1997; Knudson et al., 1995). Second, the tumor spectra displayed by *p53* knockout mice are far more diversified compared

to that of *bax* knockout mice (Yin et al., 1997). In the past decade, many more p53 dependent apoptotic genes including Noxa (Oda et al., 2000a), Puma (Nakano and Vousden, 2001), PIGs (Polyak et al., 1997), PERP (Attardi et al., 2000), and p53AIP1 (Oda et al., 2000b) have been identified using subtractive hybridization, differential display and direct cloning of p53 binding site screens. Currently, a few knockout mice that lack a p53 apoptotic target gene are available. The *puma* knockout mice are of particular interest since they recapitulate many apoptotic defects exhibited by *p53* knockout mice (Villunger et al., 2003; Yu et al., 2003). Puma is dispensable for development just like p53. However, $p53^{-/-}$ mice exhibit a wide range of tumors by 6 months of age, whereas $puma^{-/-}$ mice of the same age have not exhibited any tumor susceptibility in this study. This observation is intriguing since it indicates the defective apoptosis may not be sufficient for tumorigenesis.

Which functions of p53 contribute to tumor suppression remains an important unanswered question in cancer biology and understanding this mechanism will provide us a significant advantage in designing more effective therapeutic treatments. Traditionally, researchers favor the view that defective apoptosis gives a selective advantage in tumor development with following reasons. First, $p21^{-/-}$ mice show a defective G1/S arrest in response to genotoxic stress, however, they are not susceptible to tumor development unlike $p53^{-/-}$ mice (Deng et al., 1995). Second, Schmitt *et al*. reported that the inactivation of specific apoptotic components (such as by overexpression of dominant negative caspase 9 and/or anti-apoptotic Bcl2) downstream of p53 are sufficient to drive *Myc*-induced B-cell lymphoma that is indistinguishable from the lymphoma of p53 null mice (Schmitt et al., 2002). Furthermore, the inactivation of apoptosis downstream of p53 maintained the wild-type p53 in the B-cell lymphoma, illustrating that the pressure to mutate p53 for tumor development was no longer necessary in this model. There are a few observations that question this traditionally supported view. For example, *puma* knockout mice do not develop tumors although its apoptotic defect clearly mimics that of *p53* knockout mice. Recently, Liu

et al. generated a knock-in mouse (*Trp53*$^{515c/515c}$) that is unable to induce apoptosis but capable of inducing cell cycle arrest (Liu et al., 2004). They found that *Trp53*$^{515c/515c}$ mice did not develop thymic lymphoma which is a pronounced phenotypic trait in *p53*$^{-/-}$ mice, indicating the defective apoptotic pathway did not contribute to tumor development in their study. The evidence argues that tumorigenecity arisen from the defective p53 functions may be highly context-specific. Alternatively, other functions of p53 that were not examined in this study (such as DNA repair) may be intact in the *Trp53*$^{515c/515c}$ mice, and hence, contributed to the absence of thymic lymphoma in the mutants. Taken together, despite the years of intense research, we are still confronted with the elusiveness of p53 pathways.

***Drosophila* p53**

Drosophila p53 was identified by three independent groups (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000). The sequence analysis of the 385 amino acid Dmp53 protein revealed that many domains are conserved between mammalian p53 and Dmp53. The DNA binding domain showed the strongest homology with a 43% similarity. More importantly, the six amino acid residues of p53 that are mutated most frequently in human cancers were highly conserved (four amino acids were identical and two were similar) in *Drosophila*. p53 contacts DNA using eight residues, of which *Drosophila* contains six identical and two similar residues. One interesting difference between fly p53 and mammalian p53 is that fly p53 lacks MDM2 binding site at the N-terminus. The *Drosophila* genome does not contain any MDM2 like protein nor p19ARF which is a regulator of MDM2 (Brodsky et al., 2000b). This indicates that the p53 regulation in fly is clearly different from that of mammalian p53 and recently Brodsky *et al*. reported that the Dmp53 protein level does not change in response to IR, further ruling out MDM2 mediated regulation of the Dmp53 level (Brodsky et al., 2004).

Several checkpoint kinases play important roles in activation of p53 in mammals. ATM/ATR, Chk1 and Chk2 mediate a p53 phophorylation in response to DNA damage

(Walworth, 2000). All four kinases are conserved in *Drosophila* (*dATM* (Silva et al., 2004; Song et al., 2004), *mei41*, *grapes* and *Dmchk2* respectively), but the regulation of Dmp53 by these kinases is only partially conserved. DmChk2 is necessary for the induction of apoptosis in response to IR (Peters et al., 2002; Xu et al., 2001) and it is capable of phosphorylating Dmp53 (Brodsky et al., 2004). Mei-41 and Grapes are not needed for the induction of IR induced apoptosis (Jaklevic and Su, 2004) and the involvement of dATM in the induction of IR induced apoptosis remains to be tested.

Early studies using *Dmp53* transgenes established that an ectopic expression of Dmp53 triggers cell death. The same studies showed that the expression of dominant negative *Dmp53* transgenes prevented the induction of apoptosis in response to IR, but did not affect the induction of cell cycle arrest indicating Dmp53 is not required for the damage induced cell cycle arrest. *Dacapo* is a fly orthologue of *p21* and p21 is the primary downstream effector of p53 for the damage induced G1/S arrest in mammals. Ectopic expression of *dacapo* in fly eye discs clearly blocks the cell cycle in G1 (de Nooij and Hariharan, 1995; de Nooij et al., 1996). However, given these studies were conducted using dominant negative transgenes, validation of this observation awaits using a Dmp53 loss of function mutant.

Drosophila apoptosis and Dmp53

The cell death pathway is an evolutionally conserved pathway from worm to human. The molecular machinery of cell death was initially established in *C.elegans*. *Ced-3* and *ced-4* were identified as genes necessary for programmed cell death, and they are homologous to mammalian caspases and *apaf-1* respectively (Kaufmann and Hengartner, 2001). *Ced-9* is an anti-apoptotic Bcl2 member which binds to Ced-4 and prevents cell death. In *Drosophila,* the cell death pathway is regulated by these conserved genes, but also *Drosophila* have additional apoptosis activators. The *Drosophila* genome contains four IAP antagonists Reaper, Grim, Hid, and Sickle (Salvesen and Abrams, 2004), and as the name implies these

genes encode a protein that binds to IAPs and frees caspases from IAPs. Recent studies reported that *rpr* and *grim* might have cell death functions independent of IAP inhibition (Chen et al., 2004; Claveria et al., 2002; Holley et al., 2002; Yoo et al., 2002), however, the molecular mechanisms of how these events take place needs further investigation. Reaper, Grim, Hid, and Sickle share a N-terminal amino acid motif referred as either RHG motif or IAP binding motif (Salvesen and Abrams, 2004). These genes are located within a ~300kb cluster known as the *Reaper* region and they are transcribed in the same orientation (Christich et al., 2002). An embryo homozygous for the H99 deletion that lacks *rpr*, *hid*, and *grim* shows a complete absence of all developmental cell death (White et al., 1994), and when these genes are overexpressed, each gene is sufficient to induce apoptosis (Chen et al., 1996a; Grether et al., 1995; White et al., 1996). Although they have similar functions, each gene clearly has distinct roles and pathways they regulate since their expression profile varies. For example, *rpr* and *hid* are highly expressed in larval midgut and salivary gland during insect metamorphosis by the steroid hormone 20-hydroxyecdysone, but *grim* is not (Jiang et al., 1997).

In addition to the steroidal regulation of *rpr*, the expression of *rpr* is also regulated by IR. When I began my studies, several lines of evidence implicate *rpr* as a direct target of, and effector for Dmp53 action. First, *rpr* transcripts are strongly induced 90 minutes after IR treatment (Nordstrom et al., 1996). Second, examination of the *rpr* locus revealed that there is a 20-bp consensus p53 binding site that was similar to the site derived from mammalian counterparts. This 20-bp confers a minimal radiation response element at the *rpr* locus and a yeast one-hybrid assay demonstrated that Dmp53 can bind to this site (Brodsky et al., 2000a). Third, loss-of-function analyses uncovered a partial requirement for *rpr* in a model of x-ray-induced cell death (Peterson et al., 2002).

At the time I began my dissertation, these evidence suggested that Dmp53 plays a role in apoptosis and *rpr* may be an apoptotic target of Dmp53 in response to IR, however, the possibilities need to be directly tested using loss of function *Dmp53* animals. Previous studies led me to the following questions. First, what is the *in vivo* role of Dmp53? Second, how does Dmp53 activate the apoptotic pathway in response to IR?

CHAPTER TWO

GENERATION OF A LOSS-OF-FUNCTION *Dmp53* MUTANT

Abstract

To gain further understanding on the role(s) of p53 *in vivo*, I used a genetically tractable organism, *Drosophila Melanogaster*, to produce a targeted mutation at the *Drosophila p53* locus. A newly developed gene targeting technique through homologous recombination was used to generate $Dmp53^{-ns}$ mutants. $Dmp53^{-ns}$ mutants are viable and fertile with no overt defects as embryos, larvae, pupae and adults. However, when $Dmp53^{-ns}$ homozygous flies were crossed, a higher incidence of unfertilized embryos was observed. These results suggest that *Dmp53* is not required for normal development, but may play a role in maintenance of germ cell fidelity.

Introduction

The tumor suppressor gene *p53* plays a role as a "guardian of the genome" by initiating cell cycle arrest, DNA repair, and/or apoptosis to maintain genomic integrity in response to genotoxic damage (Lane, 1992; Levine, 1997; May and May, 1999). p53 elicits these distinct responses primarily by its transcriptional regulation (Chao et al., 2000; el-Deiry, 1998; Jimenez et al., 2000), although transcription-independent activities of p53 have been documented (Chipuk et al., 2003; Mihara et al., 2003). The human *p53* gene encodes 393 amino acid polypeptides, and contains several structurally and functionally distinct domains. p53 contains an acidic transcriptional activation domain at its N-terminus (Fields and Jang, 1990; Unger et al., 1992), a sequence-specific DNA binding domain between amino acids 94-292 (Cho et al., 1994), and a tetramerization domain at its C-terminus (Jeffrey et al., 1995). The DNA binding domain of p53 is highly mutated in human cancers, thus illustrating the importance of this protein as a transcription factor.

The evidence that connects a loss of p53 activity to tumor development came from human studies and knockout mouse studies. The *p53* null mice were generated by several groups (Clarke et al., 1993; Donehower et al., 1992; Jacks et al., 1994), and presented similar defects. The *p53* null mice are viable, however, they are highly susceptible to development of many different types of tumor. The most common type of tumor observed in these mice was lymphoma. When thymocytes and cells from the intestine were exposed to IR, wild type mice showed a robust induction of apoptosis, but p53 null mice failed to do so. These studies established the *in vivo* role of p53 in damaged induced apoptosis.

A *Drosophila* homolog of p53, Dmp53 was identified by three independent groups (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000). Like its mammalian counterparts (Cho et al., 1994; Fields and Jang, 1990; Jeffrey et al., 1995; Kraiss et al., 1988;

Unger et al., 1992), Dmp53 has a well-conserved DNA-binding domain with a transcriptional activation domain at its N-terminus, and an oligomerization domain at its C terminus. In previous studies, forced expression of wild type and variant *Dmp53* transgenes were used to establish that an ectopic Dmp53 expression triggers apoptotic cell death and possibly affects the duration of M phase (Ollmann et al., 2000). Additional experiments with Dmp53 variants showed that radiation-induced apoptosis in the wing disc was suppressed by dominant negative transgenes. However, expression of these same transgenes had no effect upon the damage induced cell cycle arrest response, even though many cell cycle regulators including fly *p21* are conserved in the fly genome and expression of fly p21 alone can trigger G1arrest (de Nooij and Hariharan, 1995). These observations supported a role for Dmp53 in apoptosis and, at the same time, suggest that Dmp53 may not engage checkpoint controls through the cell cycle like its mammalian counterparts.

To further study *in vivo* functions of Dmp53, I isolated a mutation at the *Dmp53* locus. Since there were no p-elements available that mapped near the *Dmp53* region at the time this project was initiated, I adopted a gene targeting method recently developed by Rong and Golic (Rong and Golic, 2000; Rong and Golic, 2001). This method directs mutations at the desired locus through homologous recombination events that produce aberrant duplicates of the targeted gene. Using this approach, I generated and validated one line, $Dmp53^{-ns}$, as a targeted mutation. $Dmp53^{-ns}$ mutants do not show any observable developmental defects. However, when $Dmp53^{-ns}$ homozygous flies were crossed, there was a marked increase in the number of unfertilized embryos. The egg-hatching test failed to discriminate whether this defect mapped to a maternal and/or paternal origin. In this chapter, I present one of the first published examples of homologous recombination based gene targeting in *Drosophila* using the *Dmp53* locus, and initial characterization of $Dmp53^{-ns}$ mutants in development.

Materials and Methods

Plasmid constructions. The 2.8 kb *Dmp53* donor template (Fig. 2-2A) was produced by PCR amplification of genomic DNA in two fragments using following primers. The 1.4 kb 5' end of the fragment was produced by primer I (5'-*ataagaatgcggccgc*acggaggtcgatatcaaggaggatattccg-3') and primer II (5'-attaccctgttatccctacgggccgtcgtgaaagattccgcttctcaaaggc-3'). The other 1.4 kb 3'fragment was produced by primer III (5'-tagggataacagggtaatcccgatatcgattggccaggagtccgaagacgg-3') and primer IV (5'-*ataagaatgcggccgc*catgtataacatgctgtcccacgatatcgccgc-3'). These two PCR fragments were used as templates for the second PCR amplification to make one 2.8 kb fragment using primer I and IV. Primer II and primer III contain *70I-SceI* sites (underlined), resulting in an introduction of a single *70I-SceI* site in the 2.8kb *Dmp53* donor element. *NotI* sites (in *italics*) were introduced in primer I and IV for cloning into *NotI*-cut pTV2 vector. This construct was introduced into flies using p-element mediated germline transformation.

Molecular verification of targeting. Genomic DNA was prepared from all $white^{+}$ nonmosaic flies carrying w^{+} marker on the 3rd chromosome and used to verify a disruption of the native *Dmp53* site by genomic PCR (see Fig 2-2C). The primers *1* (5'-aacattggctacggcgattgttcgcgc-3') and *a* (5'-attaccctgttatccctacgggccgtcgtgaaagattccgcttctcaaaggc-3'), and *2* (5'-caccttagcgttgagccttg-3') and *b* (5'-gacgctccgtcgacgaagcgcctctatt-3') were used to check a disruption at the 5' native *Dmp53* site. The primers *3* (5'-gtgacctgttcggagtgattagcgttac-3') and *c* (5'-ggcattggcgtacaccacgaggatatg-3') were used to check a disruption at the 3'native *Dmp53* site. For northern blot, $Dmp53^{-ns}$ cDNA was labeled with [^{32}P]dCTP using Random Primed DNA Labeling Kit (Ambion). Third instar larvae, pupae, and adults were either mock treated or γ-irradiated at 4000 rads using a Mark I Model 25 Irradiator. After the treatment, samples were

allowed to age for 1hr and 30min prior to RNA isolation.

Crosses and screens for targeting. Flies carrying the *Dmp53* donor element on the 2nd chromosome were crossed to flies carrying both *70FLP* and *70I-SceI* transgenes on the 2nd chromosome to yield *Dmp53* donor/*70FLP*, *70I-SceI*;+/+ progeny. These were heat shocked during developmental stages 0 to 3 days to induce recombinants in the germline. The primary screen for recombinations in the germline was based on eye-color mosaicism. The secondary screen for targeting events was based on a loss of *white*$^{+}$ mosaicism as described previously (Rong and Golic, 2001). A validated *Dmp53* mutant, *Dmp53*$^{-ns}$, was crossed to *yw* flies for several generations to eliminate any potential second site mutations. *Dmp53*$^{-ns}$ homozygous flies are viable and fertile. We note a marked incidence of aborted embryos in this strain, *Dmp53*$^{-ns}$.

Cytological analysis of *Dmp53*$^{-ns}$ embryos. Flies were raised at 25°C and 3~5hr embryos were collected. After dechorionation with 50% bleach, embryos were fixed in 3.7% formaldehyde in 1XPBS for 20minitues. Subsequently, embryos were devitellinized in methanol for 1 minute. After rehydration in 1XPBTA, embryos were incubated with a 1:500 sperm-tail specific antibody provided by T. Karr (Karr, 1991) followed by incubation with 1:50 FITC conjugated anti-mouse (Vector Laboratories). Embryos were then stained with 0.1 mg/ml 4,6-diamidine-2-phenylindole (DAPI) for 5 minutes.

Microscopy and imaging. All imaging was done on Axioplan 2E (Zeiss) microscope with a Hamamatsu monochrome digital camera.

Results

Generation and verification of a targeted *Dmp53* mutation.

To obtain a *Dmp53* mutant, we used a gene-targeting method developed by Rong and Golic for "ends-in" homologous recombination (Rong and Golic, 2000; Rong and Golic, 2001). The "ends-in" targeting disrupts a native targeted locus by inserting a transgenic targeting construct (donor construct) carrying engineered mutations. This results in a tandem duplication of the target gene at the native locus, with both copies containing engineered mutations (Figure 2-1).

Like its mammalian counterpart, Dmp53 protein contains a putative transcriptional activation domain at the N terminus, a DNA binding domain, and an oligomerization domain at the C terminus. A donor targeting construct using an internal 2.8kb *Dmp53* fragment as a donor template from the target gene sequence was prepared (Figure 2-2A). This internal 2.8-kb fragment in the donor construct lacks two-thirds of the transcriptional activation domain, the entire oligomerization domain, and part of the DNA binding domain (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000). Note also that five of eight residues predicted to contact DNA (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000) are absent in this donor template. Hence, an insertion of the 2.8-kb internal fragment at the *Dmp53* native locus is predicted to generate loss of function mutations at this locus.

Flies carrying the *Dmp53* donor construct on the 2nd chromosome were crossed to flies carrying both *70FLP* and *70I-SceI* transgenes to yield *Dmp53* donor/*70FLP*, *70I-SceI*;+/+ progenies. The basis of the screen for targeting is a loss of *white*$^{+}$ mosaicism from the secondary screen as shown in Figure 2-2B (Rong and Golic, 2001). We obtained seven individuals who had lost *white*$^{+}$ mosaicism from the secondary screen. Of these, we validated one line, *Dmp53*$^{-ns}$, as a targeted mutation. PCR based assays (Figure 2-2C), with several primers specific to the *Dmp53* genomic region as well as primers specific to the

marker gene w^{hs}, confirm this interpretation. For example, the primer pair 3 and c gives a product only when there is a targeting event at the *Dmp53* locus (Figure 2-2A and 2C).

We used northern blot analyses to confirm that expression of native *Dmp53* transcripts are disrupted in the $Dmp53^{-ns}$ strain. Hybridization with a *Dmp53* specific probe to RNAs from three different stages showed a mildly radiation-responsive 1.6 kb *Dmp53* transcript in wild type animals that was absent from $Dmp53^{-ns}$ homozygotes (Figure 2-2D). Instead, two aberrantly sized transcripts are evident in the mutant strain, which probably derived from one or both of the targeted variants.

$Dmp53^{-ns}$ mutants suffer from reduced viability.

Flies disrupted at the *Dmp53* locus are fertile and exhibit no overt visible defects. However, $Dmp53^{-ns}$ mutant embryos exhibited reduced viability compared to wild type flies (Table 2-1). The reduced viability maps to the defective *Dmp53* locus, since transheterozygous flies for different *Dmp53* mutant alleles retained this reduced viability. DAPI staining of early to mid stage embryos suggested that $Dmp53^{-ns}$ mutants either die at a very early embryonic stage or are possibly unfertilized (Figure 2-3A). To test whether $Dmp53^{-ns}$ embryos are unfertilized, $Dmp53^{-ns}$ embryos were stained with a sperm specific monoclonal antibody (Figure 2-3B)(Karr, 1991). $Dmp53^{-ns}$ embryos that lacked DAPI staining also lacked sperm specific antibody staining with close to 100% penetrance, indicating these embryos are unfertilized.

I next examined whether the defective fertilization in *Dmp53* mutants are of a maternal or paternal origin (Table 2-1). If this is a maternal defect, we expect that $Dmp53^{-ns}$ homozygous females crossed to $Dmp53^{-ns}$ heterozygous males will show decreased embryo viability as seen in the $Dmp53^{-ns}$ homozygous parents cross. When $Dmp53^{-ns}$ homozygous females were crossed to $Dmp53^{-ns}$ heterozygous males, the hatch rate of eggs was close to that of wild type. In the reciprocal cross, when $Dmp53^{-ns}$ homozygous males were crossed to $Dmp53^{-ns}$ heterozygous females, the hatch rate of eggs was reduced to ~80%, suggesting

there is a mild paternal defect in *Dmp53*$^{-ns}$ mutants. Although this result does not explain the severe reduced fertility observed in *Dmp53* homozygous mutant crosses, Lee *et al*. reported that *Dmp53* mutant females exhibit degenerative ovarioles that may contribute to the reduced fertility (Lee et al., 2003).

Discussion

To infer *in vivo* functions for Dmp53, previous studies relied on forced expression of either wild type or presumptive dominant negative transgenes. Though informative, conclusions from these types of studies are also confounded by considerations relating to highly expressed transgenes. To determine the precise role(s) of Dmp53 through loss-of-function genetic analyses, we produced a targeted mutation at the *Dmp53* locus. Using the homologous recombination procedure devised by Golic and colleagues, we isolated a targeted mutation at the native Dmp53 locus (Rong and Golic, 2000; Rong and Golic, 2001). As the product of "ends-in" recombination this allele, designated $Dmp53^{-ns}$, generates two variant copies of *Dmp53*- both of which - if translated- would produce nonfunctional products. For example, the disrupted template mapping to the left of the w^{hs} gene lacks most of the oligomerization and DNA binding domains, including 5 of 8 residues thought to contact DNA (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000). Likewise, the template mapping to the right of the w^{hs} gene lacks all contiguity with its native promoter and virtually all of the putative transactivation domain including the initiating methionine and a critical serine residue (Ser-4) thought to be critical for regulation by fly Chk2 (Peters et al., 2002). Given the indispensable nature of these domains, $Dmp53^{-ns}$ can be considered a null allele with respect to transcriptional activity. It is formally possible that aberrant $Dmp53^{-ns}$ - derived activity could, in principle, engender neomorphic effects (e.g. if Dmp53 encodes significant functions beyond transcription) but, given that $Dmp53^{-ns}$ phenotypes are recessive and consistent with those previously documented for the transgenic dominant negative alleles (see Chapter 3), we believe this is a remote possibility.

In agreement with Golic and colleagues (Rong et al., 2002) who briefly reported on a *Dmp53* allele, flies homozygous for $Dmp53^{-ns}$ are fertile and exhibit no overt visible defects as embryos, larvae, pupae or adults. However, a closer examination of $Dmp53^{-ns}$ embryos

revealed a high number of embryos being unfertilized. When the hatching rate of eggs was measured, the cross between homozygous *Dmp53*$^{-ns}$ mutant females and heterozygous *Dmp53*$^{-ns}$ mutant males showed close to a normal hatching rate (~91%). On the other hand, the cross between homozygous *Dmp53*$^{-ns}$ mutant males and heterozygous *Dmp53*$^{-ns}$ females showed a mild reduced viability of embryos (~80% hatching rate). However, these data did not reveal a clear maternal effect or paternal effect. Using another loss-of-function *Dmp53* mutant allele, *p53*^{e4}, Lee *et al.* observed the presence of some degenerative ovaries, which may contribute to the reduced fertility. It is possible that the fidelity of germ cells of *Dmp53* mutants is compromised. Sub-optimal *Dmp53* gametes may be rescued by wild type gametes to create healthy embryos, however, when both parents carry sub-optimal gametes, this may lead to the synergistic unfertilization effect seen in the *Dmp53* mutant cross. More detailed analyses are needed to test this possibility. It is interesting to note that Yin *et al.* observed reduced fertility and decreased germ cell death in p53$^{-/-}$ male mice (Yin et al., 1998). Knockout p53 mutations in mice are similarly viable and fertile and, hence, like its mammalian counterpart, Dmp53 function is not required for normal development. However, mice mutated at p53 also exhibit a strong predilection toward late-onset cancers, though more subtle age-dependent phenotypes might be uncovered in longevity studies not undertaken here, we observed no clear indications of late-onset 'over-growth phenotypes' in aged *Dmp53*$^{-ns}$ flies. Taken together, my results indicate that *Dmp53* is dispensable for normal development, however, it may play a role in maintenance of germ cells.

Table 2-1. Embryo viability of *Dmp53* mutants

Alleles and genotype of cross				
Maternal		Paternal	Survival rate	n
yw	X	yw	98 ± 1.7%	90
Dmp53^{-ns}	X	*Dmp53^{-ns}*	34 ± 7.2%	90
Dmp53^{1}	X	*Dmp53^{1}*	32 ± 8.5%	80
Dmp53^{-ns}	X	*Dmp53^{1}*	33 ± 9.5%	140
Dmp53^{1}	X	*Dmp53^{-ns}*	50 ± 5.3%	180
Dmp53^{-ns}	X	*Dmp53^{-ns}*/+	91 ± 7.0%	160
Dmp53^{-ns}/+	X	*Dmp53^{-ns}*	80%	60

Embryo viability was measured by scoring the number of embryos laid and hatched to larvae. *Dmp53^{1}* is another loss-of function mutant allele generated by Rong *et al* (Rong et al., 2002).

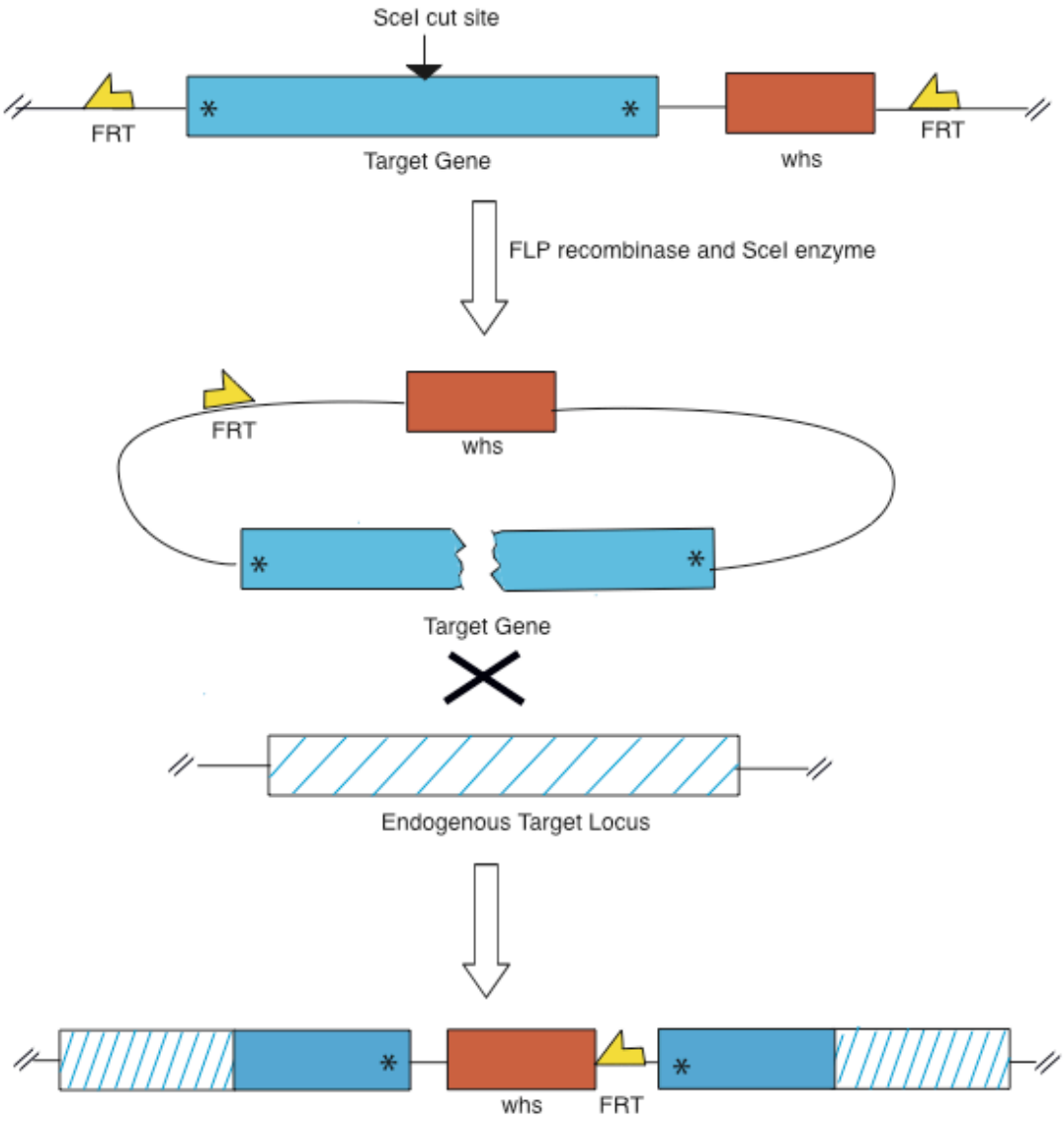
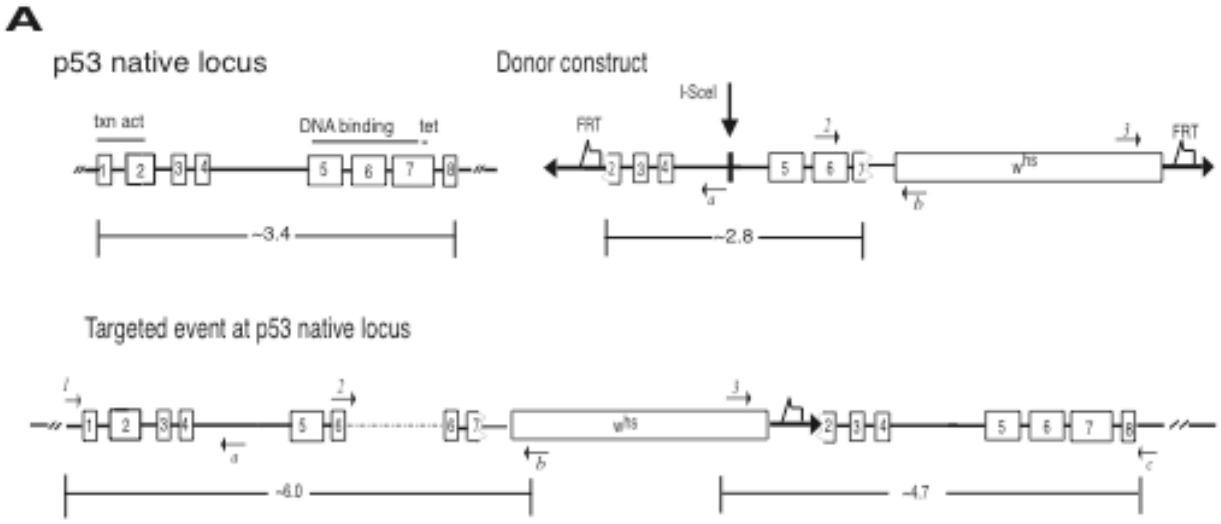
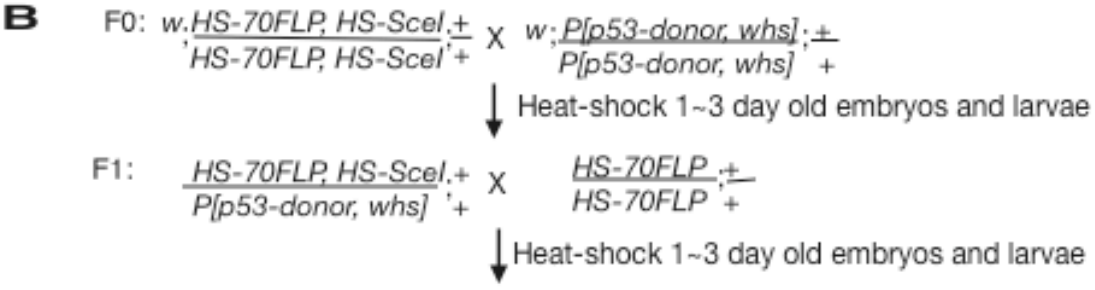
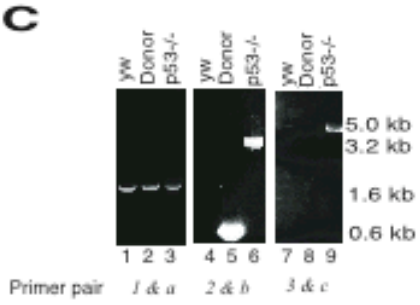


Figure 2-1. The general homologous recombination targeting scheme. A donor construct that contains two engineered mutations (denoted as *) is shown at the top. The engineered mutations can be point mutations and/or deletions. Expression of FLP recombinase and SceI enzyme leads to a production of highly recombigenic extrachromosomal donor sequence. Once a targeting event takes place by homologus recombination at the endogenous chromosomal target locus, the result is a duplication of target genes at the native locus. Engineered mutations are incorporated into both copies of target genes, thereby creating non-functional target genes.


A
p53 native locus
Donor construct
txn act
DNA binding
tet
I-SceI
FRT
FRT
~3.4
~2.8
Targeted event at p53 native locus
~6.0
~4.7


B
F0: w.HS-70FLP, HS-SceI.+ / HS-70FLP, HS-SceI.+ X w;P[p53-donor, whs];+ / P[p53-donor, whs];+
Heat-shock 1~3 day old embryos and larvae
F1: HS-70FLP, HS-SceI.+ / P[p53-donor, whs].+ X HS-70FLP;+ / HS-70FLP;+
Heat-shock 1~3 day old embryos and larvae
F2: Screen for white + progeny on 3rd chromosome


C
yw
Donor
p53-/-
5.0 kb
3.2 kb
1.6 kb
0.6 kb
1 2 3 4 5 6 7 8 9
Primer pair 1 & a 2 & b 3 & c

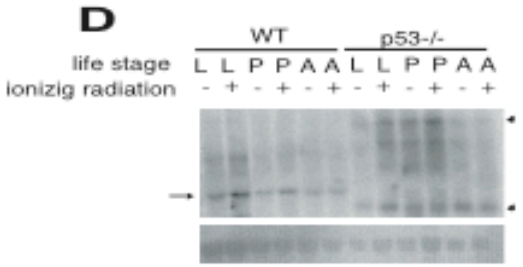

D
WT
p53-/-
life stage L L P P A A L L P P A A
ionizig radiation - + - + - + - + - + - +

Figure 2-2. Targeting scheme and molecular verification for generation of a *Dmp53* mutant. (A) Targeting scheme for the *Dmp53* gene. The donor construct illustrated next to a schematic of the native *Dmp53* locus was generated by the insertion of a 2.8 kb internal fragment of the *Dmp53* gene into the targeting vector pTV2. The 2.8 kb fragment lacks part of an amino terminus transactivation domain (due to the elimination of the entire exon 1 and part of exon 2), and part of a central DNA-binding domain and an entire tetramerization domain (due to the elimination of part of exon 7 and the entire exon 8). A germline transformant donor strain bearing this constructs on the 2nd chromosome was used for targeted mutagenesis as described (Rong and Golic, 2000; Rong and Golic, 2001). Upon heat-shock mediated induction of FLP site-specific recombinase and I-SceI endonuclease, the donor construct is excised as an extrachromosomal molecule and may create a lesion at the native *Dmp53* locus by homologous recombination. The targeted mutation at the native *Dmp53* locus, referred to as $Dmp53^{-ns}$, produced a tandem duplication of *Dmp53* variants that sandwich the w^{hs} gene. The dotted line in exon 6 shown for the targeted event at the *Dmp53* native locus indicates an unexpected insertion of partial pTV2 vector sequences (confirmed by PCR and sequencing). (B) The schematic representation of the targeting cross is shown. At F_0, flies carrying the *Dmp53* donor construct are crossed to flies carrying FLP recombinase and SceI endonuclease. The 1-3 day old progenies from F_0 cross are heat-shocked to cause targeting in the germline. The heat-shocked progenies were crossed to flies carrying only FLP recombinase at F_1cross. This step allows quick identification of the donor construct mobilization (to the target locus or at non-target loci) from the original donor construct insertion site based on the expression pattern of w^{hs} marker gene. For example, flies with an unsuccessful targeting event will show *white* + eye-color mosaicism due to their retention of two FRT sites at the original donor construct insertion site. On the other hand, flies with the mobilized donor construct to the target site (or a non-targeted site) will show non-mosaic *white* + expression since they only carry one FRT site. (C) PCR analysis of a *Dmp53* targeting event. Genomic DNA from flies was used as template DNA for PCR to verify the targeting event at the Dmp53 locus. Lanes 1-3 use a primer pair, *1* and *a*. Lanes 4-6 use a primer pair, *2* and *b*, to verify the disruption of the N-terminus *Dmp53* template. Lanes 7-9 use a primer pair, *3* and *c*, to assess the disruption at the C-terminus *Dmp53* template. All PCR fragments were partially or fully sequenced to determine the variations introduced at the native *Dmp53* locus. Locations of each primer are listed in Figure 2-2A in *italics*. Genotype of flies: *yw* refers to the parental wild type strain at the native *Dmp53* site on the 3rd chromosome; Donor refers to the *Dmp53* donor construct on the 2nd chromosome, wild type at the native *Dmp53* site on the 3rd chromosome; p53-/- refers to the disrupted native *Dmp53* site on the 3rd chromosome. (D) Northern blot analysis of a *Dmp53* targeting event. Total RNA from wild type (*yw*) and $Dmp53^{-ns}$ strains were isolated, blotted to a membrane, and hybridized with a *Dmp53* probe. The arrow indicates the 1.6 kb expected *Dmp53* transcript. In wild type larval and pupae individuals, this transcript is moderately responsive to ionizing radiation. *Dmp53* mutants lack the1.6 kb *Dmp53* transcript, and instead express aberrantly sized transcripts indicated by arrowheads. The bottom panel shows the membrane stained with Methylene Blue to show ribosomal RNA as a loading control. RNA from untreated (-) or γ-irradiated individuals (+) in the wandering third instar larvae (L); pupae (P); and adults (A) stages were analyzed.

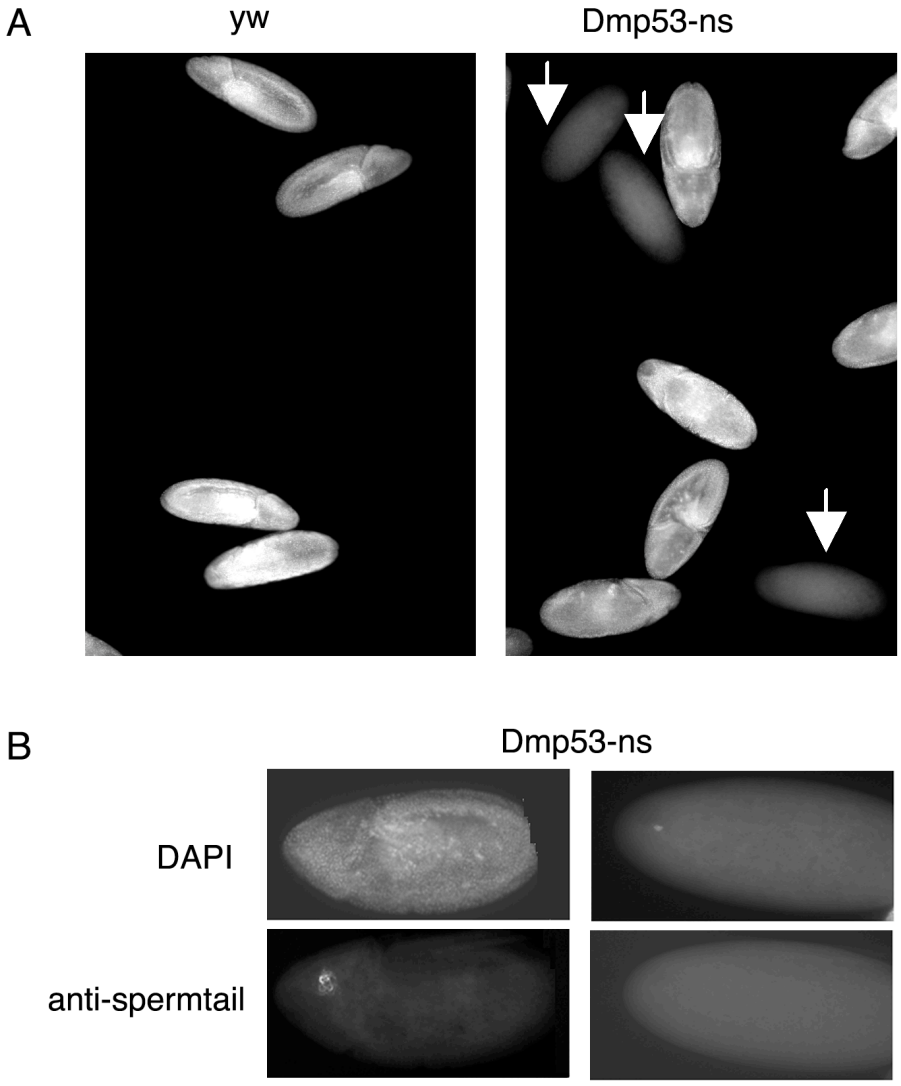


Figure 2-3. *Dmp53*$^{-ns}$ mutants show a higher incidence of unfertilization. (A) DAPI staining of wild type and *Dmp53*$^{-ns}$ early to mid stage embryos. Many *Dmp53*$^{-ns}$ mutants lack DAPI staining as shown with arrows. (B) *Dmp53*$^{-ns}$ mutants were double-labeled with DAPI and an anti-sperm tail antibody to detect a presence of fertilization. The DAPI staining positive *Dmp53*$^{-ns}$ embryos (top left panel) show the presence of coiled sperm tail in the anterior part of eggs (bottom left panel). *Dmp53*$^{-ns}$embryos that lack positive DAPI staining (top right panel) also lack the anti-sperm tail antibody staining with close to 100% penetrance (bottom right panel).

CHAPTER THREE

THE ROLE OF Dmp53 IN RESPONSE TO GENOTOXIC STRESS

Abstract

When animal cells are exposed to stressful conditions, the tumor suppressor protein p53 restrains growth by promoting an arrested cell cycle or initiating a cell death program. How these distinct fates are specified through the action of single protein is not known. To study its functions *in vivo* we produced a targeted mutation at the *Drosophila p53* (*Dmp53*) locus. We show that Dmp53 is required for damage-induced apoptosis but not for cell cycle arrest. Dmp53 function is also required for damage-induced transcription of two tightly linked cell death activators, *reaper (rpr)* and *sickle (skl)*. When challenged by ionizing radiation, *Dmp53* mutants exhibit radio-sensitivity and genomic instability. Hence, elevated mutant loads were not caused by defective checkpoint functions. Our studies support the notion that core ancestral functions of the p53 gene family are intimately coupled to cell death and possibly DNA repair as an adaptive response to maintain genomic stability.

Introduction

The tumor suppressor protein p53 is mutated in more than 50% of human cancers (Vogelstein et al., 2000). Recent studies also indicate that many tumors that retain wild-type p53, contain mutations in the genes either upstream or downstream of the p53 pathways. Therefore, the incidence of the defective p53 pathways in human cancers is much higher than 50% (Ryan et al., 2001). p53 exerts its protective mechanism against genotoxic damage primarily by either initiating cell cycle arrest, senescence, DNA repair, and/or apoptosis in mammals. However, the precise mechanism of how p53 choose to activate these various adaptive responses remain largely unknown. Furthermore, we have little knowledge of which defective pathways governed by p53 lead to a robust malignant transformation seen in human cancers.

A major goal of p53 research is to identify the downstream target genes for each pathway. So far, more than two dozen potential p53 downstream target genes have been identified (el-Deiry, 1998; Nakamura, 2004). The most well characterized p53 downstream target is *p21*, a cyclin dependent kinase inhibitor, that is required for the reversible G1/S cell cycle arrest (Deng et al., 1995; el-Deiry et al., 1993). On the other hand, there are many potential p53 proapoptotic target genes, those include *bcl2* family members, *TNF* receptor family members, and *p53-induced genes* (*PIGS*) (Polyak et al., 1997). It is unclear why so many proapoptotic genes are regulated by p53 compared to growth inhibitory genes, although, the expression of those proapoptotic genes are highly context specific. The common feature of these p53 regulated genes is that they contain one or more canonical p53 binding sites, defined as follows: 5'PuPuPuC(A/T)(T/A)GpyPyPy3'(el-Deiry et al., 1992).

Several studies using overexpression of *Dmp53* transgenes suggest that Dmp53 engages damage induced apoptosis but not damage induced cell cycle arrest (Brodsky et al.,

2000a; Jin et al., 2000; Ollmann et al., 2000). In *Drosophila*, programmed cell death (PCD) is governed by apoptosis activators mapping to a genomic interval referred to as the *Reaper* region (Abrams, 1999). Several lines of evidence implicate one gene in this region, *rpr*, as a direct target of, and effector for, Dmp53 action *in vivo*. First, *rpr* transcription is acutely sensitive to damage signals, becoming induced within 90 minutes of γ-irradiation treatment (Nordstrom et al., 1996). Second, this inductive response maps to a 20 bp radiation-responsive element that binds Dmp53 and resembles the consensus developed for mammalian counterparts (Brodsky et al., 2000a). Third, recent loss-of-function analyses uncovered a partial requirement for *rpr* in a model of gene in X-ray induced cell death (Peterson et al., 2002).

To directly examine the functions of Dmp53 and aid in the identification of direct *in vivo* targets, we isolated a mutation at the *Dmp53* locus. As there are no available p-elements that map near the *Dmp53* region, we adopted a gene targeting method recently developed by Rong and Golic (Rong and Golic, 2000; Rong and Golic, 2001). This method directs mutations at the desired locus through homologous recombination events that produce aberrant duplicates of the targeted gene. Though viable, *Dmp53* mutants exhibit pronounced defects in damage-induced apoptosis. Radiation-induced expression of two proapoptotic genes, *rpr* and *skl*, and a p53-responsive reporter transgene failed to regulate normally in these mutants. However, under these same irradiation conditions, the damage-induced cell cycle checkpoint response occurred normally. In the post embryonic stage, *Dmp53* mutants are radio-sensitive and also exhibit a pronounced mutator phenotype, manifested as high mutagenic loads in loss-of-heterozygosity (LOH) assays. Together, these results uncover important genomic consequences that the cell cycle arrest exerted by p53 proteins is dispensable for preservation of genomic stability by p53.

Materials and Methods

Cell death assay and checkpoint function assay. Cell death assay was carried out as described previously (Brodsky et al., 2000a). Checkpoint function assays were done 90 min after γ-irradiation as described previously with minor modifications (Ollmann et al., 2000). Vectastain Elite ABC Kit (Vector Laboratories) was used to stain the discs for HRP activity. The discs were incubated with biotinylated anti-rabbit secondary antibodies at 1:400 dilution for 2hrs at room temperature, followed by another 1 hr incubation in PBS/0.5% Triton containing an equal amount of reagents A and B at 1:200 dilution. The discs were visualized with a DAB/0.03% H_2O_2 solution.

Histochemical staining of *rpr*-lacZ transgenic embryos. Transgenic flies containing the150bp-*lacZ*-reporter construct (Brodsky et al., 2000a) were crossed to *Dmp53*$^{-ns}$ mutant flies to generate 150bp-*lacZ;p53*$^{-/-}$ flies. Collection of 2 to 5 hr embryos of the appropriate genotype and histochemical staining of those were carried out as described previously (Brodsky et al., 2000a).

Analysis of mRNA expression by using oligonucleotide arrays. 2.5 to 5 hr embryos from two parental wild type strains (WT^{yw} and WT^{w}) and the *Dmp53*$^{-ns}$ mutant were irradiated at 4000 rads, and 90 min later, total RNA was extracted using Trizol reagent (Gibco/BRL). Gene expression analysis was performed using the Affymetrix (Santa Clara, CA) *Drosophila* Gene Chip, and methods outlined in the Affymetrix Gene Chip expression manual. Gene expression analysis was performed using paired irradiation and control total RNA samples for each fly strain. Note that the *reaper* signal was scored as absent in control WT^{yw} embryos, but was detected as 'present' in all other samples. Likewise, the signal for *sickle* was scored as 'absent' in unirradiated WT and $Dmp53^{-ns}$ samples, but scored as 'present' in irradiated in WT^{yw} and WT^{w} embryos.

Radiation sensitivity assay. Wandering third instar larvae wild type or homozygous for the *Dmp53*$^{-ns}$ allele were collected and irradiated with increasing amount of ionizing radiation. The percentage of flies became adult after 5~6 days of radiation treatment was determined.

LOH assay. Three independent recombinant lines homozygous for *Dmp53*$^{-ns}$ and heterozygous for *mwh* were generated. Each recombinant line was mock-treated or irradiated at 250 rads during a third instar larval stage. Wings were dissected and mounted in 1:1 methyl salicylate: Canada balsam (Sigma). Cells only with 3 or more hairs were scored as a *mwh*$^{-/-}$ phenotype.

Results

Dmp53^{-ns} mutant blocks damage-induced apoptosis but not cell cycle arrest.

We examined requirements for Dmp53 *in vivo* in the context of damage-induced signaling. In the wing discs of wandering third instar larvae, apoptotic cells are rarely observed in wild type animals (Figure 3-1A) (Brodsky et al., 2000a; Ollmann et al., 2000) or in *Dmp53*$^{-ns}$ mutants (Figure 3-1C). However, after irradiation, wild type wing discs (and wing discs from *Dmp53*$^{-ns}$ heterozygotes) as well as embryos show notably increased levels of apoptotic cell death (Figure 3-1B and E). This response was clearly absent from the *Dmp53*$^{-ns}$ strain since we observed no acridine orange positive cells in irradiated wing discs and embryos from this strain (Figure 3-1D and F). Under the same conditions and in the same tissue, we next tested the requirement for Dmp53 on damage-induced cell cycle arrest. We used a phospho-histone H3 (PH3) antibody to examine the presence of cells in mitosis as in previous studies (Brodsky et al., 2000b; Hendzel et al., 1997). After irradiation, both wild type and *Dmp53*$^{-ns}$ mutant discs showed a complete absence of mitotic cells (Figure 3-2B and D) and, hence, without functional Dmp53, normal checkpoint functions were completely unaffected as assessed by this marker. Therefore, while Dmp53 status is an essential determinant of radiation induced apoptosis, the gene plays no detectable role in governing the cell cycle arrest response, at least in the developing wing discs. Together, these results demonstrate a requirement for Dmp53 in damage-induced apoptosis but not in cell cycle arrest.

Dmp53 is necessary for radiation-induced transcription of the *reaper* region.

Programmed cell death in *Drosophila* requires three apoptosis activator genes - *rpr*, *grim* and *hid*, which map to a 300 kb genomic interval (Chen et al., 1996a; Grether et al., 1995; White et al., 1994). Of these, *rpr* is acutely sensitive to damage signals, becoming transcriptionally activated within a short period after exposure to ionizing radiation

(Nordstrom et al., 1996). The minimal radiation responsive *cis*-element lies in a 150 bp fragment, 5 kb upstream of the *rpr* start codon, and within this radiation responsive enhancer, a Dmp53 binding site was identified (Brodsky et al., 2000a). To determine whether Dmp53 is required for activation of this radiation responsive enhancer *in vivo*, we used a *lacZ* reporter transgene referred as to *rpr* 150bp-*lacZ*, which contains the minimal 150 bp radiation responsive fragment. We examined β-galactosidase expression of the150bp-*lacZ* reporter gene in wild type and *Dmp53*$^{-ns}$ mutant embryos with or without irradiation treatment. When mock-treated, neither wild type nor *Dmp53*$^{-ns}$ mutant embryos showed β-galactosidase activity (Figure 3-3A and C). Following γ-irradiation treatment, wild type embryos showed robust β-galactosidase activity (Figure 3-3B). In contrast, *Dmp53*$^{-ns}$ mutant embryos showed no induction of β-galactosidase (Figure 3-3D). These data show that Dmp53 is necessary for activation of the radiation responsive enhancer upstream of the *rpr* locus.

Like other genes in the *Reaper* region, *skl* encodes an apparent IAP antagonist. This gene maps ~ 41 kb proximal to *rpr*, and, like *rpr*, is acutely radiation responsive (Christich et al., 2002). We therefore compared radiation-responsive expression of *rpr* and *skl* in embryos from two parental wild type strains and the *Dmp53*$^{-ns}$ mutants using microarray studies (Table 3-1). Microarray profiles of radiation-responsive genes confirmed that both *rpr* and *skl* are strongly induced after γ-irradiation (Christich et al., 2002). However, in the *Dmp53*$^{-ns}$ mutant strain, neither *rpr* nor *skl* were induced after γ-irradiation treatment. RT-PCR against *rpr* using larvae and pupae showed a clear upregulation of the *rpr* transcript in response to IR in wild type animals, however this radiation response failed to occur in *Dmp53*$^{-ns}$ mutant animals (Figure 3-3E). I also observed that constitutive levels of the *rpr* transcript level are significantly higher in *Dmp53*$^{-ns}$ pupae than in wild type pupae (see discussion). Although we currently do not know why the *rpr* transcript level is higher in *Dmp53*$^{-ns}$ pupae compared

to wild type pupae, the data clearly demonstrates that the radiation response of *rpr* is abolished in *Dmp53^{-ns}* larvae and pupae. These results confirm that *rpr* is a transcriptional target of Dmp53, and also indicate that *skl* could be a Dmp53 target gene as well.

Dmp53^{-ns} mutant is sensitive to ionizing radiation and shows a higher rate of genomic instability.

We assessed the *Dmp53^{-ns}* line for potential mutator phenotypes using a loss of heterozygosity (LOH) assay (Baker, 1978; Brodsky et al., 2000b). Wandering third instar larvae heterozygous for the *multiple wing hair* (*mwh*) mutation and either wild type or homozygous for the *Dmp53^{-ns}* allele were collected and irradiated. Loss of heterozygosity for the wild type *mwh* allele (arising from chromosomal aberrations or point mutations) is readily assessed by scoring the recessive *mwh* phenotype in wing cells of adult flies (Figure 3-4A and B). The absence of Dmp53 alone did not significantly affect the basal incidence of *mwh* cells (Figure 3-4C). However, if challenged by low doses of ionizing radiation (250 rads), each independent [*mwh,Dmp53*/+, *Dmp53*] line showed substantially elevated levels of *mwh* cells under conditions that had no effect in wild type animals for Dmp53. We also note that *Dmp53^{-ns}* mutant larvae exhibited a radio-sensitive phenotype, manifested as extensive lethality rates that were particularly noticeable at doses of 4000 rads (Table 3-2). These results indicate that, in the context of genotoxic damage, Dmp53 functions to preserve organismal viability and genomic stability.

Discussion

Previous studies using forced expressions of presumptive dominant negative *Dmp53* transgenes suggested roles of Dmp53 in the damage induced apoptosis but not the damage induced cell cycle arrest (Brodsky et al., 2000a; Jin et al., 2000; Ollmann et al., 2000). However, the interpretations of these studies can be complicated due to the artificial nature of experiments. To directly examine the functions of Dmp53, I generated a loss of function Dmp53 mutant, *Dmp53*$^{-ns}$. Using this *Dmp53* mutant allele, I examined the *in vivo* role of *Dmp53* in response to radiation.

Studies on dominant-negative transgenes indicated important roles for Dmp53 in damage induced apoptosis. Analyses of homozygous *Dmp53*$^{-ns}$ animals revealed profound failures in damage induced apoptosis when directly tested under conditions of genotoxic stress (Figure 3-1). If placed under the same conditions, it is worth noting that irradiated discs from *Dmp53*$^{-ns}$/+ heterozygous flies were indistinguishable from wild type flies. Hence, in this context, we uncovered no evidence for dominant-negative effects exerted through the *Dmp53*$^{-ns}$ allele. Brodsky *et al*. observed that the cis-regulatory element of the pro-apoptotic gene *rpr* contains a 150bp radiation responsive enhancer element (Brodsky et al., 2000a). Within the 150bp enhancer, there is a 20bp putative p53 binding site (p53RE). The p53RE is sufficient for radiation response and Dmp53 can activate transcription from this site in yeast. To test whether Dmp53 is necessary for transactivation of the p53RE, I placed the *rpr* 150-bp-*lacZ* reporter gene in wild type and *Dmp53*$^{-ns}$ mutant embryos. This radiation responsive transgene is entirely unresponsive in a *Dmp53*$^{-ns}$ background (Figure 3-3D). This observation is further supported by directly examining irradiated *Dmp53*$^{-ns}$ flies using microarray analyses and RT-PCR (Table 3-1 and Figure 3-3E). Together these data establish an absolute requirement for Dmp53 in radiation-induced apoptosis and also validate *rpr* as an authentic

in vivo target of Dmp53.

Recently, Peterson and colleagues studied a synthetic deletion uncovering *rpr* and found a requirement for this gene in x-ray induced cell death (Peterson et al., 2002). Hence, pathways linking damage signals to *rpr* through Dmp53 are essential for optimal apoptotic cell death in this genetic model. At the same time, however, it should also be emphasized that evidence favoring the existence of additional, pro-apoptotic Dmp53 targets is strong. For example, flies that are singly mutated for *rpr* were only partially compromised for radiation-induced apoptotic cell death and overexpression of Dmp53 in the eye was not significantly affected by the absence of *rpr*. Also, as seen here in the wing disc, removal of Dmp53 function completely abrogates radiation-induced apoptosis but removal of *rpr* only partially affects this same damage response. Together, these data argue that additional, pro-apoptotic targets of p53 exist in the *Drosophila* genome. One particularly attractive candidate in this regard, is the IAP-antagonist, *sickle* (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002) which, like *rpr*, also shows an acute, Dmp53-dependent induction provoked by damage signals (Table 3-1). This gene maps ~40kb to the 5' side of *rpr* and so it is conceivable that the Dmp53 binding site regulating *rpr* might similarly govern the radiation responsive induction of *skl* as well. Products encoded at *rpr* and *skl* provoke cell death, at least in part, by antagonizing native caspase inhibitors referred to as IAP proteins (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002) IAP antagonists with orthologous activity (e.g. Smac/Diablo, Omi/Htra2) have also been reported in mammalian systems (Jin et al., 2003) and it will therefore be of interest to determine whether any of these genes might represent p53 targets in mammals.

The RT-PCR analysis of mock-treated and IR treated wild type and *Dmp53* mutant flies from various stages revealed that constitutive levels of *rpr* transcript were significantly higher in $Dmp53^{-ns}$ pupae. I do not know currently whether this is a direct or indirect consequence of loss of Dmp53. During pupae stage, ecdysone regulates the *rpr* expression to

induce the destruction of certain tissues. The EcR/USP complex directly regulates the expression of *rpr* using a binding site located in the *rpr* promoter region (Jiang et al., 2000). It is possible that Dmp53 serves to repress a constitutive level of *rpr* in the absence of genotoxic stress, hence loss of Dmp53 may provide the EcR/USP complex an easier access to the *rpr* promoter.

As in mammalian systems, *Drosophila* cells abruptly halt progression through the cell cycle in response to genotoxic stress. In vertebrate, and possibly invertebrate models, the cell cycle regulator p21 is essential for this response and, in stressed mammalian cells, p53 is the major regulator of p21 induction. Studies with dominant negative transgenes suggested that Dmp53 may not similarly govern cell cycle progression in flies but, given the usual limitations on interpreting these types of studies, alternate technical explanations were also possible. Therefore, we directly examined the issue of damage-induced cell cycle control in the *Dmp53*$^{-ns}$ strain. Using a mitosis marker to monitor this response, we found that irradiated wing discs from *Dmp53*$^{-ns}$ homozygotes were indistinguishable from wild type samples. Hence, at least in the wing disc, Dmp53 is entirely dispensable for an arrested cell cycle and, together with earlier studies, the analyses uncover an important distinction between flies and mammals. Alternatively, since phospho-histone 3 antibody staining cannot distinguish the G1/S arrest from the G2/M arrest, we cannot exclude the possibility that *Dmp53*$^{-ns}$ mutants have the defective G1/S arrest but normal G2/M arrest. de Nooji *et al*. also reported that there was no induction of *dacapo* in response to IR (de Nooij et al., 1996). Although p21 is possibly the arresting agent in both systems, our data suggest alternative p53-independent mechanisms for regulating cell cycle arrest in the context of genotoxic damage.

There is a growing evidence to suggest that p53 is involved in DNA repair (Brodsky et al., 2004; Fei and El-Deiry, 2003; Nakano et al., 2000; Tanaka et al., 2000). Our microarray analyses also indicate Dmp53 is involved in DNA repair in response to IR

(discussed in Chapter 5). I attempted to ask the question whether Dmp53 is required for DNA repair using the Single Cell Gel Electrophoresis assay (known as the Comet Assay) that examines DNA (single and/or double strand) breaks at the single cell level. Although this technique has been widely used for assessing a DNA repair defect in mammalian systems, the small nucleus size of *Drosophila* makes a measurement of DNA breaks challenging. As an alternative approach, we are using H2Av antibody to detect a formation of double strand breaks (DSB) in *Dmp53*$^{-ns}$ mutants. H2Av is a histone variant in *Drosophila Melanogaster*, and like its mammalian counterpart H2AX, this protein becomes highly phosphorylated and localized to the sites of DSB in response to IR (Madigan et al., 2002). We hypothesized that we might observe a persistent localization of phosphorylated H2Av at DSB sites in *Dmp53*$^{-ns}$ mutants compared to wild type due to a lack of DNA repair program. A collaborative effort with Robert Glaser is underway to test this hypothesis. His preliminary observation indicates there is no difference between wild type and *Dmp53* mutant ovaries for the number of H2Av foci.

Important implications arise from the fact that p53 functions to govern apoptosis but not cell cycle arrest in *Drosophila*. From an evolutionary perspective, it can be inferred that ancestral functions of p53 were intimately coupled to the regulation of cell death and perhaps DNA repair in the face of genotoxic challenge. Hence, the core means for exerting negative growth control by this family relates to the effects of these proteins upon cell death and DNA repair. An obvious corollary here is that checkpoint arrest provoked by p53 may reflect a more recently invented function, specific perhaps to the vertebrate or mammalian lineage. In agreement with this deduction, a recent study in the nematode similarly report a role for *C. elegans* p53 in damage-induced cell death but found no role for the gene in the cell cycle arrest response (Derry et al., 2001). A second implication from these studies argues against the hypothesis that p53 launches a sequential damage response program. According to this widely held view, genotoxic stress first provokes an attempted 'repair' program (manifested

as cell cycle arrest), which is then followed by an apoptosis if repair efforts are unsuccessful. Inherent to this scenario is the assumption that p53 forces cells toward an arrested cell cycle prior to engaging a cell death program. Since *Drosophila* p53 clearly elicits apoptosis and/or DNA repair without arresting the cell cycle, our findings disfavors this notion. If there are universal effectors of this tumor suppressor, our data argue for a proximate connection between p53 signaling, cell death, and DNA repair without the need for intervening cell cycle signaling.

Perhaps the most substantive advance from these studies relates to the effect of Dmp53 on genomic stability. If exposed to moderate doses of radiation, *Dmp53^{-ns}* homozygotes harbored significantly elevated mutagenic loads that were not detectable in their wild type siblings. Interestingly, we uncovered no evidence that *Dmp53^{-ns}* promotes a mutator effect in the absence of radiation and, hence, the effect of Dmp53^{-ns} is clearly limited to conditions associated with genotoxic stress. These observations are clearly distinct from checkpoint/DNA replication/repair mutants (Brodsky et al., 2000b; Xu et al., 2001) where high mutagenic loads are detectable even in the absence of genotoxic stress. In mammalian systems, p53 is essential for the maintenance of genomic stability (Robles and Harris, 2001; Wahl et al., 1997) but the precise derivation of this activity has been difficult to resolve given the multiple functions of this protein. For example, it is not clear whether failures in cell cycle arrest, DNA repair or failures in cell death- or possibly a combination of all defective activities- is responsible for maintaining genomic stability. Since *Dmp53^{-ns}* homozygotes exhibit a mutator phenotype (and yet are normal for cell cycle arrest) we conclude that a failure in cell cycle arrest alone does not adequately account for genomic instability. Though defective cell cycle control could contribute to many p53 phenotypes seen in mammalian settings, our data are consistent with Schmitt et al. (Schmitt et al., 2002) who argue from study in mouse models that mutator defects leading to robust transformation can be traced to p53-associated apoptosis rather than defective checkpoint functions.

Table 3-1. Radiation response: Fold induction values

	WT^{yw}	WT^{w}	$Dmp53^{-ns}$
reaper	6.0 (↑)	5.4 (↑)	no change
sickle	10.6 (↑)	6.0 (↑)	no change

Expression of the indicated transcripts was examined in irradiated and control samples from 2 wild type strains (WT^{yw} and WT^{w}) and the *Dmp53^{-ns}* mutant strain. Total RNAs isolated from staged embryos were analyzed using the Affymetrix *Drosophila* Gene Chip Array. Fold induction values relative to basal levels are reported. In agreement with previous studies (Christich et al., 2002), *reaper* and *sickle* are respectively induced from ~ 6 fold to 10 fold within 90 minutes of γ-irradiation exposure. In *Dmp53^{-ns}*, levels of *reaper* and *sickle* transcripts are unchanged.

Table 3-2. Radiation sensitivity: % Survival to adult

	Exposure at 4000 rads		
	Trial 1	Trial 2	Trial 3
WT	35%	67%	78%
Dmp53^{-ns}	2%	17%	6%

Wandering third instar larvae (~50 of each genotype/trial) were irradiated and eclosion was determined 5-6 days later. When tested at 1000-3000 rads, *Dmp53^{-ns}* mutants exhibited moderate radio-sensitivity. At doses of 500 rads or lower, eclosion rates for WT and *Dmp53^{-ns}*-mutants were between 80%-90%.

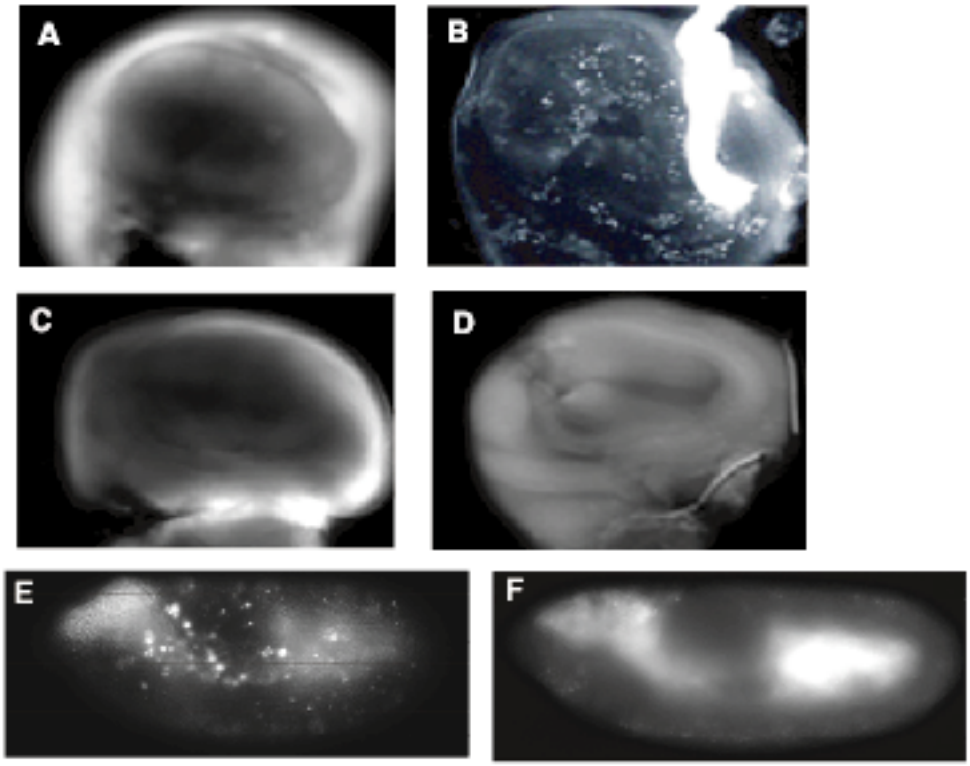


Figure 3-1. *Dmp53*$^{-ns}$ mutants are defective in damage induced apoptosis. A vital dye acridine orange, specifically recognizes apoptotic cells. Dissected wing discs from larvae and embryos from wild type (A, B and E) or *Dmp53*$^{-ns}$ (C, D and F) were mock-treated (A and C) or γ-irradiation treated (B, D, E, and F), and apoptotic cells were visualized by acridine orange. For each genotype, at least 5 wing discs were dissected and stained. The *Dmp53*$^{-ns}$ phenotype was fully penetrant.

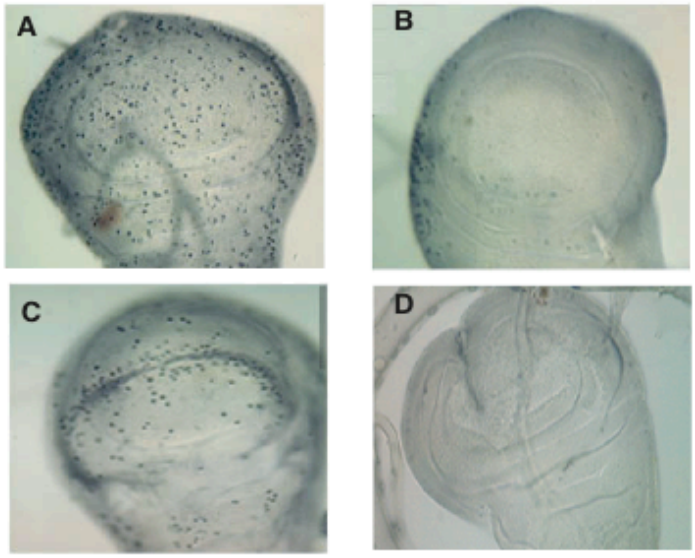


Figure 3-2. *Dmp53 $^{-ns}$* mutants exhibit a normal cell cycle arrest. Wing discs from wild type (A and B) or *Dmp53^{-ns}* (C and D) were dissected from mock (A and C) or irradiated (B and D) 3rd instar wandering larvae. Histochemical staining with phospho-histone H3 antibody detects cells undergoing mitosis. For each genotype, approximately 5 wing discs were dissected and stained. Discs from wild type or *Dmp53^{-ns}* mutant were indistinguishable with respect to the incidence of immunoreactive cells, and staining seen for the *Dmp53^{-ns}* control wing disc shown is within the normal range. The *Dmp53^{-ns}* phenotype was fully penetrant.

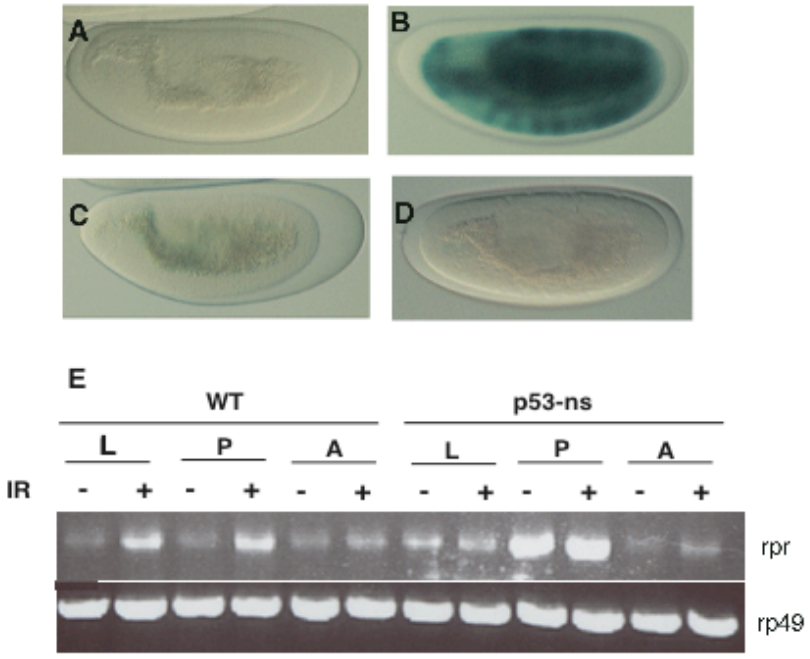


Figure 3-3. Radiation responsive enhancer at the *reaper* locus is no longer radiation responsive in *Dmp53*$^{-ns}$ embryos. Transgenic lines carrying a 150 bp radiation responsive enhancer region from the *rpr* locus was tested in wild type (A and B) or *Dmp53*$^{-ns}$ mutant embryos (C and D). Mock treated controls (A and C) or irradiated (B and D) samples were stained for β-galactosidase activity. (E) RT-PCR against *rpr*. Total RNA from wild type and *Dmp53*$^{-ns}$ strains was isolated. *rp*49 was used as a control. RNA from untreated (-) or IR treated (+) individuals in the wandering third-instar larvae (L), pupae (P), and adult (A) stages were analyzed.

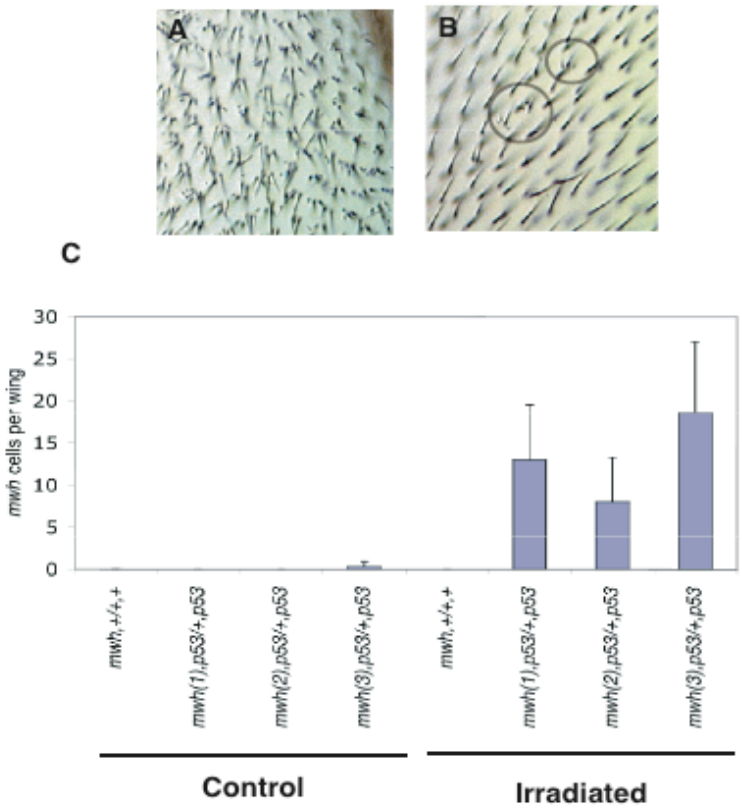


Figure 3-4. *Dmp53*$^{-ns}$ adults carry high mutation loads after exposure to ionizing radiation. Loss of heterozygosity is one measure of genomic instability. Here, the recessive *mwh* phenotype (A) was scored in animals heterozygous for *mwh* and either wild type or homozygous for *Dmp53*$^{-ns}$. (A) A wing from homozygous *mwh* animal. Each cell contains multiple hairs. (B) A wing from heterozygous for *mwh* and homozygous for *Dmp53*$^{-ns}$ animal, that has been treated with γ-irradiation. Cells within the circles have lost the wild type copy of *mwh*, uncovering the recessive *mwh* phenotype. (C) Loss of Dmp53 leads to genomic instability after irradiation. Both *mwh* and *Dmp53* genes are on the 3rd chromosome, and three independent recombinant lines, *mwh(1),p53*; *mwh(2), p53*; and *mwh(3), p53* were generated and tested. For each genotype, 3 wings were examined. The average number of *mwh* cells per wing in wild type or *Dmp53*$^{-ns}$ mutant animals is indicated in animals that had or had not been exposed to moderate levels of ionizing radiation.

CHAPTER FOUR

GENETIC INTERACTION BETWEEN *Dark* AND *Dmp53*

Abstract

Dark is a mammalian Apaf-1 homologue that is required for the activation of initiator caspases by forming an apoptosome complex. Several microarray and biochemical studies suggest *apaf-1* is a target of p53 in mammals, however, the genetic data to support this notion is currently not available. To investigate the genetic interaction between *dark* and *Dmp53,* I made $dark^{CD4}$;$Dmp53^{-ns}$ double mutants. The double mutants are born normally but develop age-dependent progressive melanoic tissues in adult wings. This phenotype is also seen in $dark^{CD4}$ mutants alone but to a lesser degree. Examination of wing epidermal cells in wild type and $dark^{CD4}$ mutants did not reveal any obvious cell death defects in $dark^{CD4}$ mutants. Together, studies undertaken here demonstrate the genetic interaction between *dark* and *Dmp53*, however, the precise mechanism of the melanoic tissue development in the wing still remains unknown.

Introduction

p53 can regulate cell death either by the death receptor mediated "extrinsic pathway" or the mitochondria mediated "intrinsic pathway". In the extrinsic pathway, *Fas/Apo1* and *Killer/DR5* are the primary targets of p53, and the expression of these receptors leads to a formation of the death inducing complex (DISC), that in turn activates initiator caspases 8 and10 (Sax and El-Deiry, 2003). Many of the downstream p53 targets for the intrinsic apoptotic pathway are pro-apoptotic Bcl2 family members such as *bax, noxa,* and *puma* (Sax and El-Deiry, 2003). Expression of these genes leads to a shift in the balance of pro and anti-survival signals at mitochondria, resulting in a release of cytochrome *c*. The released cytochrome *c* forms an apoptosome complex with apoptotic protease activating factor-1 (Apaf-1), dATP and pro-caspase 9, leading to an activation of caspase 9 followed by an activation of effector caspases.

Apaf-1 is an evolutionally conserved gene from *C.elegans* (*ced-4*) to mammals. In *Drosophila,* this gene is encoded by *dark* (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999) and is more similar to mammalian APAF-1 than CED-4 due to the presence of c-terminal WD domains where cytochrome *c* presumably binds in mammals. However, present evidence does not support the requirement of cytochrome *c* for the formation of apoptosome complex to activate initiator caspases in *Drosophila*. Genetic interaction studies showed a requirement of *dark* for the Dronc mediated cell death (Quinn et al., 2000). Dronc is one of the three initiator caspases known in *Drosophila*. Dronc is able to form a complex with Dark, suggesting Dronc is the initiator caspase required for the apoptosome formation in *Drosophila*.

Mutant animals for *dark* or *dronc* die at pupae stage (Rodriguez, unpublished data) (Chew et al., 2004) illustrating their roles in development, contrary to that of *Dmp53*.

Hypomorphic *dark*^{CD4} mutants are viable and show numerous defects including an enlarged CNS, an abnormal number of bristles, occasional melanoic tumors on the body and wing defects. Several microarray and biochemical studies indicate that *Apaf-1* is a target of p53 in mammals (Fortin et al., 2001; Moroni et al., 2001), however, *in vivo* data to support this notion is lacking. Although we currently do not know whether *dark* is a target of Dmp53, this is a likely possibility given the expression of *dark* is highly responsive to γ-irradiation (Rodriguez et al., 1999) and UV (Zhou et al., 1999). To test whether *dark* and *Dmp53* genetically interact, I constructed double mutants that are hypomorphic for *dark* and *Dmp53*. The double mutants produced age-dependent progressive melanoic tissues in adult wings with 100% penetrance. Although *dark*^{CD4} mutants alone produce melanoic tissues in the wing, the severity of dark spots was far greater and the onset of which was much earlier in the double mutants. Hence, this study establishes the genetic interaction between *dark* and *Dmp53*, and also suggests roles of these genes in the maintenance of adult tissues in the wing.

Materials and Methods

Fly strains. The *dark*^{CD4} strain was provided by Anthony Rodriguez. The *69B-GAL4,GFPN* on the 3rd chromosome stocks were generously provided by Ken-ich Kimura.

Microscopy and imaging. All imaging was done on an Axioplan 2E (Zeiss) microscope with a Hamamatsu monochrome digital camera or an Axioscope microscope.

Results

Dark^{CD4};Dmp53^{-ns} double mutants develop age-dependent melanoic tissues in the wing.

In mammals, p53 is required for the transcription of Apaf-1 in response to genotoxic stress (Moroni et al., 2001). To test whether *Dmp53* genetically interacts with *dark*, a *Drosophila* homologue of *apaf-1/ced4*, I made double mutants for *dark^{CD4}* and *Dmp53^{-ns}*. *Dmp53* mutants develop normally with no visible defects, whereas *dark^{CD4}* mutants suffer numerous developmental defects ranging from an abnormal number of bristles to a substantially larger CNS (Rodriguez et al., 1999). One of the phenotypes observed among *dark^{CD4}* mutants was a development of melanoic tissues in various parts of the body, although at a relatively low frequency. *Dark^{CD4};Dmp53^{-ns}* mutants, however, developed severe melanoic tissues with 100% penetrance in the wing (Figure 4-1A). This phenotype differs from other wing defective phenotypes reported with respect to the age-dependent nature of the phenotype, that is, the appearance of melanoic tissues is progressive (Figure 4-1B). First, flies were born without any noticeable defects in the wing. In the case of *dark^{CD4};Dmp53^{-ns}* double mutants, the noticeable dark spots appear within 24 hours approximately. Some *dark^{CD4}* flies also show this phenotype. However, the severity of melanized tissue is less obvious and the onset is delayed (~7 days after eclosion) compared to that of double mutants.

The *Drosophila* adult wing is made out of two layers of cuticle that are secreted by epithelial cells during pupation. Shortly after eclosion, these epithelial cells go through programmed cell death leading to bonding of ventral and dorsal cuticles to form an adult wing (Kiger et al., 2001; Kimura et al., 2004). We hypothesized that a failure in wing epithelial programmed cell death may lead to the persistence of undead epithelial cells, and these undead cells may somehow cause this dark spot phenotype in *dark^{CD4};Dmp53^{-ns}* double mutants. To test this, flies carrying *69B-Gal4-UAS-GFPN* transgenes on the 3rd

chromosome were crossed to *drak*^{CD4} mutants to label nuclei of epithelial cells in the wing. When the wings from wild type or *dark*^{CD4} mutants expressing GFPN transgenes were compared, there was no obvious difference in the number of GFP expressing cells within 2 hours of eclosion, the time at which the majority of epithelial cells should go through programmed cell death (Figure 4-2A and B). I also compared wings from wild type and *dark*^{CD4} mutants 24 hours after eclosion, however, no persisting cells were observed in either strain (Figure 4-2C and D), indicating almost all cells had completed programmed cell death. Hence, in this study I did not observe obvious cell death defects that might account for melanization phenotype observed in *dark*^{CD4} mutants.

***GMR-Ras*^{V12}*;Dmp53*$^{-ns}$ mutants are born with melanoic tissues in the eye.**

In human cancers, a combination of amplified survival signals and defective cell death programs leads to aggressive tumor formation. To test whether the amplification of survival signals in *Dmp53*$^{-ns}$ mutant backgrounds leads to tumor formation in flies, I expressed *GMR-Ras*^{V12} in *Dmp53*$^{-ns}$ fly eyes. These flies were born with black spots in the center of and near the anterior ridge of the eye with 100% penetrance (Figure 4-3B-D). Losing one copy of Dmp53 was sufficient to cause the black spots (Figure 4-3B &C), and in fact, losing both copies of Dmp53 resulted in lethality (Figure 4-3D). I did not observe any hypergrowh in the eye using scanning electron micrography (Figure 4-3E). These flies are very sick and only a few flies eclose, making the further studies difficult. However, studies such as sectioning of the eyes may possibly reveal the nature of the melanized spots.

Discussion

In this chapter, I report the genetic interaction uncovered between *dark* and *Dmp53*. Previous studies in mammals have shown that the *apaf-1* expression can be induced in a p53 dependent manner (Moroni et al., 2001). In addition, both *dark* and *apaf-1* transcripts can be induced in response to IR, suggesting an evolutionary conserved programmed cell death pathway in response to genotoxic stress in mammals and *Drosophila*.

When animals lack Dark and Dmp53, they develop age-dependent dark spots in the wing. This phenotype exists in *dark* mutants alone, but to a lesser degree. It is interesting to note that I did not observe enhancement of any other $dark^{CD4}$ dependent phenotypes such as melanoic tumors outside the body of the double mutants. We hypothesized that the dark spots might be developed from wing epidermal cells that failed to die, and in turn, these undead cells might become melanized in the hypotoxic wing environment. The wing epidermal cell death is presumably the last developmental programmed cell death that takes place in Drosophila, therefore, it is possible that the maternal contributed *dark* transcripts from hypomorphic $dark^{CD4}$ no longer exist to execute this programmed cell death. To investigate whether the dark spots were derived from a failure in the cell death of wing epidermal cell, I labeled wing epidermal cells with a nuclear localized GFP and observed the process of programmed cell death in wild type and $dark^{CD4}$ mutants. This experiment did not uncover any obvious programmed cell death defects in $dark^{CD4}$ mutants. The lack of any obvious cell death defects in the $dark^{CD4}$ mutants could be due to the mildness of the dark spot phenotype exhibited by $dark^{CD4}$ mutants. Expressing GFP in the wing epidermal cells in $dark^{CD4}$;$Dmp53^{-ns}$ double mutants may uncover a more pronounced cell death defect, otherwise not seen in $dark^{CD4}$ mutants alone.

Dronc is one of the *Drosophila* apical caspases that shows the strongest similarity to

mammalian caspase 9 (Dorstyn et al., 1999). Po Chen in our lab showed *dark*^{CD4}*;dronc*^{51}*/TM3* mutants also developed the dark spots in the wing (Chew et al., 2004). Clonal analysis conducted by Fatih Akdmir in our lab showed that both *dronc*$^{-/-}$ clones and *dark*$^{-/-}$ clones in the wing developed dark spots indicating that Dark and Dronc regulate this cellular event in the same pathway. On the other hand, *Dmp53*$^{-ns}$ homozygous mutants alone do not develop dark spots. Dmp53 does not seem to play a role in developmental programmed cell death unlike other apoptotic genes, but rather its roles are focused on the damage induced cell death and DNA repair. Therefore, it is an intriguing question how the loss of Dmp53 significantly enhances the dark spot phenotype seen in *dark*^{CD4} mutants. One possible explanation for this observation is that Dmp53 might detect the dark spots as aberrant damaged tissues and activate a damage-induced Dark-independent cell death program to eliminate the aberrant tissues. In the absence of Dmp53, the aberrant tissues might not be removed, and hence, it might lead to the emergence of observed dark spots. It is worth noting that an enhancer screen conducted in our lab identified 24 mutants out of 1200 mutant lines on the X chromosome that enhanced the dark spots in the *dark*^{CD4} mutant backgrounds (Kathaleen Galindo, unpublished observation). On the other hand, I did not identify a single mutant out of ~900 X chromosome mutant lines that caused the dark spots in the *Dmp53*$^{-ns}$ backgrounds (Naoko Sogame, unpublished observation). These data suggest: First, these interactions we observed with *dark-dronc* and *dark-Dmp53* are very specific. Second, *Dmp53* is a classic enhancer of *dark* in this respect.

During my dissertation, I also sought other possible genetic interactors of *Dmp53*. Two examples particularly worth mentioning are *MJDtr-Q78* and *Ras*^{V12}. *MJD* is a gene with unknown functions and mutations in this gene result in the most common dominantly inherited ataxia (Warrick et al., 1998). This gene contains 12-40 polyglutamine repeats at the C-terminus, and becomes pathogenic when repeats expand to 55-84. Transgenic flies carrying a truncated form of *MJD* (*MJDtr*) followed by 78 glutamine repeats show late-onset

neurodegeneration. A similar model of this neurodegeneration can be suppressed by a *dark* mutation (Sang et al., 2005). When I drove an expression of MJDtr-Q78 in the fly eye, a few flies showed a mild reduction in neurodegeneration in the *Dmp53*$^{-ns}$ mutant backgrounds, indicating the neuronal cell death caused by polyglutamine repeats is likely activated by the *Dmp53-dark* pathway. We were unable to follow up with this observation since the flies that carry the *MJDtr-Q78* transgenes in *Dmp53*$^{-ns}$ mutant backgrounds are very rare, and even when they are born, they die within a few hours of eclosion. George Jackson, our collaborator, is currently following-up on with this issue. *Ras* is an oncogenic gene and its roles are implicated in cell proliferation and differentiation. When a constitutively activated form of Ras, Ras^{v12}, is ectopically expressed in the *Drosophila* eye using an eye specific driver, this results in a rough eye phenotype due to a transformation of non-neuronal cone cells into ectopic R7 cells (Fortini et al., 1992). We tested whether expressing *Ras*^{v12} in *Dmp53*$^{-ns}$ mutant backgrounds would give cells a growth advantage leading to overgrowth of eye tissues. Flies carrying the *Ras*^{v12} transgenes in the *Dmp53*$^{-ns}$ mutant backgrounds did not show a sign of overgrowth in the eye, however, they were born with black spots in the center of and near the anterior ridge of the eye. Scanning electron micrographs of the eyes did not reveal any hypergrowth where the black spots were observed. As seen in the case of *GMR*-MJDtr-*Q78;Dmp53*$^{-ns}$ flies, *GMR-Ras*^{v12};*Dmp53*$^{-ns}$ flies are very sick and many of them fail to eclose, therefore limiting further studies. Although the GMR promoter has been suggested to be eye specific, its leakiness of expression has been reported. The toxicity of these transgenes combined with the GMR leaky expression system may be exacerbated in the absence of Dmp53 resulting in the higher incidence of lethality observed here.

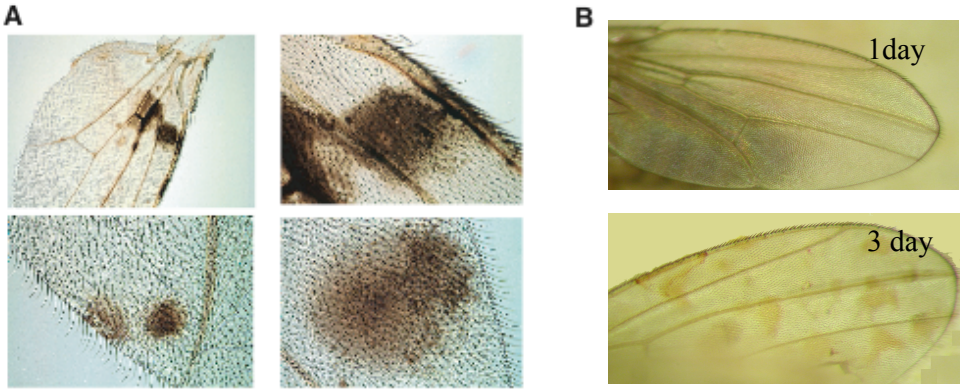


Figure 4-1. Interaction of *dark*^{CD4} and *Dmp53*$^{-ns}$ in adult wing tissues leads to a development of age-dependent melanoic tissues. (A) Wings from *dark*^{CD4}*; Dmp53*$^{-ns}$ mutants. Wings are normal at the time of eclosion. (B) On the top panel, a wing from *dark*^{CD4}*; Dmp53*$^{-ns}$ mutant shortly after eclosion is shown. Notice the absence of dark spots. On the bottom panel, a wing from *dark*^{CD4}*; Dmp53*$^{-ns}$ mutant 3 days after eclosion is shown.

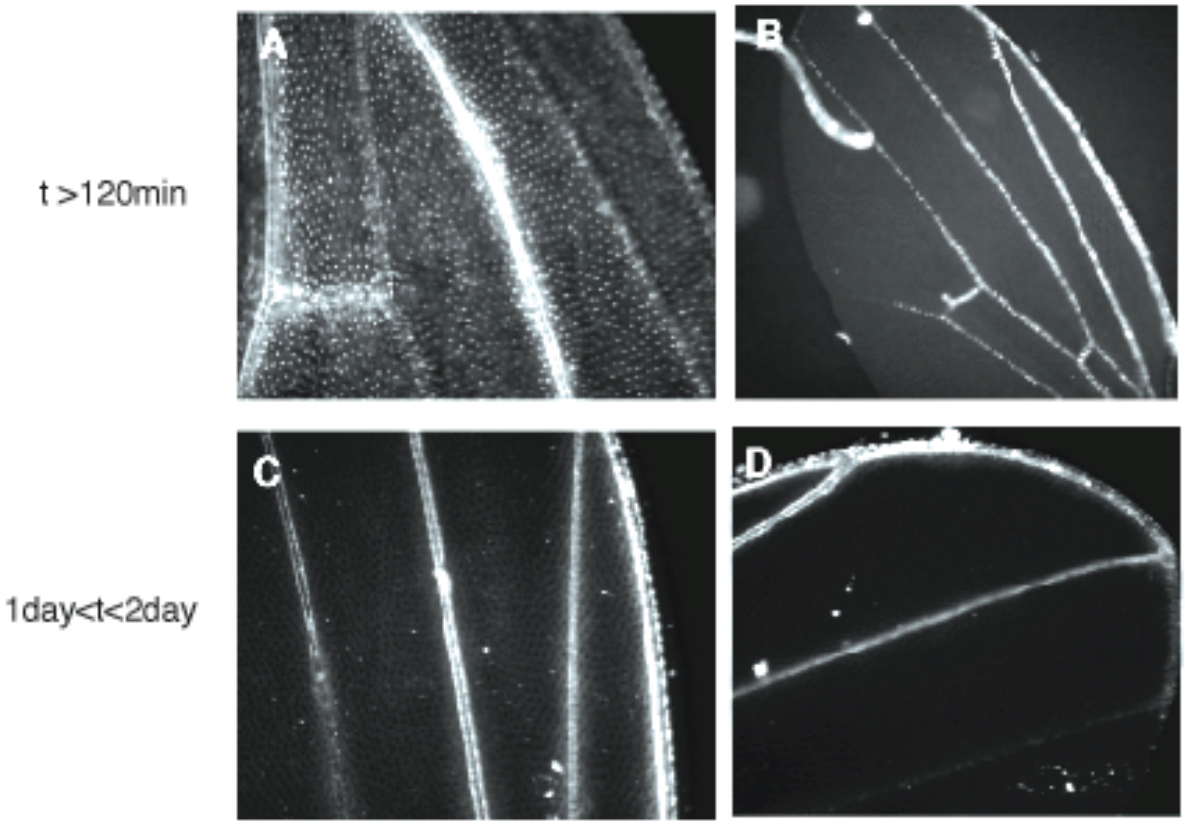


Figure 4-2. GFP-labeled epidermal cells show normal programmed cell death in *dark*^{CD4} mutants. GFP labeled wing epidermal cells in wild type (A & C) and *dark*^{CD4} mutants (B & D) are shown. Wings were dissected from adult flies within 120 minutes (A & B) or more than 1day but less than 2 days (C & D) after eclosion. Most programmed cell death of epithelial cells takes place within 60 minutes of eclosion. The difference observed between panels A and B is that A is that approximately 30 minutes after eclosion and B is close to 120 minutes after eclosion. Notice the absence of any persisting GFP positive epithelial cells in wild type or *dark*^{CD4} mutants in C and D.

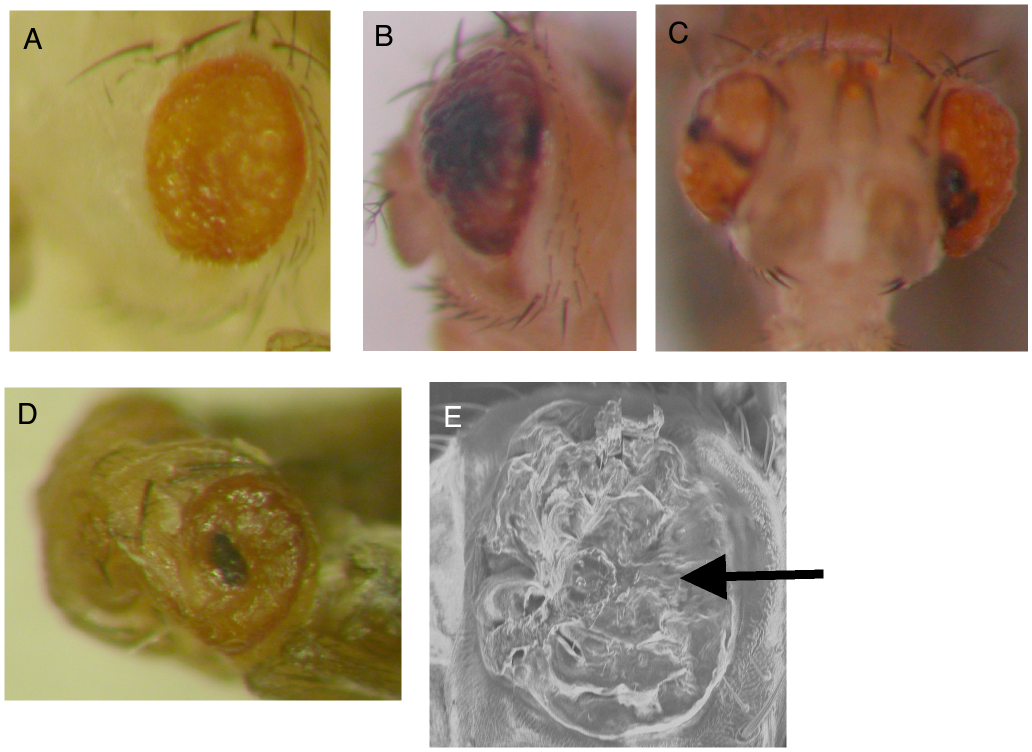


Figure 4-3. Overexpression of oncogenic Ras^{v12} in *Dmp53^{-ns}* mutant eyes results in development of melanoic tissues. Bright field images (A-D) and a scanning electron micrograph image (E) of fly eyes are shown. (A) *GMR-Ras^{v12}/Cyo*. Overexpression of *Ras^{v12}* causes rough eye phenotype. (B & C) *GMR-Ras^{v12}/Sp;Dmp53^{-ns}/TM3,Sb* (D) A dead *GMR-Ras^{v12}/Sp;Dmp53$^{-ns/-ns}$* fly was dissected out from the pupae case. (E) *GMR-Ras^{v12}/Sp;Dmp53^{-ns}/TM3,Sb*. The black arrow indicates where the black spot was developed.

CHAPTER FIVE

GENOME-SCALE ANALYSIS OF THE RADIATION RESPONSE AND IDENTIFICAITON OF CANDIDATE Dmp53 EFFECTORS

Abstract

We used microarray analysis to study radiation response in *Drosophila*. The IR response in wild type embryos was limited in scope, comprising only 35 (~0.25% of the genome) genes that consistently responded to IR. Dmp53 was required for the induction of the majority of radiation responders (30 out of 35). Functional analyses of the p53 dependent radiation responders using RNAi revealed that RnrL, a subunit of the ribonucleotide reductase enzyme complex, is involved in induction of caspase activation. In mammals, p53 regulates a different subunit of ribonucleotide reductase in response to DNA damage. These findings indicate an evolutionarily conserved radiation response pathway which directly impacts a rate-limiting determinant that controls nucleotide pools.

Introduction

The *p53* gene is an evolutionally conserved gene from *C. elegans* to mammals and its function is to limit propagation of damaged DNA caused by genotoxic stress. In mammals, p53 exerts this function by halting cell proliferation (perhaps followed by DNA repair) and inducing apoptosis. In contrast, *C. elegans* and *Drosophila* p53 exert their protective mechanisms by regulating apoptosis without promoting cell cycle arrest (Brodsky et al., 2000a; Derry et al., 2001; Ollmann et al., 2000; Schumacher et al., 2001; Sogame et al., 2003). We currently do not know which functions of p53, cell cycle arrest, apoptosis or perhaps both functions, are essential to prevent oncogenesis in mammals. However, the fundamental importance of apoptosis in preservation of the genome is obvious given that apoptosis is the evolutionally conserved function of p53 in both flies and worms and loss of Dmp53 causes a mutator phenotype, which likely contributes to oncogenesis in humans.

Current studies suggest that the primary mechanism by which p53 engages cell cycle arrest and apoptotic responses is a transcriptional regulation of p53 target genes (Espinosa et al., 2003). This notion is supported by the observation that most human cancers contain a mutation in the DNA binding domain of p53 (Burns and El-Deiry, 2003; Sax et al., 2003). Contrary to the well-defined p53 dependent cell cycle arrest pathway that targets *p21*, understanding the p53 dependent apoptosis pathway remains at a preliminary stage. Identification of numerous p53 apoptotic target genes in a context-specific manner (i.e., tissue specificity, stress specificity, etc…) makes the dissection of this pathway very complex. Recent advancement in the genome-wide search for p53 target genes continues to increase the number of "putative" p53 targets (Burns and El-Deiry, 2003; Polyak et al., 1997; Sax et al., 2003; Unger et al., 1992). However, whether these are a direct or indirect target of p53, and how these genes specify a stress response still remain to be understood.

Dmp53 regulates apoptosis partly by transcribing the IAP antagonists, *reaper* and *sickle* (Brodsky et al., 2000a; Sogame et al., 2003), leading to a release of IAP inhibition from caspases. This regulation of IAP antagonists by p53 is also conserved in mammals (Jin et al., 2003), perhaps indicating the relevance of this pathway to human cancers. In an effort to gain an understanding of the p53 dependent apoptotic pathways that are conserved, Anna Christich in John Abrams' Lab and I conducted a genome-scale radiation analysis using *Dmp53*$^{-ns}$ mutant embryos. First, our analysis of wild type embryos revealed that consistently radiation-responsive genes in *Drosophila* are limited in number, corresponding to 35 genes (0.25%) in the genome. Second, the majority of radiation responders required Dmp53 for their expression. To test their functional significance, I developed a novel *Drosophila* cell culture system that triggers massive apoptosis in response to IR. RNAi against Radiation Induced p53 Dependent (RIPD) genes using the newly established IR responsive cell culture system, I showed *ribonucleotide reductase large subunit* (*RnrL*) is required for the induction of caspase activation. Previous studies using mice identified a small subunit of ribonucleotide reductase *p53R2* as a p53 target, indicating conservation of the p53 dependent radiation response pathway that regulates the ribonucleotide reductase enzyme between *Drosophila* and mammals (Tanaka et al., 2000). Taken together, these studies identified the ancient radiation response pathway controlled by Dmp53.

Materials and Methods

Embryo collection and mRNA isolation for microarray analyses. Embryos from two parental wild type strains (*yw* and w^{1118}) and $Dmp53^{-ns}$ were collected and the total RNA from each sample was isolated as described in Chapter 3. Analyses of mRNA expression by using the Affymetrix (Santa Clara, CA) Gene Chip are outlined in Sogame *et al*. (manuscript in preparation).

dsRNA synthesis. PCR templates containing T7 polymerase binding sites at both ends were generated by amplification of cDNA by PCR (for *Dmp53*, *dronc, Dmchk2* (Open Biosystems), *bcn92* and *gfp*), or amplification of genomic DNA (Clontech) by PCR (for *RnrL*, *RnrS, mre11, cg17836, corp, eIF6, esc1, ku80, skl* and *rpr*) using the following gene specific primer sets: *Dmp53* (FWD: 5'-accacggaacccatggccttcttgcaggga-3'; REV: 5'-ttcggcggcttccggcacggacttgcg-3'); *dronc* (FWD: 5'- gccatattgggcacatataagatgcaatcacg-3'; REV: 5'- gtctgtgtcgtgggcatgatcggccatcacttg-3'); *Dmchk2* (FWD: 5'-ccgaggagattaacaagacgt-3'; REV: 5'- ttcttggtgtaggcttctcgg-3'); *RnrL* (FWD: 5'-gaaacggaaagatcgcagag-3'; REV: 5'- atccacagggcgtaaaacag-3'); *RnrS* (FWD: 5'-taccagagaatcagagaagcgcc-3'; REV: 5'- gatgtcgtggtactggatgggaa-3'); *mre11* (FWD: 5'-acggacttagccacatccac-3'; REV: 5'- atcattgcctccactcgttc-3'); *cg17836* (FWD: 5'-tcccatcttaatggcatc-3'; REV: 5'- ttattgttatgccagccgcgc-3'); *corp* (FWD: 5'-gaccaacggcaaggactacgtg-3'; REV: 5'- ggcttcgaacagacgtccacg-3'); *eIF6* (FWD1: 5'-ggctctacgcgtccaattcgag-3'; FWD2: 5'-acacttcgataggcagccaatc-3'; REV: 5-' gtgcctggttaagcttgaagacgc-3'; REV2: 5'-ctcgaattggacgcgtagagcc-3'); *esc1* (FWD: 5'-cacatgcgcttggtcctacgac-3'; REV: 5'-ggcgttgaaggcgaagcgtacgaa-3'); *ku80* (FWD: 5'-aaatggttgtgctactgccc-3'; REV: 5'-ttctctgctggttccgactt-3'); *skl* (FWD: 5'-gagcgactcaaatatggcca-3'; REV: 5'-gttgtatcccgacggataag-3'); *rpr* (FWD: 5'-

gtgtgtgcgccagcaacaaagaac-3'; FWD2: 5'-gcagtggcattctacatacccgatcaggcg-3'; REV: 5'-agccaacttcgactcatcttcg-3'; REV2: 5'-atcgagtcgcgtcggctgatcagtgg-3'); *bcn92* (FWD: 5'-ttcgcggagatcgatcgccaaa-3'; REV: 5'-gtttcatgaatgcaagtgcc-3'); *gfp* (FWD: 5'-cgacgtaaacggccaca-3'; REV: 5'- caggaccatgtgatcgcg-3'). Both forward and reverse primers contain a T7 polymerase binding site (ttaatacgactcactatagggaga) at the 5'ends. Multiple dsRNA were generated for *eIF6* and *rpr* to obtain a maximum knockout efficiency. The PCR products were purified and used to synthesize RNA using a MEGASCRIPT T7 Transcription Kit (Ambion). To anneal ssRNA to dsRNA, the samples were incubated for 30 minutes at 65°C and cooled to room temperature.

dsRNA and irradiation treatment of Kc167 Cells. Kc167 (Drosophila Genomics Resource Center) were maintained in Schneider's Drosophila media (Gibco) supplemented with 10% FBS (Gibco) and 1:200 dilution of Penicillin-Streptomycin (Gibco) at 25°C. Prior to dsRNA treatments, Kc167 cells were diluted to $1X10^6$ cells/ml in serum-free CCM3 media (Hyclone). 1ml of the cell suspension was plated in a 35mm culture dish with 15 μg of the specified dsRNA and cells were incubated for 48 hours. After a 48-hour incubation, the cell density of the sample was adjusted to $1X10^6$ cells/ml, and divided into three 250μl aliquots of the cell suspension. These aliquots were plated into 24-well plates with an additional 3.75μg of the specified dsRNA. The cells were incubated for 1 hour before adding 250μl of CCM 3 media containing a 1:500 dilution of 10mM Ecdysone analog, Muristerone A (Invitrogen) followed by a 72-hour incubation. After 72 hours of incubation, two of the 250μl aliquots were irradiated at 28K rads, and one 250μl aliquot was used for isolation of RNA for RT-PCR. The *gfp* dsRNA treated cells were used as a control and the DEVDase activity values were normalized to that of the *gfp* treated sample.

Caspase activity assays. Six hours after γ-irradiation treatment, cell extracts were collected

from dsRNA treated samples as described in Chen *et al* (Chen et al., 2004). For each sample, 5μg of a protein extract was incubated with 10μM Ac-DEVD-AFC (Calbiochem) substrate in a final volume of 100μl in a 96 well microtiter plate. Fluorescence was monitored over time with excitation at 360nm and emission at 465nm in a Spectra Fluor Plus plate reader (Tecan).

RT-PCR. Total RNA from each sample was isolated using a High Pure RNA isolation kit (Roche). 0.1~0.3ug of total RNA was used for RT-PCR reactions. Primers used for the reactions were: *Dmp53* (FWD: 5'-cgcttgatcagatatagccgac -3'; REV: 5'-cgcctccttaatcatgccctcgatgctctgcag-3'); *dronc* (FWD: 5'-gccggagctcgagattgg-3'; REV: 5'-ggaccgtcaacgacacccac-3'); *Dmchk2* (FWD: 5'-gcgggcaaactgcgaact -3'; REV: 5'-ggcagagtgccactcagg-3'); *RnrL* (FWD: 5'-tgccgcctcttgtga-3'; REV: 5'-gagcagataggaacgc-3'); *RnrS* (FWD: 5'-ttcccatccagtaccacgacatc-3'; REV: 5'-cagacctcgcttcttaagccag-3'); *mre11* (FWD: 5'-ggtagcccaacacac-3'; REV: 5'-aacaaggtctccgga-3'); cg17836 (FWD: 5'-tcccatcttaatggcatc-3'; REV: 5'- gtcgtcgacacaagttcc-3'); *corp* (FWD: 5'-gaccaacctcatcacttgc-3'; REV: 5'- gactcggtctggcccagactg-3'); *eIF6* (FWD1: 5'-agcaggctcagtcaggccagt-3'; REV: 5-' ttcgcttaggacatgtcctcg-3'); *esc1* (FWD: 5'-ctccgagcgaagagccaccgg-3'; REV: 5'-gtcgtaggaccaagcgcatgtg -3'); *ku80* (FWD: 5'-ctggatgtgcgaacatgtg-3'; REV: 5'-tggtcaccttgaagac-3'); *skl* (FWD: 5'-ttgcgcctccgagtgccgagg-3'; REV: 5'- cacatttgcgcttatcc-3'); *rpr* (FWD: 5'-gtgtgtgcgccagcaacaaagaac-3'; REV: 5'-agccaacttcgactcatcttcg-3'); *bcn92* (FWD: 5'-aatacgcgacacattccgcgcca-3'; REV: 5'-cttcatctggagacgcctcctgc-3'); *rp49* (FWD: 5'-atgaccatccgcccagcataca -3'; REV: 5'-acaaatgtgtattccgaccagg -3').

Results

Radiation responsive genes in *Drosophila* are limited in scope and most require Dmp53 for induction.

In the absence of Dmp53, apoptotic radiation response is totally abrogated in embryos as shown in Figure 3-1E & F. To obtain a genome wide profile of the gene expression change associated with a loss of Dmp53 in response to IR, Anna Christich in John Abrams' Lab and I conducted microarray analyses using $Dmp53^{-ns}$ mutant and wild type (*yw* and w^{1118}) embryos. Since the $Dmp53^{-ns}$ mutants were generated in a mixed wild type background, two different wild type strains were used in these experiments. Using the Affymetrix Gene Chip Expression Array system, nearly 14,000 probes were assayed in each experiment. We identified a core set of 35 genes (corresponding to 0.25% of genes in the genome) that are consistently IR responsive with $\geq$ 1.6 fold changes in three independent trials (one trial of *yw* and two trials of w^{1118}) using wild type embryos as shown in Figure 5-1. The vast majority of genes were unaffected by IR treatment regardless of the genotype. Only 1.2-3.0% of genes were responsive to IR in each experiment with the exception of one trial of w^{1118}, showing a wider scope of IR response (~6%) (unpublished observation, Anna Christich). The majority of IR responsive genes failed to respond in the absence of Dmp53. Here, we report a core set of 30 Radiation Induced Dmp53 Dependent genes (RIPD) that are detailed in Table 5-1. The RIPD genes were consistently up regulated in all three wild type samples but consistently failed to respond in two mutant samples. The RIPD genes include genes implicated in apoptosis and DNA repair as well as genes with unknown functions. Although *bcn92*, an uncharacterized gene on X chromosome, was IR responsive only in two out of three trials (responsive in one *yw* trial and one w^{1118} trial), it merits further studies since it resides near the computed Dmp53 binding site that is similar to the RRE near *rpr* described previously

(Brodsky et al., 2000a).

RnrL is a novel Dmp53 effector that is required for effector caspase activation.

To test the potential roles for RIPD genes, each gene was silenced by RNAi using a new cell culture model I developed. We examined whether any of the RIPD genes are required for induction of apoptosis using RNAi with an embryonic *Drosophila* cell line, Kc167. Many of the existing *Drosophila* cell lines are highly resistant to γ-irradiation (IR) (Sogame, unpublished observation). However, we observed pretreatment of Kc167 cells with 20-hydroxyecdysone, an active form of ecdysteroid that is required for insect metamorphosis (Echalier, 1997), dramatically increased sensitivity of Kc167 cells to γ-irradiation (Figure 5-2A~F). At 4 hours after IR treatment, we detected an increased DEVDase activity in the IR & ecdysone treated cells (Figure 5-2G). After 24 hours, the DEVDase activity of IR & ecdysone treated cells was approximately 20 times higher than the cells treated only with IR or ecdysone.

We next tested whether Dmp53 is required for the activation of DEVDase in the ecdysone & IR treated Kc167 cells. Figure 5-3A is a schematic representation of RNAi treatment followed by ecdysone & IR treatment of Kc167 cells. RNAi mediated knockdown of *Dmp53* lowered the DEVDase activity by ~65% (Figure 5-3B). DmChk2 is indispensable for the activation of the apoptotic function of Dmp53 (Peters et al., 2002), perhaps by activating Dmp53 through a direct phosphorylation at Ser-4. Knocking down the level of *Dmchk2* in Kc167 cells also lowered the DEVDase activation equal to the reduction observed in *Dmp53* RNAi treated cells. In *Drosophila*, the apical caspase Dronc plays a role in stress-induced apoptosis (Chew et al., 2004; Daish et al., 2004). As we expected, the expression of *dronc* is required for the DEVDase activation. The caspase activation was lowered by ~70% in $dronc^{-/-}$ cells. Taken together, our results indicate that the activation of DEVDase in the IR & ecdysone treated Kc167 cells is dependent on the DmChk2/Dmp53/Dronc stress response

pathway.

Using the ecdysone & IR treated Kc167 cells, we examined several candidate RIPD genes for a role in the activation of DEVDase. Our rationale for selecting candidate RIPD genes to be tested was whether they have clear human orthologues and/or they are located within 20kb from a computed p53 binding sites. Among the 10 RIPD genes tested (Figure 5-3C&D and Table 5-2), the cells knocked-down for *ribonucleotide reductase large subunit* (*RnrL*) showed a reduction in the DEVDase activity comparable to that of *Dmp53*, *Dmchk2*, and *dronc* (Figure 5-3B&C). On the contrary, the cells knocked-down for *mre11*, a gene involved in DNA repair, retained a high level of DEVDase activation indicating Mre11 is not required for the DEVDase activation (Figure 5-3D). *RnrL* encodes a large subunit of ribonucleotide reductase enzyme which catalyzes the conversion of ribonucleotides to deoxyribonucleotides for DNA replication and repair (Jordan and Reichard, 1998). Ribonucleotide reductase is a tetrameric enzyme composed of two dissimilar subunits, a large subunit R1 and a small subunit R2. R1 and R2 subunits are further divided into two subunits (R1) and subunits (R2). Tanaka *et al*. (Tanaka et al., 2000) identified a new member of the R2 subunit, *p53R2* as a novel p53 target gene. Although *RnrS*, the only existing *Drosophila* orthologue of R2 in the *Drosophila* genome, was not radiation responsive in our microarray studies, we tested whether RnrS is also required for the activation of DEVDase. RNAi mediated knockdown of *RnrS* also resulted in a significant reduction in DEVDase activity compared to that of the *gfp* dsRNA treated cells (Figure 5-3E). Since ribonucleotide reductase is involved in DNA replication, it is possible that the caspase activity reduction observed in *RnrL* and *RnrS* dsRNA treated cells is a secondary consequence of the S-phase associated inhibition. However, we believe this is an unlikely possibility since ecdysone treatment alone causes Kc167 cells to cease proliferation (Echalier, 1997), and therefore proliferative capacity is already quite low in this cell culture model. BrdU incorporation assays on RNAi treated cells showed very little incorporation of

BrdU in all samples (data not shown), supporting the notion that Kc167 cells lose a proliferative capacity after ecdysone treatment. However, we observed a reduction in the total number of cells in the *RnrS* RNAi treated sample (~45% reduction, data not shown), indicating that the proliferative capacity of the *RnrS* RNAi treated cells may have been affected during the 48-hour incubation prior to ecdysone treatment. Taken together, our studies suggest that *Dmp53* activates *RnrL* directly or indirectly in response to IR, and RnrL is required for the effector caspase activation. The small subunit of ribonucleotide reductase, RnrS, is also necessary for the effector caspase activation, perhaps forming a tetrameric enzyme complex with RnrL.

Discussion

Recent advancements in microarray technology identified many potential p53 downstream target genes. However, since p53 controls multiple distinct pathways in response to genotoxic stress, understanding the mechanism of how potential p53 target genes contribute to preservation of genomic stability (or tumor suppression) has been difficult. In this study, we conducted a genome-wide microarray analysis to examine stress responses governed by Dmp53. Previous studies using *Drosophila* and *C.elegans* suggest that regulation of cell death but not cell cycle arrest is the evolutionally conserved function of p53 (Brodsky et al., 2000a; Derry et al., 2001; Ollmann et al., 2000; Schumacher et al., 2001; Sogame et al., 2003). Therefore, *Dmp53*$^{-ns}$ mutants are ideal to study the conserved stress induced programmed cell death pathway.

The IR response in *Drosophila* was very limited in scope, comprising only 0.25% of the genes responding in three trials we tested using wild type embryos. Moreover, Dmp53 was required for induction of a vast majority of the genes (30 genes out 35). Interestingly, in the absence of Dmp53, we observed approximately the same number of genes responding to IR (2.9% in Dmp53^{-ns} set 1, 2.0% in Dmp53^{-ns} set 2). However, these responses were rather stochastic and unpredictable compared to the responses seen in wild type embryos, and we did not uncover a shared core set of radiation responders in the absence of Dmp53 in two independent trials. Hence, the role of Dmp53 is likely to orchestrate a focused radiation response. As expected from previous studies, the RIPD genes include those implicated in apoptosis and DNA repair as well as genes with unknown functions. We did not observe *dacapo*, the *Drosophila p21* homologue, as one of the radiation responders, confirming previous data that Dmp53 does not engage the damage induced cell cycle arrest. Our results are corroborated by Brodsky *et al*. (Brodsky et al., 2004) who also observed a similar

radiation response using another *Dmp53* mutant allele. The shared radiation responsive Dmp53 targets include members of IAP antagonist *rpr, skl*, and *hid,* the TNF family member *eiger* (Igaki et al., 2002; Kauppila et al., 2003), and a DNA repair gene *mre11*. Using microarray analysis, Brodsky and I both failed to detect upregulation of *dark*, the *Drosophila* Apaf-1 homologue, that has been reported to be IR and UV responsive (Rodriguez et al., 2002; Zhou et al., 1999; Zhou and Steller, 2003), perhaps due to a fairly low abundance level of the *dark* transcript. These data suggest that the shared radiation responsive genes by Brodsky's and our studies likely represent a core set of genes governed by p53, and in fact a mammalian IAP antagonist HTRA2 (Jin et al., 2003) and the TNF receptor *fas* (Munsch et al., 2000) have been identified as p53 targets. It is worth noting that we did not observe *ku80* as a p53 downstream effector, contrary to Brodsky's report. The differences in two studies could be explained by the use of different *Dmp53* mutant alleles and/or technical issues related to microarray procedures.

In the absence of Dmp53, IR induced cell death is completely blocked in embryos and wing discs (Sogame et al., 2003). However, animals with both copies of *rpr* deleted show only a partial reduction in the amount of cell death (Peterson et al., 2002), suggesting additional apoptotic effectors exist. Brodsky *et al*. observed a full induction of IR induced apoptosis in animals heterozygous for the *rpr* and *skl* chromosomal deficiency, while on the other hand, animals that lost one copy of *hid* showed a greater decrease in IR induced apoptosis, suggesting the dose-sensitive effects of *hid*. Since the transheterozygote deletion used in Peterson's study takes out two copies of *rpr* as well as one copy of *hid* and *grim,* Brodsky suggests that the effect of *rpr* deletion seen in Peterson's study might be partly due to the haploinsufficiency effect of *hid*. Therefore, to what extent each proapoptotic gene contributes to induction of apoptosis remains unclear. Using the RNAi technique, we sought to identify additional Dmp53 dependent apoptotic genes from our list of RIPD genes. Here I describe identification of *RnrL* as a Dmp53 dependent apoptotic effector. RnrL is part of a

ribonucleotide reductase complex that catalyzes a conversion of rNDP to dNDP that is ultimately converted to dNTP used for DNA replication and DNA repair. In mammals, the catalytically active RNR is composed of two large subunits known as R1 and two small subunits known as R2 for DNA replication. During DNA repair, however, R1 forms a catalytically active RNR complex with a different small subunit called p53R2 (Guittet et al., 2001). *p53R2* is induced in response to DNA damage in a p53 dependent manner (Nakano et al., 2000; Tanaka et al., 2000) and the RNAi mediated knockdown of *p53R2* impaired its DNA repair ability and sensitized cells to DNA damaging agents. As a result, *p53R2* silenced cells showed a higher incidence of apoptosis in response to DNA damage. This observation is contrary to the result obtained from my study using *Drosophila*, that is, silencing *RnrL* reduces DEVDase activation. Silencing *RnrS*, the small subunit of RNR complex, also reduces DEVDase activation, indicating the RNR complex is important for effector caspase activation. The result also suggests that *Drosophila* uses only one type of the RNR complex, composed of RnrL and RnrS, for DNA replication and repair to supply dNTP source, consistent with the observation that the *Drosophila* genome only encodes a single form of the small and large RNR subunits.

How the RNR complex is involved in caspase activation in *Drosophila* is currently unknown. However, it is unlikely that the DEVDase reduction observed here in *RnrL* and *RnrS* silenced cells is merely a secondary consequence of the DNA repair defect, since silencing *mre11,* a DNA repair gene involved in nonhomologous end joining, does not affect DEVDase activation. Alternatively, it is possible that silencing RnrL and RnrS may be affecting the ecdysone sensitization process rather than caspase activation. One of the criteria used in this study to ensure that the cells were sensitized to ecdysone was a morphological change associated with ecdyone treatment. Kc167 cells formed an extension upon ecdysone treatment. Although the effect of *RnrL* and *RnrS* silencing on the ecdysone sensitization process cannot be ruled out, it is worth mentioning that silencing of these genes

did not affect this morphological change. One possibility on how the RNR complex is involved in caspase activation in *Drosophila* is that the RNR complex may provide dATP needed for formation of an active apoptosome complex. Previous *in vitro* studies demonstrated the requirement of dATP or ATP for formation of an active apoptosome, however, we do not know whether it is ribo- or deoxyribo-ATP that is physiologically relevant for the formation of apoptosome. It is worth mentioning that dATP is more efficient in catalyzing the procaspase cleavage than ATP by 10-fold in the *in vitro* study (Li et al., 1997). In *Drosophila,* silencing of *RnrL* and *RnrS* perhaps shuts down the synthesis of dATP completely, hence the observed reduction of DEVDase activity. In mammals, when *p53R2* is silenced, R2 may be able to substitute the lack of p53R2 to supply just enough dATP for caspase activation. However, this R1/R2 complex may be insufficient to provide enough nucleotide pools for DNA repair, and therefore, leads to the increased cell death in p53R2$^{-/-}$ cells. Consistent with this idea, a recent study by Lin *et al.* showed that when p53(-/-) HCT-116 cells were treated with Triapine, a RNR inhibitor, there was a significant decrease in the levels of dATP, a moderate decrease in the level of dGTP, but no changes in the level of dCTP and dTTP (Lin et al., 2004). He also provided evidence that R2-RNR could supply dNTP necessary for the repair of damaged DNA in this cell line in which p53 dependent induction of *p53R2* is impaired.

Figure 5-4 shows a working model for the damage-induced apoptosis in *Drosophila*. The similar cell death defect seen in *Dmchk2* and *Dmp53* mutants likely indicates a sequential pathway governed by these genes. The activation of these two genes leads to induction of three IAP inhibitors, *rpr, skl* and *hid*, and a subunit of RNR complex, *RnrL*. The RNR complex may provide dATP necessary for the formation of apoptosome to process procaspases. Interestingly, cells treated with *RnrL* and *RnrS* RNAi failed to activate DEVDase, however, cells still died. On the other hand, cells treated with *dronc* RNAi failed to activate DEVDase and failed to die. This suggests caspase activation and cell viability can

be uncoupled, and Dronc may participate in cell death in caspase-dependent and independent manners. The involvement of Dronc in caspase independent cell death may include activation of necrotic death, or perhaps Dronc may be involved in the release of nucleases that cause DNA fragmentation independent of caspases as seen in mammalian Endonuclease G (Li et al., 2001). On the other hand, RnrL and RnrS might only participate in caspase dependent cell death.

Studying IR response using a cell culture model has several advantages compared to the classic genetic approach, because silencing a gene using RNAi is simple and quick, and also multiple genes can be silenced at the same time. In addition, advancements in Genome-scale RNAi technology allow one to dissect the genome-wide IR response at one time. However, until now, *Drosophila* cell lines (Mel2, S2, KC, mbn, and ML-DmBG1 lines) tested in our lab were known to be highly resistant to ionizing radiation. I discovered that pre-treatment of Kc167 cells with ecdysone highly sensitizes cells to IR. I employed the Kc167 ecdysone treated cells to dissect the radiation induced cell death pathway. Although our data indicates that the IR induced caspase activation observed in Kc167 cells requires DmChk2, Dmp53, and Dronc, silencing *rpr* did not affect caspase activation. Consistent with this observation, we did not detect upregulation of *rpr* transcripts in this cell culture model using microarray analysis. We considered the possibility that *skl* may compensate for the lack of *rpr* in these cells, however silencing *skl* alone or *skl*/*rpr* together did not reduce caspase activation. Since we were unable to detect the *skl* transcript (perhaps due to its low abundance in these cells) even in the control, we cannot rule out this possibility. The inherent differences in the animal versus the cell culture may explain our low success rate of identifying additional RIPD genes that are involved in the cell death pathway. For example, animals are made of many different types of tissues and it is not surprising if each tissue may respond to DNA damage differently. In the case of cell culture, the tissue diversities have been lost, and therefore, we may only be focusing on a subset of DNA damage responses. I

also note that studies undertaken here using RNAi only uncover the genes that are rate-limiting for induction of apoptosis, since RNAi, in many cases, leads to a hypomorphic effect rather than a loss of function effect. The newly available ~29,000 transposon insertion strains from Exelexis together with existing mutant strains will permit us to further study the involvement of the RIPD genes in cell death. Ultimately comparing p53 targets in human, mice, flies, and worms may allow us to arrive at a core set of a conserved generic cell death program.

Table 5-1. List of RIPD genes.

Gene and/or CG #	WT fold change range	Putative functions
cul-2 (CG1512)	1.6-2.6	ubiquitin ligase complex
CG1718	1.6-2.2	ABC transporter complex
mus205 (CG1925)	2.2-3.8	DNA polymerase Zeta
bcn92 (CG3717)*	2.0-3.2	
rpr (CG4319)	4.8-6.0	Apoptosis Activator
w (hid) (CG5123)	2.8-3.6	Apoptosis Activator
esc1 (CG5202)	2.2-3.0	Transcription repression and integrin interaction
RnrL (CG5371)	1.6-2.2	Ribonuleoside diphosphate activity
CG5664	2.0	DNA binding protein
Pka-C3 (CG6117)	3.8-4.6	Serine/threonine kinase
CG6171	1.8-2.6	
CG6272	3.0-4.8	bZIP transcription factor family
halo (CG7428)	1.6-2.2	
mus210 (CG8153)	1.6-2.0	Nucleotide excision repair
CG9386	2.2-4.4	Aminohydrolase, nitrogen metabolism
Cyp307a1 (CG10594)	1.6-4.4	Cytochrome P450 activity
corp (CG10965)	3.8-4.6	
CG11897	2.2-4.0	
CG12171	2.2-8.6	Oxidoreductase lipid metabolism
CG12194	3.4-5.4	General substrate transporter
GstD5 (CG12242)	2.2-3.0	
eiger (CG12919)	2.4-4.4	
CG13204	1.6-4.6	
skl (CG13701)	3.4-10.6	Apoptosis activator
CG15479	1.8-5.4	
mre11 (CG16928)	2.0-5.6	
eIF6 (CG17611)	1.6-2.0	Transcription initiation factor
CG17836	7.4-11.2	bZIP transcription factor
CG18596	2.0-4.0	SpoU methylase
pyd (CG31349)	1.6-2.2	Guanylate kinase activity

A total of 30 genes are identified as RIPD genes by comparing three independent WT embryo and two independent *Dmp53*$^{-ns}$ embryo microarray experiments. *bcn92 was responsive to IR in two out of three WT embryo microarray experiments.

Table 5-2. RNAi against RIPD genes that are not rate-limiting for radiation induced DEVDase activity.

RNAi	DEVDase Activity (A.F.U*)± S.E**
gfp	1
CG17836	1.178 ± 0.276
corp (CG10965)	2.28 ± N.D***
eIF6 (CG17611)	0.814± 0.158
esc1 (CG5202)	0.72 ± N.D
ku80 (CG18801)#1	0.74 ± N.D
skl (CG13701)	0.83 ± N.D
skl&rpr	0.83 ± N.D
rpr (CG4319)	1.216 ± 0.18
bcn92 (CG13717)#2	1.273 ± 0.171

The RIPD genes were tested for its involvement in caspase activation using Kc167 Ecdysone & IR cell culture model. Data shown are representative of multiple trials ranging from a minimum of two trials to a maximum of six trials for each gene tested. Each data point was normalized as described in Figure 5-3.
*A.F.U., Arbitrary Fluorescence Unit.
**S.E., Standard Errors.
***N.D., Not Determined.
#1, Not a RIPD gene in our study. However, it is a RIPD gene in Brodsky's study (Brodsky et al., 2004).
#2. Not a RIPD gene in our study. It is IR responsive in two out of three wild type embryo trials. It is located ~21kb away from a computed p53 binding site.

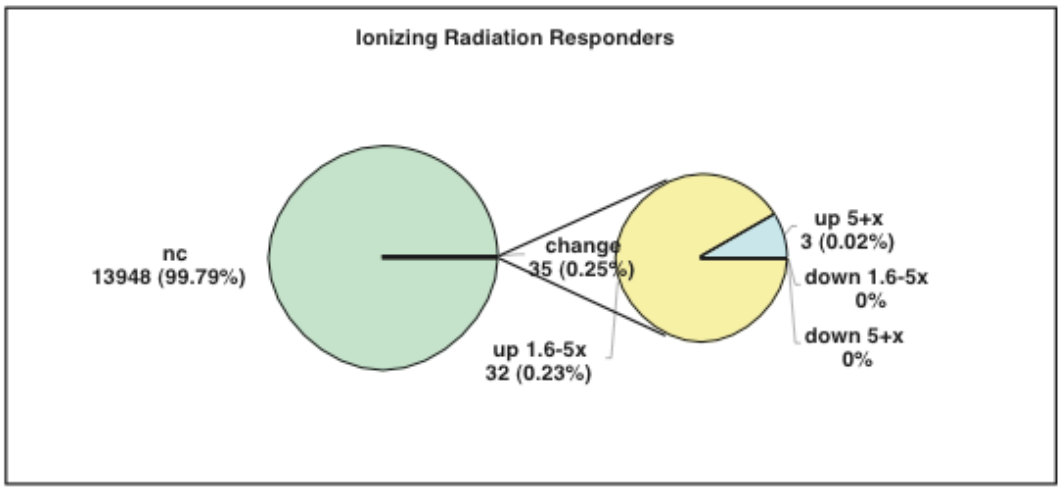


Figure provided by Anna Christich

Figure 5-1. IR response in Drosophila is a narrow focused response. A pair-wise comparison of control and irradiated wild type embryos with transcript fold changes of 1.6 or greater is shown. The result is a compilation of three independent trials using one *yw* and two w^{1118} samples.

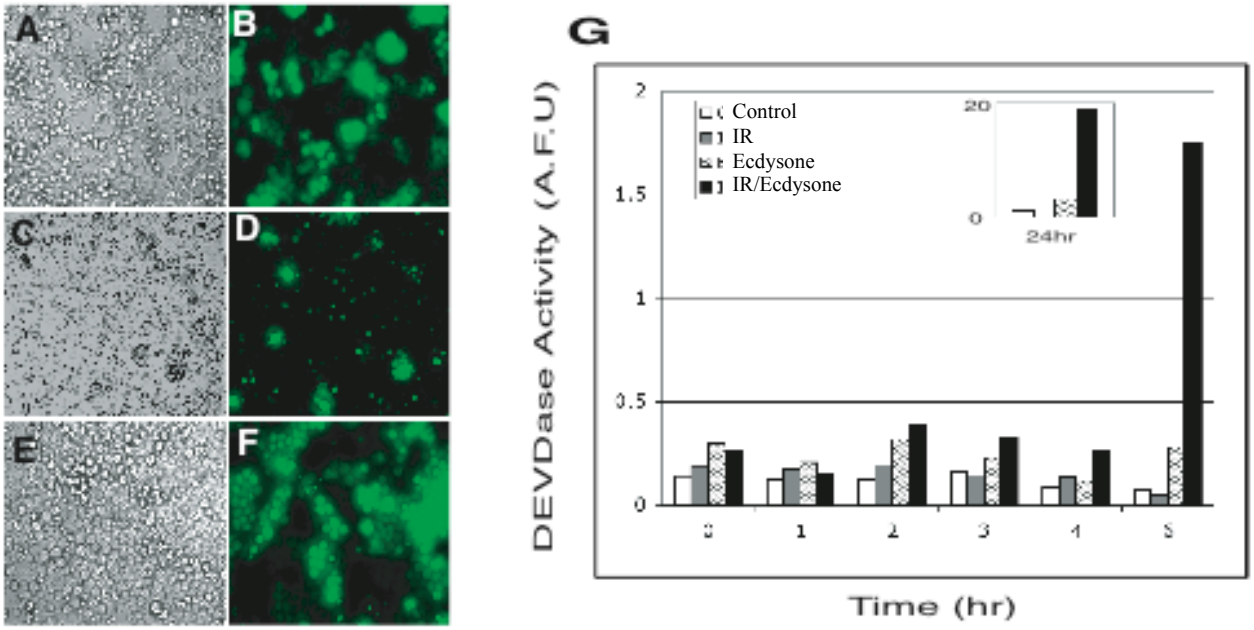


Figure 5-2. Establishment of IR responsive tissue culture model. Kc167 cells are unresponsive to γ-irradiation even at high doses (28K rads). Pre-treatment of Kc167 cells with an insect metamorphic hormone "Ecdysone" sensitizes cells to γ-irradiation dramatically as shown in increased effector caspase activity. (A-F) Kc167 cells were transfected with an Armadillo-GFP expressing plasmid and cells were either mock-treated (A&B), IR & Ecdysone treated (C&D) or IR treated (E&F) (Figures kindly provided by Saila Kauppila). Images of cells are shown in a bright filed (A, C, and E) and a fluorescent field (B, D and F). (G) DEVDase activation is significantly higher in the IR & Ecdysone treated sample. A.F.U., Arbitrary Fluorescence Unit.

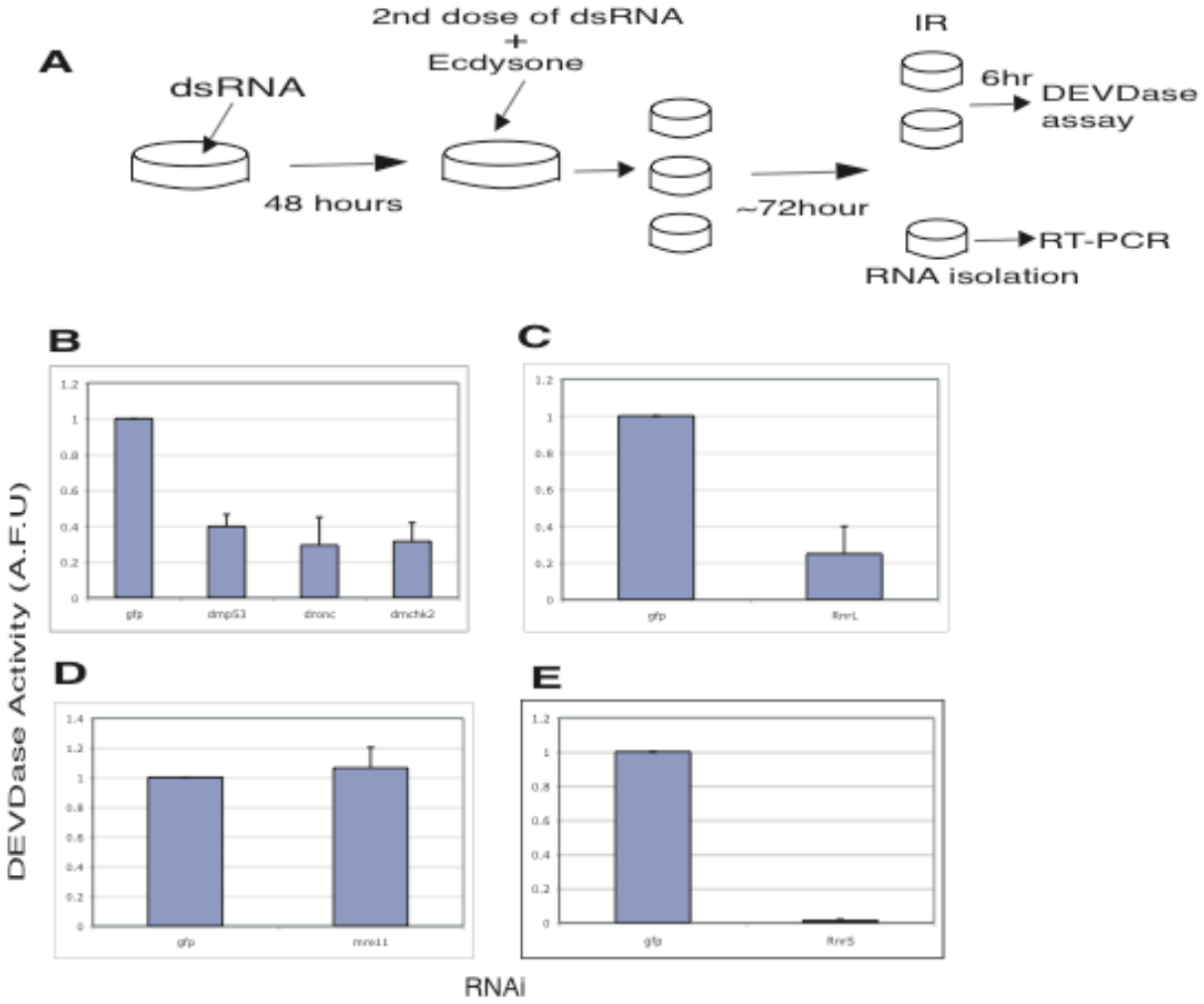

A
dsRNA
48 hours
2nd dose of dsRNA
+
Ecdysone
~72hour
IR
6hr
DEVDase
assay
RT-PCR
RNA isolation
B
C
D
E
DEVDase Activity (A.F.U)
gfp
dmp53
dronc
dmchk2
RnrL
mre11
Rnr5
RNAi
F
p53
dronc
chk2
rnrl
rnrs
mre11
rp49
dsRNA
–
+

Figure 5-3. RnrL and RnrS are required for effector caspase activity. (A) Schematic representation of RNAi treatment and Ecdysone & IR treatment using the Kc167 cell culture system. (B-F) Measurement of DEVDase activity in ecdysone/γ-irradiation treated Kc167 cells that are silenced for the genes indicated. Data shown are representative of multiple trials, ranging from a minimum of three trials (Dmchk2 & Mre11) to a maximum of five trials (Dmp53) for each gene tested. The *gfp* dsRNA treated cells were used as a control and the DEVDase activity values for each gene tested were averaged and normalized to that of the *gfp* dsRNA treated cells. A.F.U. Arbitrary Fluorescence Units. (B) RNAi mediated knockdown of known death genes established that Dmp53, Dronc, and DmChk2 are required for activation of effector caspases in response to γ-irradiation in ecdysone-sensitized Kc167 cells. (C) & (D) RNAi against candidate RIPD genes. RnrL is necessary for effector caspase activity (C), but Mre11 is not necessary (D). (E) RANi against *RnrS*, a heteromeric partner of RnrL to form an active ribonucleotide complex, revealed that RnrS is also required for activation of effector caspases. (F) The efficacy of silencing by RNAi was checked by RT-PCR. rp49 was used as a control in the RT-PCR reactions.

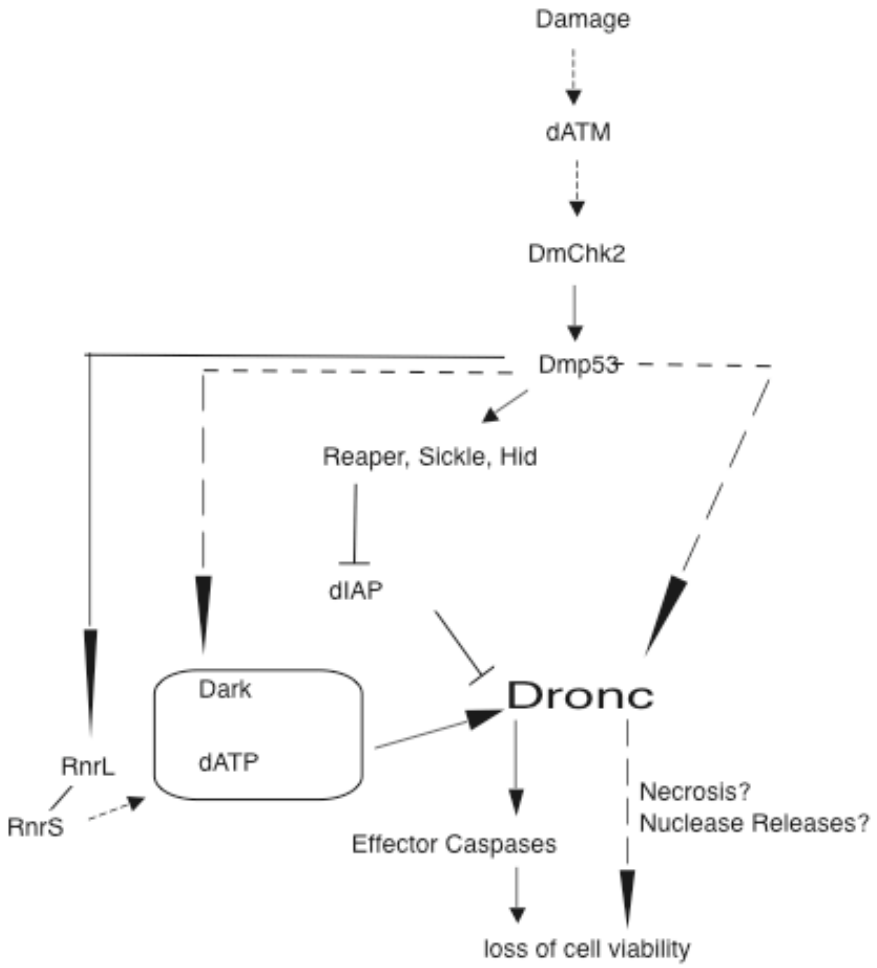


Figure 5-4. A working model for Dmp53 dependent cell death pathway in response to IR. In response to IR, Dmp53 is activated by DmChk2. Dmp53 activates IAP inhibitors Reaper, Sickle and Hid, and a component of RNR, RnrL, directly or indirectly. IAP inhibitors release dIAP inhibition on Dronc. The RnrL & RnrS complex may provide dATP source necessary for activation of apoptosome. Current lines of evidence suggest that cytochrome c proteins (DC4 &DC3) are not required for caspase activation in *Drosophila* (Dorstyn et al., 2002). The IAP inhibition pathway as well as the apoptosome pathway lead to the activation of an initiator caspase Dronc. My data suggests that Dronc may activate the cell death pathway in caspase dependent and caspase independent manners.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Four decades after the discovery of X-rays, H. J. Muller established *Drosophila* as a preeminent model for an emerging field of *radiation biology*, receiving a Nobel Prize for showing a cause and effect relationship between radiation and heritable mutations (Muller, 1927). As a metazoan, *Drosophila* offers fundamental insights regarding the ways in which developmental programs exert constraints on adaptive responses to radiation damage. One of the central players in DNA damage response is p53, a gene conserved from worm to human. p53 earned its status as a "guardian of the genome" due to its multifunctional roles in eliminating damaged DNA. p53 prevents propagation of damaged DNA by specifying cell cycle arrest possibly followed by a DNA repair program and/or apoptosis. How p53 can specify such a distinct response is not clear.

To study how *Drosophila* p53 functions in response to genotoxic stress with aims to gain a better understanding of pathways that are shared, and equally importantly an understanding of pathways that are not shared by common descent, I generated a $dmp53^{-ns}$ mutant using the newly developed homologous recombination mediated ends-in targeting strategy (Rong and Golic, 2000; Rong and Golic, 2001). To our knowledge, this is one of the first published examples that uses this methodology to generate a mutant in *Drosophila* outside of Kent Golic's Lab. My work presented here demonstrates that Dmp53 engages damage-induced apoptosis but plays no part in damage-induced cell cycle arrest. Our microarray analysis of $Dmp53^{-ns}$ mutants further supports the role of Dmp53 in apoptosis, and in addition, indicates a role of Dmp53 in DNA repair. This evidence contradicts the

commonly held notion of p53 executing a sequential linear damage response pathway, that is, first p53 engages cell cycle arrest to repair any damage, and if the damage is too extensive then p53 subsequently activates the apoptosis pathway. At least in *Drosophila*, Dmp53 can specify the DNA repair and apoptotic fate without first engaging cell cycle arrest. An evolutionary corollary related to these observations is that, among the damage responses governed by p53, cell cycle arrest could be a more recently acquired pathway. In mammalian systems, p53 is essential for keeping genomic stability (Robles and Harris, 2001; Wahl et al., 1997). However, mapping the precise function of p53 important for maintenance of genomic stability is difficult, given the multiple functions of p53 in mammals. Because *Dmp53*$^{-ns}$ exhibits a mutator phenotype without a defective cell cycle arrest, I conclude that a failure in apoptosis and/or DNA repair alone is sufficient to contribute to genomic instability.

How p53 engages the apoptotic pathway remains ill defined compared to the well established p53 mediated cell cycle arrest pathway. The complexity arises from the context-specific regulation of apoptotic genes by p53. The work of myself and others indicates Dmp53 induces cell death partly by removing IAP inhibition from caspases (Brodsky et al., 2000a; Sogame et al., 2003). Several lines of evidence suggest that the removal of IAP from caspases is accomplished by Reaper, Sickle and perhaps Hid. First, they are acutely responsive to IR. Second, they fail to respond to IR in the absence of Dmp53. Third, they can bind to IAP (Christich et al., 2002; Vucic et al., 1998). Brodsky and Peterson *et al.* reported that reducing the level of each gene alone is not sufficient to block apoptosis completely, contrary to the complete absence of apoptosis in *Dmp53*$^{-ns}$ mutants (Brodsky et al., 2004; Peterson et al., 2002; Sogame et al., 2003), suggesting the expression of multiple IAP antagonists is necessary for induction of full apoptosis. There is a p53 binding site located at ~6kb upstream of *rpr* and ~30kb downstream of *skl* (Brodsky et al., 2000a). We currently do not know whether this binding site is responsible for transcribing both *rpr* and *skl* (and perhaps *hid*) or alternatively there is another p53 binding site for the regulation of

skl. Recent evidence suggests the existence of chromosomal domains that harbor a cluster of similarly regulated genes (Boutanaev et al., 2002; Spellman and Rubin, 2002), presumably to allow the most efficient physiological responses in a given time period. A closer examination of the ~300 kb radiation responsive region harboring *rpr, skl* and *hid* may allow us to gain an understanding of gene territories and how one transcription factor affects the regulation of multiple genes in a given territory.

In my dissertation project, Anna Christich and I utilized microarray technologies to study the genome-wide radiation response in *Drosophila* and the effect of loss of Dmp53 in radiation response. Our study demonstrates that Dmp53 consistently regulates an expression of 30 genes in response to IR out of 35 IR responsive genes in the Drosophila genome. It is not known how many RIPD genes represent a direct target of Dmp53, but in the near future, Chromatin immuno-precipitation (ChIP) analysis with an antibody which recognizes endogenous Dmp53, will allow us to address this issue. Sequence analysis of these genes indicates many of them function in DNA repair and apoptosis, and others are unknown. To test their functional significance in induction of apoptosis, I developed a novel *Drosophila* cell culture system that triggers massive apoptosis in response to IR. This system takes advantage of my discovery that, although existing *Drosophila* cell lines are highly resistant to IR, pre-treatment of Kc167 cells with an insect metamorphic hormone "Ecdysone" sensitizes cells to IR dramatically. My functional studies of RIPD genes uncovered one RIPD gene, *RnrL*, as a p53 apoptotic effector. Although the apoptotic response seen in irradiated Kc167 cells is dependent on some of known death regulators such as Dmp53, Dmchk2, and Dronc, the radiation response in this cell culture model clearly differs from that of an animal model (discussed in Chapter 5). For example, induction of *rpr* does not occur in this model. Silencing *rpr* and *skl* does not affect caspase activation in Kc167 cells but a loss of *rpr* in wing discs reduces the incidence of apoptosis (Peterson et al., 2002). Therefore, it is possible that examining a defective apoptosis in mutant animals for RIPD genes may uncover

additional p53 apoptotic effectors that were not obtained in this study.

Mammalian p53, at least in part, induces apoptosis by transcribing pro-apoptotic Bcl-2 family members such as *bax*, *noxa*, and *puma*. So far, one pro-apoptotic Bcl-2 member, *debcl*, has been identified in the *Drosophila* genome (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000). In the current study undertaken here, I was unable to detect any evidence of the *debcl* upregulation in response to IR. Does Dmp53 not use pro-apoptotic Bcl-2 to activate the apoptotic pathway? This is an unlikely scenario given the newest development from *C. elegans* study. Schumacher *et al.* reported that a BH3 only gene, *ced-13*, is induced in response to IR and this is a CEP-1/p53 dependent process (Schumacher et al., 2005). The annotated *Drosophila* genome currently does not reveal any obvious BH3 only Bcl2 members. This is because the consensus sequence used to search a BH3 only gene is a very short sequence, hence, identification of the true BH3 only gene requires additional functional studies. The *C.elegans* study proves that the regulation of pro-apoptotic Bcl2 genes by p53 is a conserved process. A closer examination of RIPD genes may reveal unidentified *Drosophila* BH3 only genes.

Drosophila holds its position strongly as a genetically tractable organism and allows us a rapid characterization of genes. The newly developed ~29,000 transposon insertion strains from Exelixis (Parks et al., 2004) with existing p-insertion lines will permit us to further study RIPD genes *in vivo* and ultimately uncover how these genes are integrated to specify the apoptotic response under control by p53. Continued elucidation of shared and distinct features of radiation–induced damage responses in *Drosophila* model should uncover important insights that help us understand human disease.

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VITAE

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