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**DNA DAMAGE-INDUCED APOPTOSIS IN THE PRESENCE AND ABSENCE
OF THE TUMOR SUPPRESSOR p53**

A Dissertation Presented

By

Laura Michelle McNamee

**Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of**

DOCTOR OF PHILOSOPHY

October 22, 2008

Interdisciplinary Graduate Program

DNA DAMAGE-INDUCED APOPTOSIS IN THE PRESENCE AND ABSENCE OF
THE TUMOR SUPPRESSOR p53

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Interdisciplinary Graduate Program

October 22, 2008

Acknowledgements

My deepest appreciation and thanks to my graduate advisor, Mike for investing so much time in my education and giving me some great opportunities. I would also like to acknowledge the other members of the Brodsky lab, Sarah and Adam, and thank them for making work an enjoyable place to be.

I thank my committee members, Tim, Eric, Neal, and Tony for their helpful discussions and suggestions, as well as their support along the way. I especially thank Kristin for taking time out of her busy schedule to be my outside member today.

Many thanks to my friends both in and out of graduate school. My graduate school friends, Mary, Kristen, Kelly, and Vivian always made the work a little more enjoyable and were encouraging through the “tougher” times. I especially thank Mary, who was a mentor to me when I started graduate school and since has become a great friend. I would also like to thank my non-graduate school friends, especially Leah, Noelle, Crafts, Theresa, and Ryan for always listening and occasionally pulling me away from science.

None of this would have been possible without the support of my family. I thank my parents, Michael and Mary Ann Lemoine, for always encouraging me to follow my dreams and providing the means for me to do so. I also thank my Gram and my siblings, Lucas, Lisa, Doug, Lee, and Lindsay. I am also very thankful for my great in-laws, John and Marie McNamee, who have always taken an interest in my career.

Finally, I would like to acknowledge my husband, James McNamee. I met Jim just before I started my third year of graduate school, it may be a coincidence, but

graduate school seemed to get much easier after finding him. He is a constant source of strength and always cheers me on. There are no words to describe how grateful I am to have him in my life.

Abstract

A key regulator of DNA damage-induced apoptosis is the tumor suppressor gene, p53. p53 is a transcription factor that upregulates genes involved in cell cycle arrest, apoptosis, and senescence. How p53 decides to activate one of these responses in response to DNA damage is largely unanswered. Many have hypothesized it is due to interaction with various signaling pathways and post-translational modification. The p53 tumor suppressor can be modified by SUMO-1 in mammalian cells, but the functional consequences of this modification are unclear. Conjugation to SUMO is a reversible post-translational modification that regulates several transcription factors involved in cell proliferation, differentiation, and disease. In Chapter II, we demonstrate that the *Drosophila* homolog of human p53 can be efficiently sumoylated in insect cells. We identify two lysine residues involved in SUMO attachment, one at the C-terminus, between the DNA binding and oligomerization domains, and one at the N-terminus of the protein. We find that sumoylation helps recruit *Drosophila* p53 to nuclear dot-like structures that can be marked by human PML and the *Drosophila* homologue of Daxx. We demonstrate that mutation of both sumoylation sites dramatically reduces the transcriptional activity of p53 and its ability to induce apoptosis in transgenic flies, providing *in vivo* evidence that sumoylation is critical for *Drosophila* p53 function.

Many therapeutic cancer treatments rely on DNA-damaging agents to induce apoptosis in cancer cells. However, fifty percent of all human tumors lack functional p53 and p53 mutant cells are partially resistant to damage-induced apoptosis. Therefore, it is

important to identify mechanisms to induce apoptosis independent of p53. *Drosophila* provides a good model system to study p53-independent apoptosis because it contains a single p53 homolog. In Chapter III, we describe a p53-independent mechanism that acts in parallel to the canonical DNA damage response pathway in *Drosophila* to activate apoptosis in response to inappropriately repaired chromosome breaks. Induction of chromosome aberrations by DNA damage followed by cell division results in segmental aneuploidy and reduced copy number of ribosomal protein genes. We find that activation of the pro-apoptotic gene *hid* by the JNK pathway acts in a p53-independent mechanism to induce apoptosis and limit the formation of aneuploid cells. Mutations in *grp*, the *Drosophila* Chk1 homolog, and *puc*, a negative regulator of the JNK pathway sensitize p53 mutant cells to IR-induced apoptosis. We propose a model in which the death of cells with reduced copy number of genes required for cell survival helps maintain genomic integrity following chromosome damage.

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CHAPTER I
INTRODUCTION

Preserving the integrity of the genome is essential for maintaining cellular fitness. Loss of critical genes results in cells that are less fit, and therefore tissues with overall decreased fitness. Upon induction of DNA damage, cells use repair mechanisms to protect integrity of the genome and to return the chromosome back to its original form. However, one consequence of this repair is that is not always accurate and can lead to mutations at the site of the break or cause chromosome rearrangements. It is an accumulation of these mutations that can eventually lead to cancer.

Cells have evolved several mechanisms to efficiently deal with DNA damage. Unicellular organisms rely on cell cycle checkpoints and repair mechanisms to resolve the damage. Multi-cellular organisms also rely on these mechanisms, and can additionally eliminate the damaged cell from the tissue by activating apoptosis. However, how a cell is selected to undergo apoptosis following DNA damage is largely unanswered. Additionally, the functional consequence of damage-induced apoptosis *in vivo* is not well understood.

A key step in the induction of apoptosis is the activation of the tumor suppressor gene, p53. It is well established that p53 is a transcription factor that is required for the immediate and efficient response to DNA damage by upregulating genes involved in cell cycle arrest, apoptosis, and senescence. How p53 selects the transcriptional program to induce apoptosis is not well understood. Likely, it is due to interactions with various signaling pathways and post-translational modifications triggered by various stimuli. However, under what conditions these interactions and modifications occur and exactly how they impact p53 function *in vivo* is still unclear.

DNA-damage induced apoptosis is of also clinical importance because many DNA damaging agents are used to kill cancer cells. However, approximately 50% of all human tumors are mutant for functional p53 and are partially resistant to damage-induced apoptosis. Therefore, it is not only important to understand how p53 is regulated after DNA damage, but also mechanisms of damage-induced apoptosis that are independent of p53. In mammals, two other p53 homologs, p63 and p73, induce p53-independent apoptosis after DNA damage. However, many layers of complexity exist within the p53 family. For example, certain mutant forms of p53 can inhibit p63 and p73. Identifying damage-induced apoptotic pathways that are independent of p53, p63, and p73 will be important for treating tumors lacking functional p53.

Cell signaling in response to DNA damage leading to apoptosis has primarily been studied in cell culture. How cells within a tissue are selected to undergo apoptosis after DNA damage is unclear. Because the apoptotic response to DNA damage is conserved from insects to mammals, *Drosophila melanogaster* provide a genetically tractable model system to study the regulation of damage-induced apoptosis in the context of a tissue. In *Drosophila*, there is only a single p53 homolog, which is also called p53 and has a conserved function for damage-induced apoptosis. Induction of DNA damage by IR or unprotected telomeres rapidly induces apoptosis in the *Drosophila* developing wing. Interestingly, *Drosophila* lacking the p53 tumor suppressor gene delay apoptosis following induction of DNA damage. These results suggest that there are two apoptotic responses activated by DNA damage: (1) an early and immediate response that is p53-dependent and (2) a later response that is p53-independent. Thus, *Drosophila*

provides a useful model system to study the regulation of p53-dependent apoptosis, as well as to identify mechanisms that induce apoptosis independent of p53.

DNA damage-induced apoptosis in mammals

This section provides an overview of DNA damage-induced apoptosis in mammals. It is divided into 3 subsections: DNA damage signaling and cell cycle arrest, the tumor suppressor gene p53, and the canonical apoptosis pathway.

DNA damage signaling and cell cycle arrest. Upon induction of double strand breaks (DSBs) by ionizing radiation (IR), there are proteins that sense the damage and activate downstream signaling pathways to elicit the appropriate cellular response. These responses include DNA repair, cell cycle arrest, and apoptosis. Members of the MRN complex, Mre11, Rad50, and Nbs1, are the sensors of a DSB, which recruit and activate the phosphoinositide 3-(PI-3) kinase, ATM. In turn, ATM rapidly recruits another PI-3 kinase, ATR and its binding partner ATR-interacting protein (ATRIP)(Lee and Paull 2004; Lee and Paull 2005; Myers and Cortez 2006). ATM and ATR phosphorylate many downstream substrates that mediate responses to DNA damage. Interestingly, ATM and NBS1 deficiencies result in the human disorders, Ataxia telangiectasia and Nijmegen Breakage syndrome, respectively. These diseases have many overlapping phenotypes, including increased sensitivity to IR and defective telomeres, demonstrating that both genes play a critical role in the DNA damage response (Harper and Elledge

2007).

ATM and ATR phosphorylate many downstream substrates including checkpoint kinases, Chk1 and Chk2, and the transcription factor p53 to induce cell cycle arrest (Harper and Elledge 2007). ATR primarily phosphorylates Chk1, and in turn, Chk1 phosphorylates the phosphatases, Cdc25A and Cdc25C (Sanchez et al. 1997; Liu et al. 2000). Once phosphorylated, Cdc25A and Cdc25C are inhibited from activating their respective cyclin dependent kinases and cyclin counterparts, resulting in a G1 and G2 arrest (Peng et al. 1997; Mailand et al. 2000). ATM and Chk2 primarily phosphorylate p53, which upregulates genes involved in cell cycle arrest and apoptosis (Fridman and Lowe 2003).

Mammalian p53. p53 belongs to a small family of transcription factor proteins, including p63 and p73. p63 and p73 have clear roles in normal development and immune function, while p53 has evolved to prevent tumour development by killing unstable cells (Vousden and Lu 2002). p53 is 393 amino acids with three domains, a N-terminal transactivation domain, a central DNA binding domain, and a C-terminal tetramerization domain. Two p53 homodimers bind to form a tetramer that can transactivate genes in response to a variety of cellular stresses. Although additional functions of p53 have been suggested, p53 primarily functions as a transcription factor that upregulates genes involved in cell cycle arrest, apoptosis, and senescence. The importance of p53 as a tumor suppressor and transcriptional activator is highlighted by the fact that 50% of all human cancers contain a mutation in p53 and more than 80% are located in the DNA-

binding domain (Das et al. 2008).

In mammals, p53 protein levels are regulated by the E3 ligase, Mdm-2. In unstressed cells, Mdm-2 can monoubiquitinate p53, which allows for p53 to be exported from the nucleus, or it can polyubiquitinate p53, resulting in proteasomal degradation (Li et al. 2003; Carter et al. 2007). ATM and Chk2 phosphorylate p53 at the N-terminus in response to DNA damage. Phosphorylation of p53 inhibits its ability to interact with Mdm-2, allowing for its stabilization and ability to transactivate many genes involved in cell cycle arrest and apoptosis (Bode and Dong 2004). Additionally, Mdm-2 is a transcriptional target of p53 in response to DNA damage, indicating an autoregulatory feedback mechanism between these two proteins (Fridman and Lowe 2003).

To induce cell cycle arrest, p53 upregulates p21, 14-3-3 σ , and GADD45 (growth arrest and DNA damage inducible protein). The cyclin dependent kinase inhibitor, p21 induces a G1 arrest by inhibiting cyclin dependent kinases cdk2 and cdk4. 14-3-3 σ and GADD45 induce a G2 arrest by simultaneously inhibiting cdc2/cyclinB complexes. 14-3-3 σ is a scaffold protein that sequesters cdc2/cyclinB into the cytoplasm, while GADD45 destabilizes the cdc2/cyclinB complex (Olsson et al. 2007).

To induce apoptosis, p53 upregulates genes that extrinsically and intrinsically activate the apoptotic pathway. Extrinsic apoptosis is induced by binding of a ligand to its receptor. Members of the tumor necrosis factor (TNF) family, including FAS ligand and Death Receptor 5, are induced by p53 to extrinsically induce apoptosis. Although these proteins contribute to the induction of apoptosis, mouse models lacking individual components of this pathway have a normal apoptotic response to DNA damage,

suggesting that individual components are not required for damage-induced apoptosis (Danial and Korsmeyer 2004). The pro-apoptotic Bcl-2 family members, Bax, Puma, Noxa, and Bid are upregulated by p53 to intrinsically induce apoptosis (Fridman and Lowe 2003). In some cell types, upregulation of these pro-apoptotic genes is also dependent on p63 and p73, which are thought to aid in the ability of p53 to bind NOXA and bax promoters (Figure 1.1) (Flores et al. 2002).

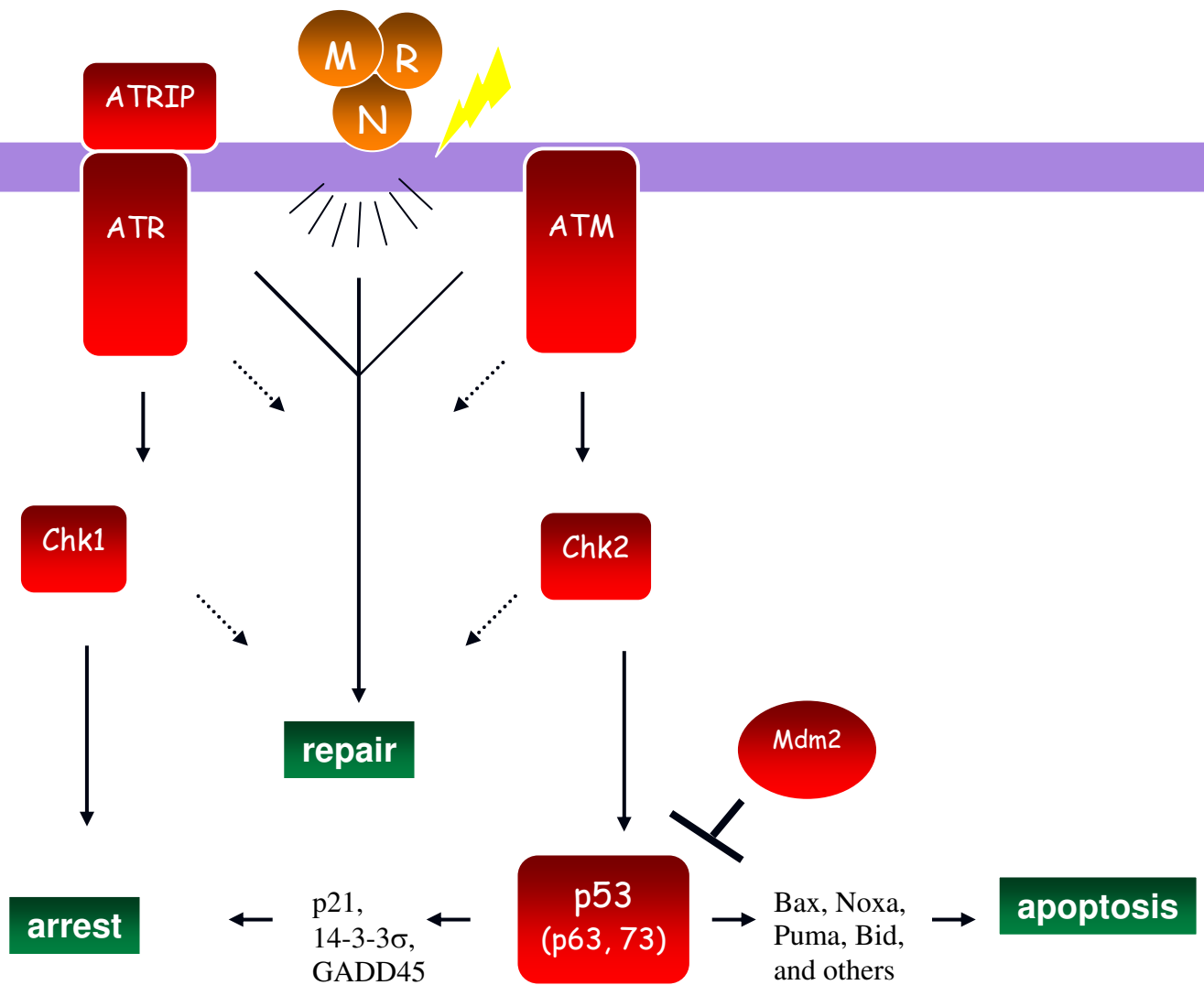


Figure 1.1. Mammalian DNA damage response pathway. Members of the MRN complex act as the sensors of DSBs. The MRN complex activates ATM and ATR, which in turn activates downstream checkpoint proteins. Chk1 induces cells cycle arrest, while Chk2 primarily phosphorylates and activates p53. Normally, p53 levels are kept low through interaction with Mdm2, however, phosphorylation of p53 inhibits this interaction and allows p53 to stabilize and upregulate genes involved in apoptosis and cell cycle arrest.

One key question is how does p53 select the transcriptional program for cell cycle arrest versus apoptosis. Post-translational modification of p53 is one factor that influences transcriptional outcome. p53 undergoes many post-translational modifications including phosphorylation, ubiquitination, sumoylation, acetylation, and methylation. For example, p53 can be acetylated at several different lysine residues. Although it is controversial how acetylation alters p53 function, acetylated p53 at lysine 120 accumulates on promoters of pro-apoptotic genes. Mutation of this residue affects pro-apoptotic activity, but does not impair cell cycle arrest. Several lysine residues that are acetylated can also be methylated. The affect of methylation on p53 largely depends of which residue is methylated. Methylation of lysine 372 enhances p53 stability and restricts it to the nucleus, whereas methylation of lysine 370 represses transcriptional activity (Olsson et al. 2007; Das et al. 2008).

Lastly, p53 can be modified by SUMO-1 (small ubiquitin like modifier-1) on lysine 386. Like ubiquitination, SUMO-1 is covalently attached to the lysine residue of a target protein. Sumoylation affects localization, activity, and stability of a protein (Muller et al. 2004). The affect of sumoylation of p53 is controversial in mammals. Some have reported that sumoylation of p53 has been shown to stimulate pro-apoptotic function (Gostissa et al. 1999; Rodriguez et al. 1999b; Muller et al. 2000), while others have shown no impact on p53 function (Kwek et al. 2001; Schmidt and Muller 2002). In Chapter II, we demonstrate that sumoylation of *Drosophila* p53 modulates its transactivation function. We show for the first time *in vivo* that the sumoylation sites of *Drosophila* p53 are required for efficient induction of apoptosis following DNA damage.

Canonical apoptosis pathway. In mammals, a cell's decision to commit to apoptosis in response to DNA damage is primarily mediated through mitochondrial outer membrane permeabilization (MOMP). MOMP is dependent on the Bcl-2 family of proteins, consisting of pro-apoptotic and anti-apoptotic members. The Bcl-2 family is divided into three groups based on the presence of up to four Bcl-2 homology domains (BH1-4). The first group consists of anti-apoptotic Bcl-2 proteins, which contain BH domains 1-4. The anti-apoptotic proteins are primarily located in the outer mitochondrial membrane, where they bind and inhibit pro-apoptotic Bcl-2 proteins. The last two groups, effectors and BH3 only proteins, are pro-apoptotic Bcl-2 proteins. Effectors include BAK and BAX, which contain BH domains 1-3, and BH3 only proteins, BID, BIM, BAD, Noxa, and PUMA. Upon induction of cellular stress, cells upregulate BH3 only proteins that bind and inhibit anti-apoptotic Bcl-2 proteins, resulting in free BAX and BAK. Once liberated, BAX and BAK homo-oligomerize and form proteolipid pores in the outer membrane of the mitochondria, resulting in MOMP. Overexpression of anti-apoptotic Bcl-2 or mutation of pro-apoptotic bax renders cells resistant to apoptosis, demonstrating the importance of the Bcl-2 family of proteins and the loss of mitochondrial integrity in the apoptotic pathway (Chipuk and Green 2008).

Once the mitochondrial membrane has been permeabilized, proteins from the intermembrane space are released. These proteins include SMAC/Diablo, Omi/HTRA, and cytochrome c. In unstressed cells, Inhibitors of Apoptosis (IAPs) bind and inhibit caspases. Once SMAC/Diablo and Omi/HtrA are released from the mitochondrial intermembrane space, they sequester IAPs. Binding of SMAC/Diablo and Omi/HTRA to

IAPs results in the autoubiquitination and degradation of IAPs, allowing for the subsequent activation of caspases (Srinivasula and Ashwell 2008).

Caspases are cysteine proteases that execute cell death through cleavage of many substrates, resulting in apoptotic phenotypes such as nuclear condensation, DNA laddering, and cellular blebbing. Caspases are synthesized as inactive zymogen precursors, containing a large and a small subunit preceded by an N-terminal prodomain. Cleavage of the zymogen precursors allows the small and large subunit to associate and form the active site. Caspases are divided into 2 groups; initiators and effectors. Although seven mammalian caspases play a role in apoptosis (four initiator and three effector), the initiator caspase-9 and effector caspase-3 are the predominant caspases involved in apoptosis after DNA damage. Initiator caspase-9 has a long pro-domain that contains a caspase recruitment domain (CARD), which is required for interaction with Apaf-1 (apoptotic protease activating factor 1). Caspase-9 autocatalytically cleaves itself into its active form. However, caspase-9 does not have optimal catalytic activity until it forms the apoptosome with Apaf-1 and cytochrome c. Once the apoptosome is formed, caspase-9 can cleave and activate effector caspase-3. The effector caspase-3 can then cleaves numerous cellular substrates to induce cell death (Figure 1.2)(Bao and Shi 2007).

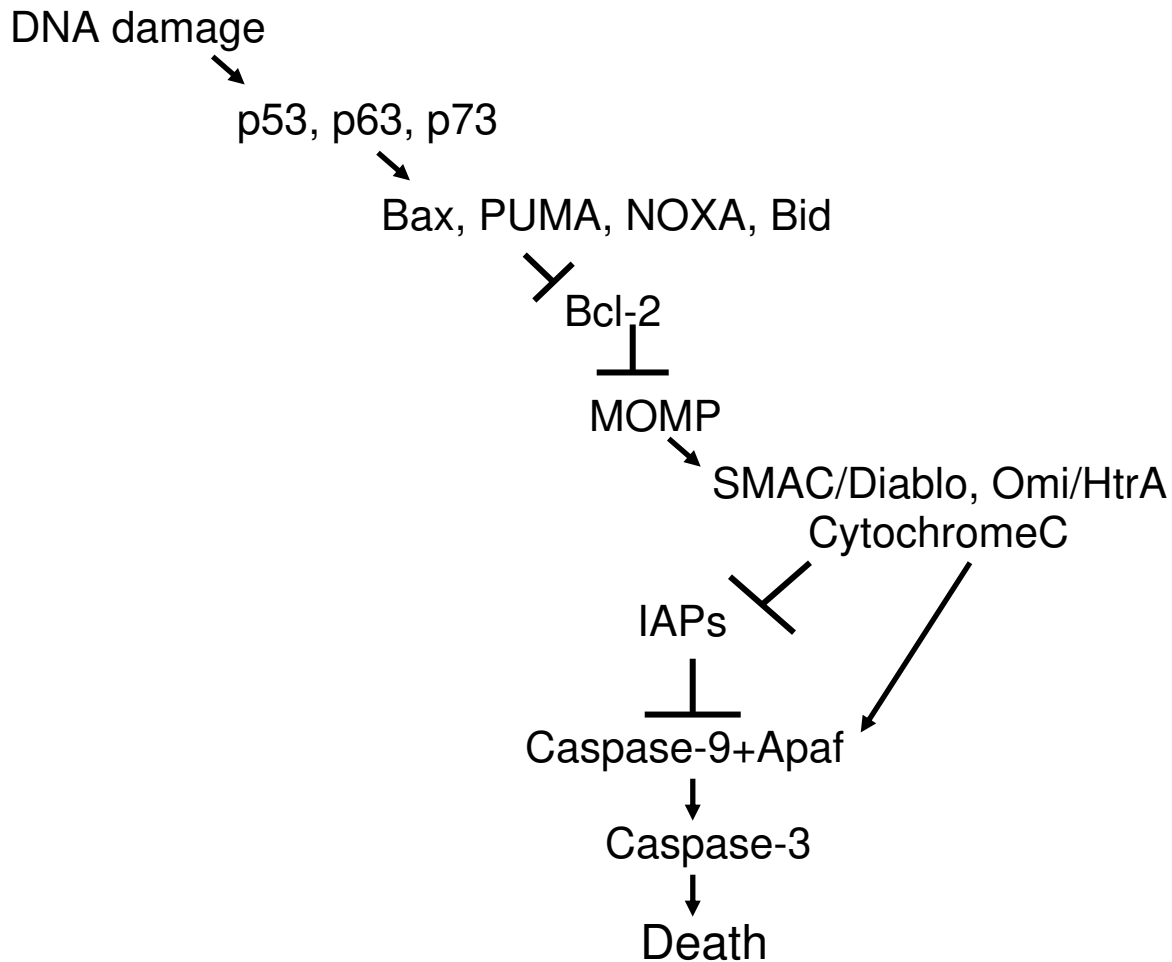


Figure 1.2. Canonical apoptosis pathway in mammals. In response to cellular stress, such as DNA damage, p53 becomes activated and upregulates the pro-apoptotic Bcl-2 genes Bax, PUMA, NOXA, and Bid. These proteins antagonize the anti-apoptotic protein Bcl-2, allowing for Bak and Bax to induce MOMP. Once the outer mitochondrial membrane is permeabilized it releases SMAC/Diablo, Omi/HtrA, and cytochrome c. SMAC/Diablo and Omi/HtrA antagonize IAPs, allowing for the activation of caspases. Cytochrome c associates with initiator caspase-9 and Apaf-1 to form the apoptosome. Formation of the apoptosome allows caspase-9 to cleave and activate the downstream effector caspase-3 to kill the cell.

DNA damage-induced apoptosis in *Drosophila*

This section provides an in-depth view of the *Drosophila* DNA damage response and apoptosis pathways. Sections include DNA damage signaling and cell cycle arrest, *Drosophila* p53, and the canonical apoptotic pathway in flies.

DNA damage signaling and cell cycle arrest. In response to DSBs induced by IR, cells in the imaginal wing disc undergo a G2 arrest for approximately 4-8hrs. *mei-41* (ATR orthologue), *grapes* (Chk1 orthologue), and *mus304* (ATRIP orthologue) are required for damage-induced cell cycle arrest, but not apoptosis (Hari et al. 1995; Fogarty et al. 1997; Brodsky et al. 2000b; Brodsky et al. 2004). Flies with mutations in *mei-41*, *grps*, and *mus304* have defects in meiotic recombination and females show reduced fertility (Baker and Carpenter 1972; Brodsky et al. 2000b). Additionally, developing embryos normally go through 13 synchronized nuclear divisions in a syncytial environment. After the 13th syncytial division a long interphase occurs to allow for cellularization and the initiation of zygotic transcription. *mei-41* and *grps* mutant embryos fail to terminate the final syncytial division, initiate zygotic transcription, and cellularize (Sibon et al. 1997; Sibon et al. 1999). *mus304* mutants also show cell cycle defects and aberrant nuclear morphology at early stages of embryogenesis (Brodsky et al. 2000b). These results demonstrate that *mei-41*, *grps*, and *mus304* function in the same genetic pathway, which is required for cell cycle arrest.

Like mammals, mutants of *Drosophila tefu* (ATM homologue) have high

frequencies of telomere fusions and spontaneous apoptosis (Bi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). Members of the MRN complex, *Drosophila mre11*, *rad50*, and *nbs1*, also show similar phenotypes, indicating that these proteins are required for telomere protection (Bi et al. 2004; Ciapponi et al. 2004; Ciapponi et al. 2006; Oikemus et al. 2006). Additionally, these mutants have defects in DNA repair and are defective for DNA damage responses. For instance, *Drosophila* ATM and Nbs1 are required for IR-induced apoptosis. *Drosophila nbs1* is also required for IR-induced cell cycle arrest and ATM plays a role in cell cycle arrest at low dose of IR (Oikemus et al. 2004; Ciapponi et al. 2006; Oikemus et al. 2006). These results indicate that ATM and members of the MRN complex play a role in telomere protection, as well as DNA damage responses.

Finally, *Drosophila mnk* (homolog of Chk2) and *p53* are both required for damage-induced apoptosis at early time points (Brodsky et al. 2000a; Ollmann et al. 2000; Xu et al. 2001; Sogame et al. 2003; Brodsky et al. 2004). In response to DNA damage, p53 is phosphorylated in a MNK-dependent manner (Brodsky et al. 2004). Imaginal tissues deficient for *mnk* fail to induce apoptosis at early time points after DNA damage and show a partial defect in cell cycle arrest (Xu et al. 2001; Brodsky et al. 2004). Additionally, syncytial embryos treated with DNA damaging agents undergo MNK-dependent “mitotic catastrophe”. This mitotic response to DNA damage eliminates cells with genomic instability and prevents aneuploid cells from being formed (Takada et al. 2003).

Drosophila p53. *Drosophila* p53 is a 385 amino acid protein that has a similar structure to mammalian p53, consisting of a N-terminal transactivation domain, a central DNA binding domain, and C-terminal tetramerization domain. The highest amount of sequence homology is in the DNA binding domain, including conservation of frequently mutated residues. *Drosophila* p53 has the ability to bind human p53 recognition sites, and mutation of conserved sites within the DNA binding domain inhibits DNA binding (Brodsky et al. 2000a; Jin et al. 2000; Ollmann et al. 2000). Despite little sequence similarity in the other domains, there are several pieces of evidence that indicate conserved activities. For instance, like human p53, the transactivation domain of *Drosophila* p53 shows a high proportion of acidic residues and the tetramerization domain contains several basic residues that aid in sequence specific DNA binding. Additionally, yeast one and two-hybrid assays also show conserved activities for the amino and carboxyl terminal domains (Brodsky et al. 2000a). Finally, crystal structures reveal that *Drosophila* p53 has a similar secondary structure in the tetramerization domain of mammalian p53 (Jin et al. 2000; Ollmann et al. 2000; Ou et al. 2007).

Unlike mammals, *Drosophila* p53 does not induce cell cycle arrest after DNA damage (Brodsky et al. 2000a; Jin et al. 2000; Ollmann et al. 2000). Additionally, there is no evidence for the upregulation of the p21 homolog, *decapo*, after damage (Brodsky et al. 2004). Another difference between mammalian and *Drosophila* p53 is the regulation of p53 protein levels. *Drosophila* embryos treated with IR show no increase in p53 protein levels by western blot analysis (Brodsky et al. 2004). Furthermore, *Drosophila* p53 does not appear to contain an Mdm2 binding motif and there is no

obvious Mdm2 homolog in the genome. Regulation of p53 protein levels may have developed later in evolution to better mediate levels of p53-dependent transcription (Brodsky et al. 2000a; Ollmann et al. 2000; Brodsky et al. 2004).

Microarray analysis showed that *Drosophila* p53 upregulates the pro-apoptotic genes *head involution defective* (*hid*), *reaper* (*rpr*), *sickle* (*skl*), and the *Drosophila* TNF ligand, *eiger* to induce apoptosis (Brodsky et al. 2000a; Sogame et al. 2003; Brodsky et al. 2004). The *Drosophila* TNF ligand, Eiger, induces apoptosis extrinsically through cell-to-cell signaling (Igaki et al. 2002; Moreno et al. 2002b). Although overexpression of *eiger* is sufficient to induce apoptosis, genetic experiments show that it is not required for IR-induced apoptosis (Brodsky et al. 2000a). p53 intrinsically induces apoptosis through upregulation of the pro-apoptotic genes *hid*, *rpr*, and *skl*. An irradiation responsive enhancer region (IRER) is located upstream the *rpr* promoter. The IRER is required for induction of *rpr* and the adjacent gene *hid* after DNA damage (Zhang et al. 2008). Within the IRER, p53 binds and transcriptionally induces *rpr* expression (Brodsky et al. 2000a). *hid* is required for damage-induced apoptosis in imaginal tissues, whereas *rpr* plays a smaller role (Brodsky et al. 2004; Moon et al. 2008) (Figure 1.3).

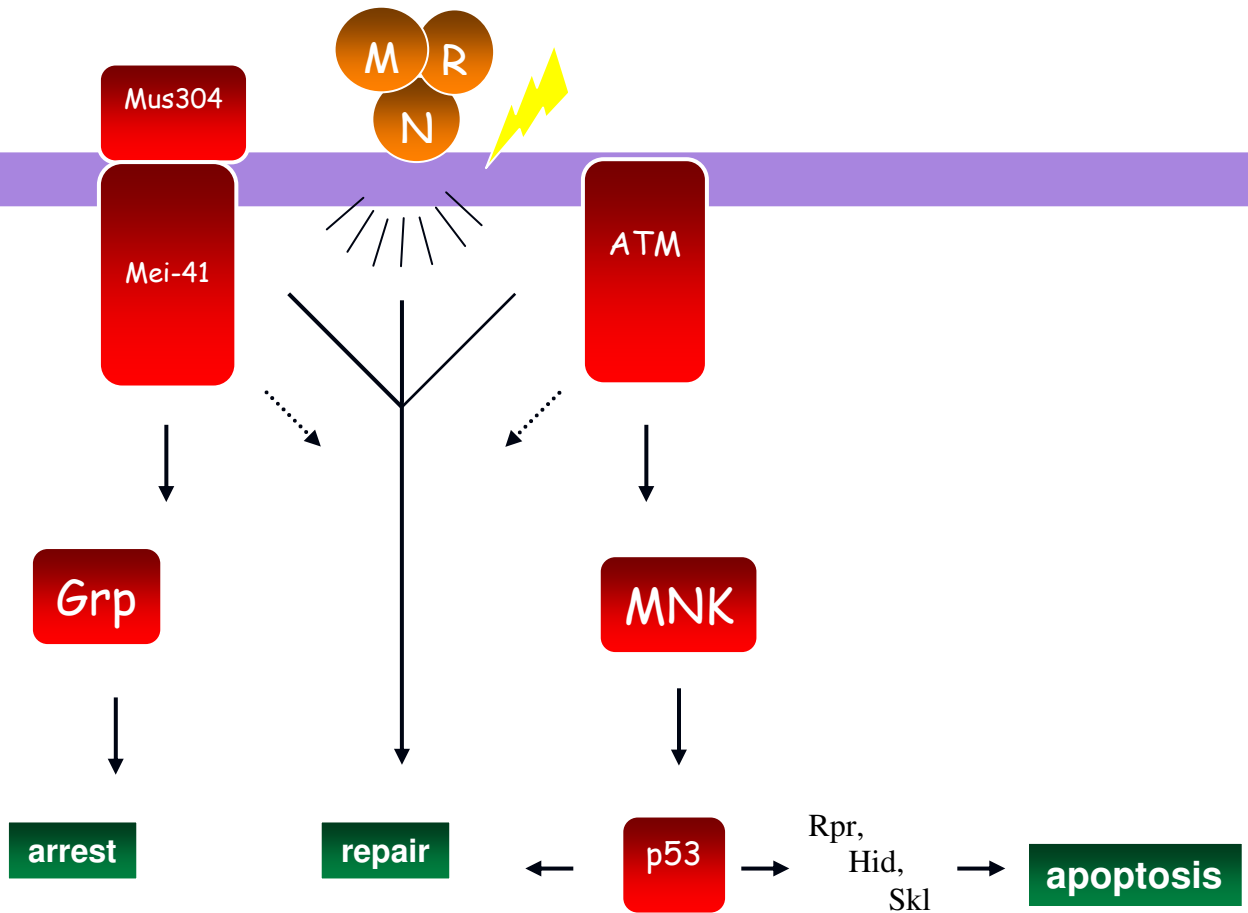


Figure 1.3. *Drosophila* DNA damage response pathway. In response to IR, ATM, Chk2, and p53 induce apoptosis. At the same time, Mei-41 and Mus304 activate Grp to induce cell cycle arrest. Members of the MRN complex contribute to repair as well as both the apoptotic and cell cycle arrest pathways.

The JNK pathway. Similar to mammals, the *Drosophila* JNK pathway contributes to induction of apoptosis in response to many stimuli including DNA damage. The gene, *hemipterous* (*hep*), is the upstream MAPKK that activates the MAPK, *Bsk*, and in turn phosphorylates D-Jun (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). D-Jun heterodimerizes with D-Fos to form the AP-1 transcription factor complex (Perkins et al. 1988). A target of the JNK pathway and AP-1 is the MAPK phosphatase, *puckered*, which forms a negative feedback loop by dephosphorylating JNK (Martin-Blanco et al. 1998). In response to ultra violet light, the JNK pathway induces cell death by activating the downstream transcription factors D-Fos and Foxo (forkhead Box O), which induces transcription of *hid* (Luo et al. 2007). Also, in response to IR, JNK is activated in a p53-dependent manner and is required for the efficient induction of apoptosis (McEwen and Peifer 2005)

Canonical apoptosis pathway. Although the core apoptotic machinery is conserved from *Drosophila* to mammals, the key points of control in the pathway are different. The *Drosophila* genome contains two Bcl-2 homologs *debcl*/*dBorg1*/*Drob1*/*dBok* and *buffy*/*dBorg2*. *Debcl* promotes apoptosis, while *buffy* has anti-apoptotic functions (Brachmann et al. 2000; Colussi et al. 2000; Igaki et al. 2000; Quinn et al. 2003). *Debcl* and *buffy* mutants have normal apoptotic patterning in the embryo and *debcl* mutants show reduced amounts of apoptosis following IR. Despite the conservation of Bcl-2 proteins in flies, these results demonstrate that Bcl-2 proteins are not essential regulators of apoptosis (Sevrioukov et al. 2007).

In *Drosophila*, the pro-apoptotic genes *hid*, *rpr*, and *grm* control the induction of apoptosis. Hid, Rpr, and Grim are found in the cytoplasm and are functionally similar to mammalian Smac/Diablo and Omi/HtrA, which all contain an IAP binding motif (IBM) at the N-terminus that is required for their pro-apoptotic function (Bergmann et al. 2003). Overexpression of any one of these pro-apoptotic genes is sufficient to induce apoptosis (Grether et al. 1995; Chen et al. 1996; White et al. 1996). Interestingly, *hid*, *rpr*, and *grm* are all located in the same genomic region (White et al. 1994; Grether et al. 1995; Chen et al. 1996). The H99 deficiency spans this region and H99 deficient embryos lack developmental cell death and show reduced IR-induced apoptosis (White et al. 1994). *sickle (skl)*, another cell death protein that contains an IBM motif and is located just outside of the H99 region, can also induce apoptosis when overexpressed (Christich et al. 2002; Srinivasula et al. 2002; Wing et al. 2002).

Although the pro-apoptotic proteins *hid*, *rpr*, and *grm* are functionally similar, there are differences in the way they are regulated. For instance, *rpr* and *grm* expression is limited to cells that are destined to die, suggesting they are controlled primarily by transcriptional regulation (White et al. 1994; Chen et al. 1996). *hid* can also be transcriptionally induced, however, it is also expressed in living cells, and is subject to post-translational modification to suppress its pro-apoptotic function (Bergmann et al. 1998; Kurada and White 1998).

Overexpression of *hid* or *rpr* in the *Drosophila* eye imaginal disc results in an eye ablation phenotype due to excessive apoptosis (Grether et al. 1995; White et al. 1996). *thread*, which encodes *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1) was initially

identified in a screen searching for modifiers of the eye ablation phenotype (Hay et al. 1995). While overexpression of DIAP1 or gain of function alleles suppressed the eye ablation phenotype, loss of *thread* led to increased cell death and caspase activation (Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000). These results indicate that *hid* and *rpr* induce cell death by inhibiting DIAP1.

IAPs were first identified in baculovirus, and were later discovered in *Drosophila* and mammals. IAPs play an important role in suppressing activation of caspases in the living cell (Bao and Shi 2007). DIAP1 contains two baculovirus IAP repeats (BIR), and a RING domain, which encodes an E3 ubiquitin ligase that is required for ubiquitin-mediated degradation (Hay et al. 1995). In living cells, BIR2 domain of DIAP1 binds to the prodomain of *Drosophila* homolog of caspase-9, Dronc, and ubiquitinates it, causing its degradation (Meier et al. 2000; Muro et al. 2002; Wilson et al. 2002; Chai et al. 2003). In addition, the BIR1 domain of DIAP1 binds to the *Drosophila* homolog of caspase-3, and inhibits its catalytic activity (Yan et al. 2004).

Upon apoptotic stimulation, several mechanisms act to deplete DIAP1 levels, thus allowing for caspase activation. Rpr, Hid, and Grm bind to one of the BIR domains of DIAP1 via their IAP Binding Motif (IBM). Binding of the IBM to the BIR domain results in the autoubiquitination and degradation of DIAP1. Additionally, Rpr and Grm have also been shown to limit DIAP1 levels through suppression of protein translation (Joazeiro and Weissman 2000; Yang et al. 2000b; Holley et al. 2002; Yoo et al. 2002; Olson et al. 2003). Lastly, activation of caspases can also limit DIAP1 protein levels. Normally, the N-terminus of DIAP1 contains a stabilizing residue. However, a caspase

cleavage site on DIAP1 at residue 20 results in a destabilizing Asn residue at the N-terminus, causing its rapid degradation, a process known as the N-end rule pathway (Ditzel et al. 2003). Once DIAP1 levels are depleted, caspases can be activated and begin cleaving downstream substrates.

The *Drosophila* genome contains seven caspase genes. The caspases Dredd, Strica, and Dronc encode initiator caspases, while Decay, Damn, Drice, and DCP-1 are effector caspases (Salvesen and Abrams 2004). Dronc is essential for most developmental and stress-induced apoptosis (Chew et al. 2004; Daish et al. 2004; Xu et al. 2005). Dronc contains a caspase recruitment domain (CARD) in its prodomain (Dorstyn et al. 1999), and is similar to mammalian caspase-9 (Kornbluth and White 2005). The CARD domain, which is required for most developmental cell death, binds to the CARD domain of the *Drosophila* Apaf-1 homolog, Dark, to make up the apoptosome. Binding of Dronc to Dark is required for its auto-activation (Kanuka et al. 1999; Rodriguez et al. 1999a; Zhou et al. 1999; Muro et al. 2004; Xu et al. 2005). Dronc can also cleave and activate the downstream effector caspase Drice in vitro (Hawkins et al. 2000). *Drosophila* Drice and DCP-1 are functional homologs of mammalian caspase-3 (Fraser and Evan 1997; Fraser et al. 1997; Song et al. 1997). In embryos, *drice* is required for cell death in some cell types, whereas either *drice* and *dcp-1* can induce apoptosis in other cell types, indicating that they have partially redundant function (Xu et al. 2006). In the imaginal wing disc, *drice* is required for IR-induced apoptosis, whereas *dcp-1* is dispensable (Kondo et al. 2006).

Until recently it was unclear if the mitochondrial pathway played a role in the

induction of apoptosis in *Drosophila*. Although the release of cytochrome c from the mitochondria is not required (Dorstyn et al. 2002; Zimmermann et al. 2002; Means et al. 2006), mitochondrial fragmentation does contribute to the induction of apoptosis. Localization of Hid, Rpr, and Grim to the mitochondria is required for optimal cell death (Haining et al. 1999; Claveria et al. 2002; Olson et al. 2003). At the mitochondria, Hid and Rpr causes changes in the mitochondrial ultrastructure and is required for full induction of apoptosis. These results suggest a conserved role for the mitochondria in cell death (Figure 1.4)(Abdelwahid et al. 2007; Goyal et al. 2007).

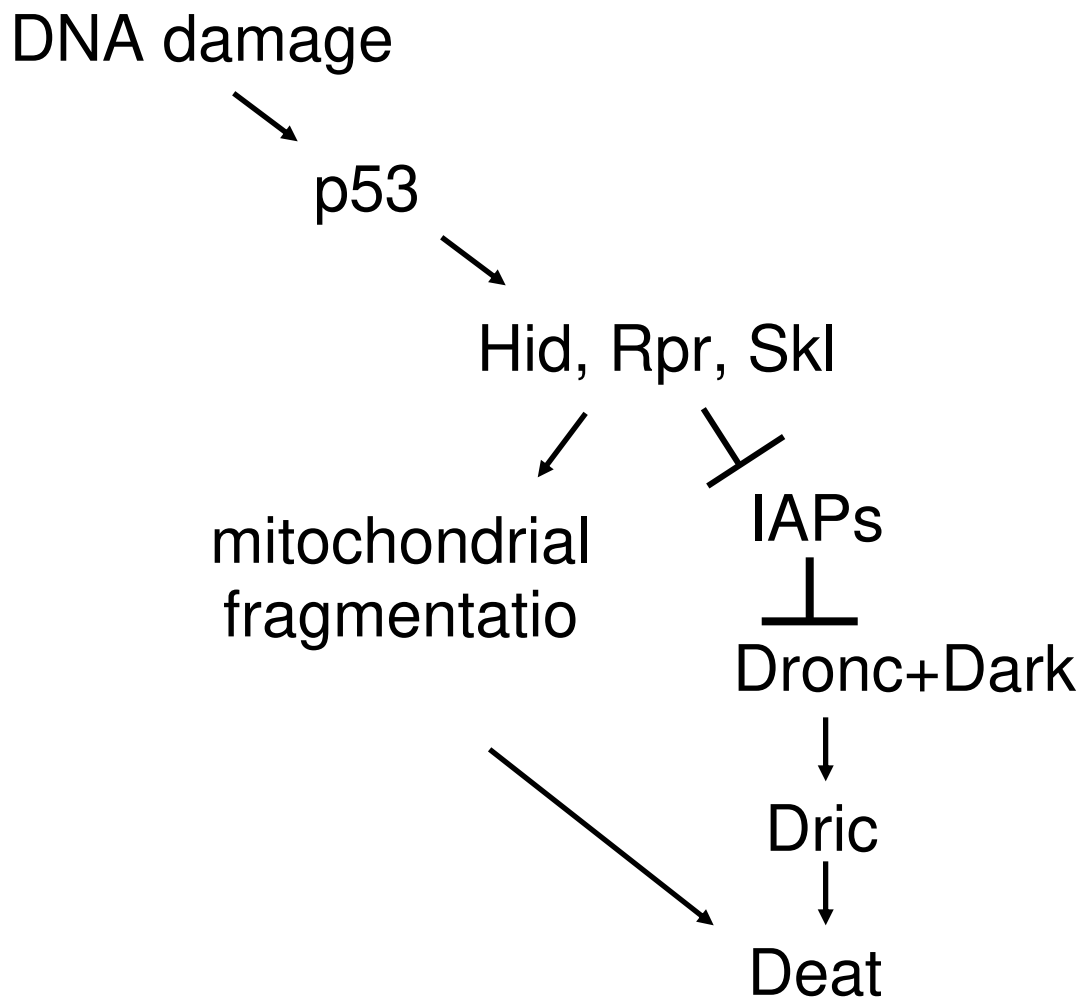


Figure 1.4. Canonical apoptosis pathway in *Drosophila*. In response to DNA damage, p53 becomes activated and upregulates the pro-apoptotic genes *hid*, *rpr*, and *skl*, resulting in the inhibition and degradation of DIAP1. Once degraded, DIAP1 can no longer inhibit caspases, allowing Dronc to form the apoptosome with Dark and cleave downstream effector caspase, Drice to induce death.

Compensatory Proliferation. A substantial number of cells are eliminated when an imaginal wing disc is treated with a DNA damaging agent, yet it can still develop into a normal size adult wing. This result suggests that cells within the tissue communicate with one another to eliminate damaged cells and increase proliferation to replace the dying cells (Milan et al. 1997). Dying cells transiently signal surrounding cells to proliferate, a process known as compensatory proliferation. When dying cells are kept alive by the caspase inhibitor p35, they propagate the compensatory signal, resulting in overgrowth of the tissue. These dying cells begin to accumulate the morphogens Decapentaplegic (Dpp) and Wingless (Wg). Accumulation of these morphogens is dependent on p53, JNK pathway activation, and dronc (Huh et al. 2004; Perez-Garijo et al. 2004; Ryoo et al. 2004; Perez-Garijo et al. 2005; Kondo et al. 2006; Wells et al. 2006). It has been suggested that upregulation of Dpp is required for proliferation, while upregulation of Wg is necessary to prevent excessive proliferation, indicating that these morphogens play opposing roles to optimize growth after DNA damage (Perez-Garijo et al. 2005).

DNA damage induced, p53-independent apoptosis

Approximately 50% of all human cancers are mutant for p53 and partially resistant to damage-induced apoptosis. Because many cancer therapies rely on DNA damaging agents to induce cell death, it is important to identify pathways that induce apoptosis in the absence of p53. This section provides an overview of previously

described mechanisms of p53-independent apoptosis.

The most well defined example of p53-independent apoptosis is mediated by the p53 homolog, p73 (Agami et al. 1999; Gong et al. 1999; Yuan et al. 1999). Isoforms of p73 share significant homology with p53, particularly in the DNA binding domain (Kaghad et al. 1997; Kartasheva et al. 2002). However, p73 and p53 seem to have different functions *in vivo* and knock out mice display very different phenotypes. For example, p53 deficient mice are tumor prone, while p73 deficient mice show defects in neuronal development and immune function, but no increase in tumor formation (Donehower et al. 1992; Yang et al. 2000a). Despite these phenotypic differences, p53 family members aid in the induction of apoptosis after DNA damage, in both the presence and absence of p53 (Roos and Kaina 2006).

In response to DNA damage, Chk1 and Chk2 phosphorylate and activate the transcription factor, E2F1, which increases levels of p73 mRNA and protein (Irwin et al. 2000; Stiewe and Putzer 2000; Urist et al. 2004). p73 is further stabilized by c-Abl mediated phosphorylation (Agami et al. 1999; Gong et al. 1999; Yuan et al. 1999). Once phosphorylated and stabilized, p73 mediates the transcriptional upregulation of PUMA, which causes BAX mitochondrial translocation and apoptosis (Melino et al. 2004). However, many tumors that express mutant forms of p53 inhibit p73, rendering cells resistant to cancer therapies that induce DNA damage (Di Como et al. 1999; Gaididon et al. 2001; Bergamaschi et al. 2003; Irwin et al. 2003). Additionally, certain squamous cell carcinomas suppress p73-dependent apoptosis through interaction with p63 (Rocco et al. 2006). Finally, p73 is rarely mutated and often overexpressed in tumors (Roos and Kaina

2006). These results indicate that many layers of complexity exist within the p53 family, it is important to identify alternate pathways that induce apoptosis independent of the p53 family.

Inhibiting cell cycle checkpoints after treatment with DNA damage is another mechanism that induces p53-independent apoptosis. In response to DSBs, p53-deficient cells rely on ATM/ATR dependent activation of the p38MAPK/MK2 checkpoint for cell survival. Mutation of the p38MAPK/MK2 pathway in p53 mutant cells, but not p53 wild-type cells, results in a mitotic catastrophe (Reinhardt et al. 2007). Zebrafish and mammalian cell culture models have shown that cells can also undergo p53-independent apoptosis by mutation of Chk1. This Chk1-independent pathway requires atm, atr, and caspase-2. Interestingly, this pathway is unaffected by p53 deficiency, inhibition of caspase-3, or overexpression of Bcl-2 (Sidi et al. 2008).

Drosophila provides an excellent model system to study p53-independent apoptosis because it contains a single p53 homolog. Flies with unprotected telomeres have increased amounts of spontaneous apoptosis, which can mostly be suppressed by a mutation in *p53*. However, some apoptosis still remains, indicating there is a p53-independent apoptotic response (Oikemus et al. 2004; Oikemus et al. 2006). Consistent with this observation, imaginal wing discs treated with IR can undergo p53-independent apoptosis, however, the amount of apoptosis is reduced and it occurs with greatly delayed kinetics (Wichmann et al. 2006). Additionally, this study also indicated that one or more of the pro-apoptotic genes in the genomic region Df(3L)H99 deletion is likely to

contribute to p53-independent apoptosis (Wichmann et al. 2006). However, it is still unclear how these cells activate apoptosis in the absence of p53.

Previously, it has been shown that cells treated with IR leads to various chromosome rearrangements (Gatti et al. 1974). When these cells go through mitosis they become heterozygous for large regions of a chromosome, which is referred to as segmental aneuploidy (Lindsley et al. 1972; Baker et al. 1978). In Chapter III, we demonstrate that p53-independent apoptosis limits the production of cells with segmental aneuploidy after DNA damage. Additionally, it has been shown that significant segmental aneuploidy is required for p53-independent apoptosis (Titen and Golic, unpublished result). Ribosomal protein genes are distributed throughout the genome. Therefore, cells with segmental aneuploidy often results in reduced copy number of at least one ribosomal protein gene (Baker et al. 1978). Because loss of a ribosomal protein gene is sufficient to induce apoptosis, we favor the model that segmental aneuploidy triggers p53-independent apoptosis through loss of a ribosomal protein gene.

Haploinsufficiency of ribosomal protein genes

This section provides an overview of haploinsufficiency of ribosomal protein genes in flies and the phenomenon of cell competition. Conservation of haploinsufficiency of ribosomal protein genes is also reviewed in this section.

Haploinsufficiency of ribosomal protein genes in *Drosophila*. The most extensive analysis of haploinsufficiency of ribosomal protein genes has occurred in *Drosophila*. Flies that are heterozygous for a ribosomal protein gene were first described as a dominantly inherited class of mutants exhibiting varying degrees of developmental delay, short and thin bristles, and reduced viability and fertility. These mutations were linked to 50 different loci scattered across the genome and were termed *Minutes* (Schultz 1929). Recently, it was shown that the *Drosophila* genome contains highly conserved orthologs of all 79 mammalian ribosomal proteins. Of the 79 ribosomal protein genes, 64 of them encode *Minute* loci. Only one of the *Minute* loci did not encode a ribosomal protein gene, rather it corresponded to a translation initiation factor (Marygold et al. 2007).

Minute flies develop normal wings. Interestingly, clones of *Minute* cells in the wing are out-competed by their wild type neighbors, a phenomenon known as cell competition (Morata and Ripoll 1975). Slower growing *Minutes* have increased competition, compared to faster growing *Minutes*. However, cell competition can be suppressed when *Minute* clones are induced into *Minute* background with a similar growth rate or when flies are starved (Simpson 1979; Simpson and Morata 1981). These data demonstrate that it is a difference in growth rates that results in the elimination of *Minute* clones.

Originally, cell competition was thought to be solely due to the difference in cell autonomous growth rates (Morata and Ripoll 1975). Later, it was shown that *Minute* cells undergo apoptosis due to inability to compete with wild-type neighbors for the morphogen, Decapentaplegic (Dpp) (Moreno et al. 2002a). *dpp* is expressed at the

anterior/posterior boundary of the imaginal wing disc and forms a long range morphogen gradient to the outer edges (Zecca et al. 1995). Dpp transcriptionally represses *brinker*, which is expressed in the outer regions of the wing disc, where levels of Dpp are low (Campbell and Tomlinson 1999; Jazwinska et al. 1999; Minami et al. 1999; Muller et al. 2003; Martin et al. 2004). *Minute* clones are unable to sequester the morphogen Dpp, resulting in the aberrant expression of *brinker*, and activation JNK dependent apoptosis (Moreno et al. 2002a). Additionally, it has demonstrated that aberrant expression of morphogens in the imaginal wings disc, including *brinker*, is sufficient to activate JNK dependent apoptosis (Figure 1.5)(Adachi-Yamada et al. 1999; Adachi-Yamada and O'Connor 2002).

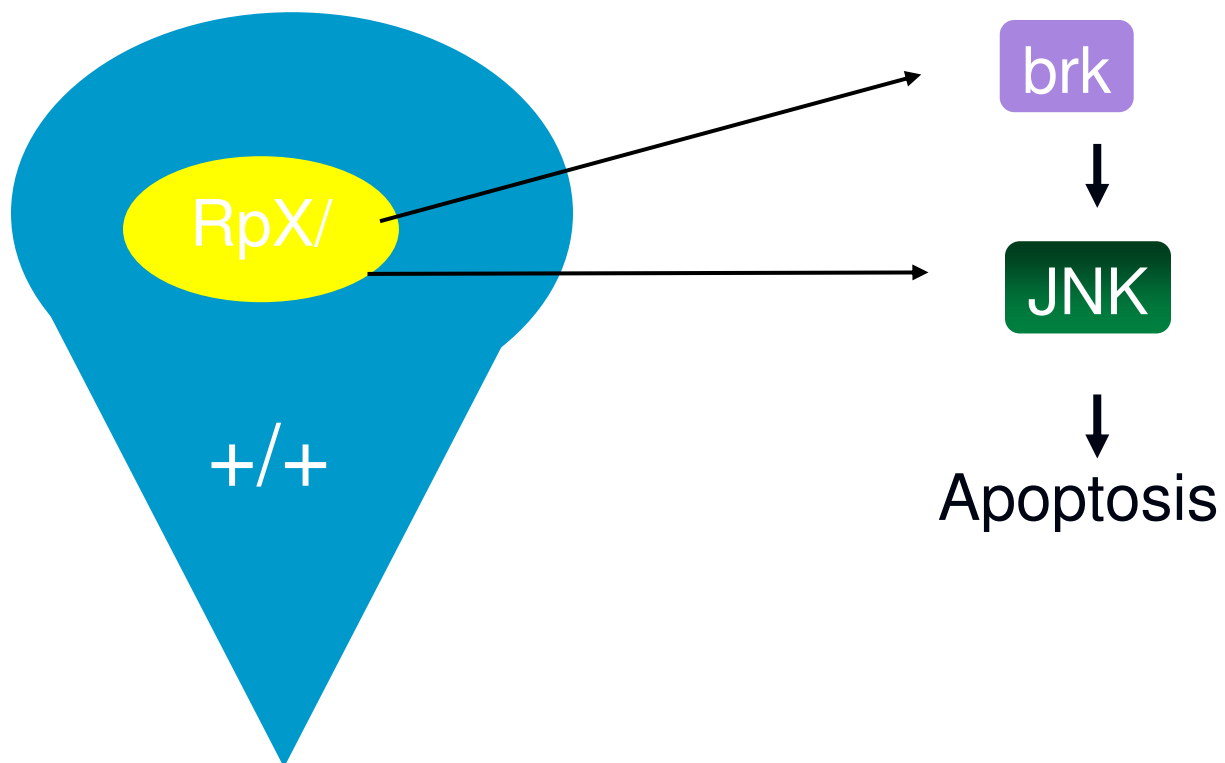


Figure 1.5. Model for *Drosophila* cell competition. Flies heterozygous for ribosomal protein genes have some developmental defects, but normally give rise to a normal adult wing. However, clones of cells heterozygous for a ribosomal protein gene in a wild-type wing disc undergo apoptosis, a process known as cell competition. This process is facilitated by ectopic expression of *brk* and JNK dependent apoptosis.

A genetic screen was performed to identify other genes that affect cell competition. Genes in the hippo pathway were originally identified in a clonal screen for mutations that result in an overgrowth phenotype (Tapon et al. 2002; Harvey et al. 2003; Lai et al. 2005). Mutation of genes in the hippo pathway including *hippo*, *warts*, *sav*, and *mats* allowed *Minute* clones to survive (Tyler et al. 2007). *Minute* clones containing mutations in *hippo*, *warts*, or *mats* increased Dpp signaling to rescue the growth defect. Interestingly, *salvador* was able to rescue the growth defect independent of Dpp signaling (Tyler et al. 2007). Other genes required for cell competition include genes in the engulfment pathway such as *draper*, *wasp*, and *phosphatidylserine receptor*. Wild-type cells begin engulfing *Minute* cells, which is required to activate the apoptotic program (Li and Baker 2007). Although engulfment previously has been shown to contribute to cell death (Hoepfner et al. 2001; Reddien et al. 2001), these results indicate that engulfment is required during cell competition and the cell-autonomous cell-death program is insufficient to remove *M/+* cells by itself (Li and Baker 2007). Engulfment genes are required for wild-type cells to activate apoptosis in neighboring *Minute* cells, suggesting that wild-type cells “eat” their way through mosaic compartments (Li and Baker 2007).

Clones of cells overexpressing *myc*, a transcription factor that regulates many genes involved in growth, acts as super competitors in a wild-type background, resulting in over-proliferation of *myc* clones and elimination of their wild-type neighbors. Wild-type neighboring cells fail to effectively compete for Dpp and undergo JNK and *hid*-dependent apoptosis (de la Cova et al. 2004; Moreno and Basler 2004). It is important to note that clones overexpressing *myc* can no longer act as super competitors if the cells

are also heterozygous for a ribosomal protein gene. This result is consistent with the model that ribosomal proteins act downstream of myc and efficient protein synthesis is required to out compete neighboring cells (Moreno and Basler 2004).

Haploinsufficiency of ribosomal protein genes in mice. Mouse embryos heterozygous for ribosomal protein gene S6 die during gastrulation (E5.5) and have increased p53-dependent apoptosis and cell cycle arrest. Mutation of p53 allows them to survive past gastrulation, but they are smaller than their littermates. They eventually die at E12.5, showing decreased expression of D-type cyclins, diminished fetal liver erythropoiesis, and placental defects (Panic et al. 2006). Examination of ribosomal subunits showed an accumulation of the 34S precursor, a phenotype previously characterized in S6 deficient livers (Sulic et al. 2005; Panic et al. 2006). Although these animals show aberrant translation machinery, it is the p53 checkpoint that initially eliminates them. These results suggest that mammals have evolved a strong selective pressure to eliminate cells heterozygous for a ribosomal protein gene in a p53-dependent manner, however p53-independent mechanisms can eventually terminate the animal (Volarevic et al. 2000; Sulic et al. 2005; Panic et al. 2006).

Another example of haploinsufficiency of a ribosomal protein gene is the Belly spot and tail (Bst) mouse. This mutation is semidominant and homozygous lethal. Phenotypes of Bst/+ include decreased pigmentation, kinked tail, and retinal abnormalities. Mapping of Belly spot and tail (Bst) mouse showed a deletion in the RpL24 ribosomal protein

gene. Bst/+ cells have decreased protein synthesis, slower proliferation, and are out-competed by wild type cells in a chimeric mouse (Oliver et al. 2004).

Haploinsufficiency of ribosomal protein genes in human disease. Diamond-Blackfan anemia (DBA) is a congenital hypoplastic anemia. Patients exhibit low numbers of erythroid precursors in the bone marrow. Fifty percent of the patients have short stature, various physical abnormalities, and a predisposition to leukemias (Flygare and Karlsson 2007). Approximately 25% of DBA patients have a mutation in RpS19, 2% have a mutation in RpS24, and a small number have been linked to a mutation in RpS17 (Draptchinskaia et al. 1999; Gazda et al. 2006; Gazda and Sieff 2006; Cmejla et al. 2007; Choismel et al. 2008). Recently, a small number of patients showed a mutation in RpL35A, which is the first example of large subunit mutation contributing to the disease. This results supports the hypothesis that this disease is likely due to an overall defect in ribosomal function, rather than a specific function of a ribosomal protein gene (Farrar et al. 2008).

Cells deficient for RPS19 show a reduction of 40S in subunits, have fewer mature 40S ribosomes, and an increase in apoptosis (Choismel et al. 2007; Idol et al. 2007). Global gene expression analysis of bone marrow cells from patients with DBA show misregulation of genes involved in apoptosis, DNA repair, and cancer (Gazda et al. 2006). Recently, it has been shown that mice heterozygous for RPS19 have increased apoptosis in bone marrow, low red blood cell count, and growth retardation. Many of these phenotypes could be suppressed in a p53 null background (McGowan et al. 2008).

Another human disease linked to haploinsufficiency of a ribosomal protein gene is 5q- syndrome. This somatic chromosomal deletion results in a subtype of a myelodysplastic syndrome caused by defective erythroid differentiation (Van den Berghe et al. 1974). These patients have an increased risk of developing acute myeloid leukemia. Recently, it was identified that this disease is linked to haploinsufficiency of RPS14 and shows similar defects in pre-rRNA processing as DBA and increased levels of apoptosis (Ebert et al. 2008).

CHAPTER II

**MODIFICATION OF DROSOPHILA p53 BY SUMO MODULATES ITS
TRANSACTIVATION AND PRO-APOPTOTIC FUNCTIONS**

Foreword

The work presented in this chapter has been published: Federico Mauri*, Laura M. McNamee*, Andrea Lunardi*, Fulvio Chiacchiera, Giannino Del Sal, Michael H. Brodsky and Licio Collavin. 2008. Modification of *Drosophila* p53 by SUMO modulates its transactivation and pro-apoptotic functions. *J Biol Chem.* 283(30): 20848-56.

* authors contributed equally to this work.

Specific contributions are as follows: Fredrico Mauri mapped the sumoylation sites and performed colocalization experiments in S2 cells. DNA-binding and transactivation assays were performed by Andrea Lunardi. Laura McNamee performed all the in vivo analysis.

Introduction

The p53 tumor suppressor is a highly regulated transcription factor that coordinates cellular responses to DNA damage, activation of oncogenes, and a variety of other stress signals (Vousden and Lane 2007); accordingly, p53 inactivation is the most common mutation found in human cancers (Harris and Levine 2005). A complex array of post-translational modifications regulate stability, localization, conformation, and transcriptional activity of p53, with crucial implications for its tumor suppressive function (Bode and Dong 2004; Toledo and Wahl 2006; Watson and Irwin 2006; Horn and Vousden 2007).

SUMO-1 belongs to a family of small ubiquitin-related proteins that are covalently linked to lysine residues of protein substrates (Muller et al. 2001; Hay 2005). In contrast to ubiquitination, sumoylation does not target modified proteins for degradation, but can affect their localization, stability, and functions (Muller et al. 2001; Muller et al. 2004; Gill 2005; Hay 2005). Human p53 can be modified by SUMO-1 on a single C-terminal lysine (K386) but the effects of this modification are controversial (Hoeller et al. 2006; Watson and Irwin 2006). Initial studies indicated that SUMO stimulates the activity of p53 (Gostissa et al. 1999; Rodriguez et al. 1999b; Muller et al. 2000). In contrast, other work suggested that sumoylation does not affect p53 transcriptional activity (Kwek et al. 2001; Schmidt and Muller 2002). In addition, conflicting reports indicate that the SUMO E3 ligase PIAS1 can either stimulate or inhibit p53 activity (Megidish et al. 2002; Schmidt and Muller 2002). Overexpression of SUMO-1 stimulates recruitment of p53 to PML Nuclear Bodies (NBs), with implications for p53 pro-apoptotic activity, but mutation of the SUMO acceptor site does not prevent p53 localization to NBs (Fogal et al. 2000; Kwek et al. 2001). Two knock-in mouse models have been generated in which all C-terminal lysine residues in p53 have been mutated, including the sumoylation site; despite extensive cell culture data indicating critical roles of these residues for p53 function, these mice are similar to wild type, and MEFs and thymocytes derived from these animals display normal apoptotic responses after DNA damage (Feng et al. 2005; Krummel et al. 2005). These results suggest that several post-translational modifications of the C-terminus, including sumoylation, may not be crucial for p53 function in mammalian cells (Toledo and Wahl 2006).

Other members of the p53 family are also sumoylated at their C-terminus (Watson and Irwin 2006). In cell culture, sumoylation of p63a destabilizes the protein and decreases its transactivation function (Huang et al. 2004; Ghioni et al. 2005) while sumoylation of p73a modulates its nuclear localization and turnover (Minty et al. 2000). Therefore, although the biological effects of sumoylation may vary among p53-related proteins, modification with SUMO is a common feature of the p53 family, suggesting an ancient regulatory mechanism inherited from a common ancestor gene.

In *Drosophila melanogaster* there is a single p53 family member, with the same domain structure of mammalian p53 proteins. The core DNA binding domain has the greatest sequence similarity, while the N- and C-terminal domains show little sequence conservation but retain similar structural and functional features (Brodsky et al. 2000a; Jin et al. 2000; Ollmann et al. 2000; Ou et al. 2007). *Drosophila* p53 binds the same consensus sequence as human p53, and transactivates reporter constructs driven by p53 responsive elements (Brodsky et al. 2000a; Jin et al. 2000; Ollmann et al. 2000). *Drosophila* mutants lacking p53 function are viable and fertile, but are defective for induction of apoptosis by DNA damage or unprotected telomeres (Brodsky et al. 2000a; Ollmann et al. 2000; Lee et al. 2003; Sogame et al. 2003; Brodsky et al. 2004; Oikemus et al. 2004). *Drosophila* p53 induces cell death when overexpressed in eye imaginal discs (Brodsky et al. 2000a; Ollmann et al. 2000), upregulates pro-apoptotic genes including *reaper*, *sickle*, *hid* and *Eiger*, and binds a specific DNA damage responsive element within the *reaper* promoter (Lee et al. 2003; Sogame et al. 2003; Brodsky et al. 2004; Akdemir et al. 2007). Activation of *Drosophila* p53-dependent apoptosis following DNA

damage depends on the protein kinase Mnk/Chk2, which phosphorylates p53 (Peters et al. 2002; Brodsky et al. 2004). Other post-translational modifications of *Drosophila* p53 have not been demonstrated.

Here we show that *Drosophila* p53 can be modified by SUMO on two independent residues. We present evidence that a sumoylation-defective p53 mutant is markedly less active than the wild-type counterpart, in cell culture and *in vivo*, implicating sumoylation in the biochemical circuitry that positively regulates *Drosophila* p53 function.

Results

Identification of two functional sumoylation sites in *Drosophila* p53. In yeast two-hybrid screens, we and others have found interactions between *Drosophila* p53 and lesswright/dUbc9 (an E2 SUMO ligase), Su(var)2-10/dPIAS (an E3 SUMO ligase) and Ulp1 (a SUMO specific peptidase), suggesting that p53 may be sumoylated (Stanyon et al. 2004; Formstecher et al. 2005)(M.H.B. and Garson Tsang, unpublished results). Within the p53 sequence there are two consensus sites for sumoylation, one on lysine 302 in the C-terminal region of the protein, the other on lysine 26 within the N-terminal transactivation domain (Fig. 2.1A). These sites do not directly correspond to the single site identified at the extreme C-terminus of mammalian p53, p63 or p73 (Watson and Irwin 2006).

To test if *Drosophila* p53 can be sumoylated, *Drosophila* S2 cells were transfected with His-tagged p53 (HT-Dmp53) alone, with *Drosophila* SUMO fused with GFP (GFP-dSUMO), or with a non-conjugatable version of SUMO lacking the C-terminal glycines necessary for attachment to substrates (GFP-dSUMO Δ C). Transfected p53 was visualized by immuno-blotting with an antibody to the RGS-His tag. As shown in Figure 2.1B-C, transfected p53 migrates as one primary band and two slower migrating bands; the apparent molecular weights are compatible with attachment of one or two SUMO molecules. In cells transfected with GFP-dSUMO, the upper bands shift to higher molecular weights, compatible with covalent attachment of one and two GFP-dSUMO molecules. This shift is not observed in cells transfected with the non-conjugatable GFP-dSUMO Δ C (Fig. 2.1B).

To test the requirement of lysine 302 and lysine 26 for conjugation, they were replaced with arginine by site-directed mutagenesis. When either lysine 26 or lysine 302 are altered, the resulting proteins (p53 K26R and p53 K302R) display a single slower migrating band (Fig. 1B). When both residues are mutated, the resulting protein (p53 KRKR) is no longer modified. Comparison of the various mutants suggests that lysine 302 may be sumoylated more efficiently than lysine 26. In addition, modification at K302 apparently induces a greater shift in migration than modification at K26 (Fig. 2.1C).

To verify that endogenous SUMO is covalently attached to p53, HT-Dmp53 was immuno-precipitated from transfected S2 cells and probed with an antibody to *Drosophila* SUMO (Fig. 2.1D). Based on these results, we conclude that a significant

fraction of Dmp53 is sumoylated when expressed in S2 cells, with lysine 302 being the primary modification site.

Figure 2.1

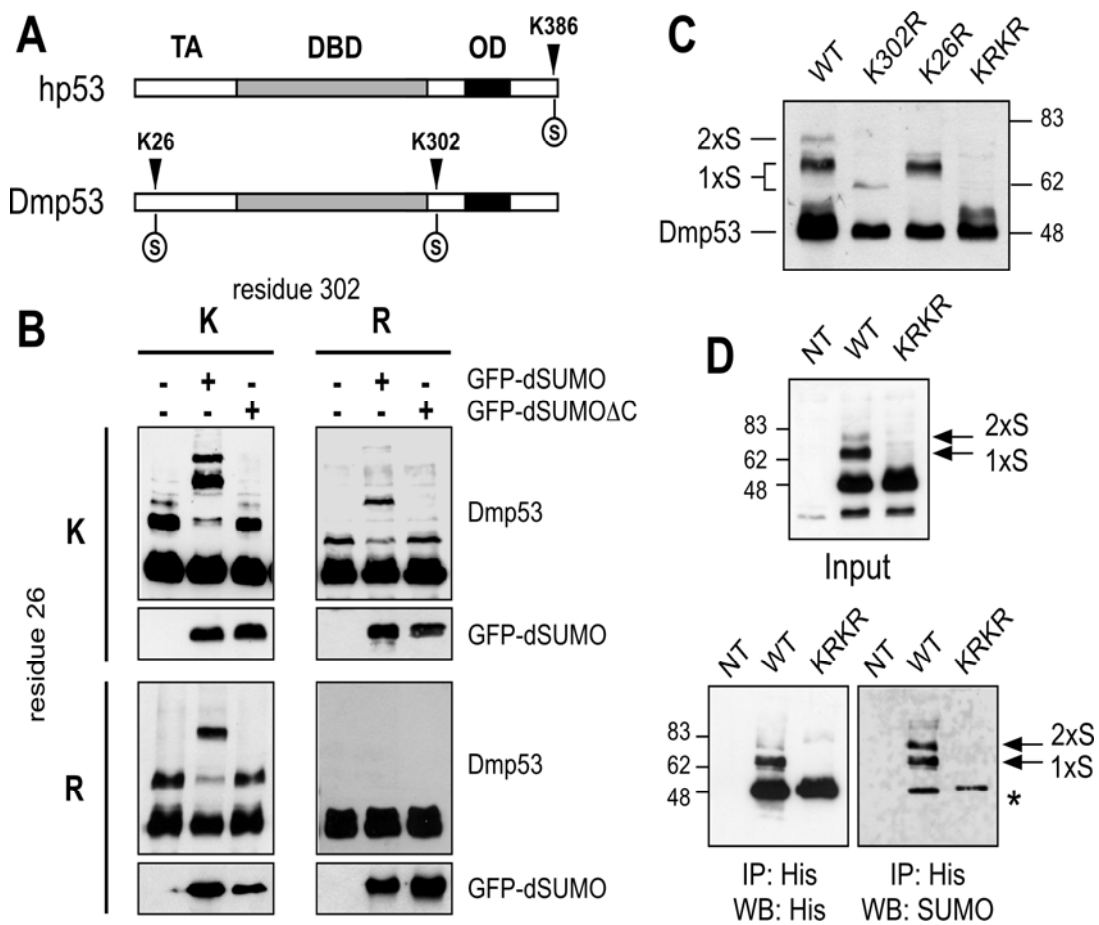


Figure 2.1. Identification of two sumoylation sites in *Drosophila* p53. (A) Schematic structure of human and *Drosophila* p53, with respective sumoylation sites. The transactivation (TA), DNA binding (DBD) and oligomerization (OD) domains are also indicated. (B) Sumoylation of p53. Wild-type HT-Dmp53 and the indicated mutants were transfected in S2 cells with or without plasmids expressing GFP-dSUMO or its non-conjugatable version GFP-dSUMODC. Lysates were separated by SDS-PAGE. HT-Dmp53 and GFP-dSUMO were detected by immunoblotting. (C) The various sumoylated forms migrate differently. The indicated p53 mutants were transfected in S2 cells and analyzed by immunoblotting in the same gel. (D) p53 is conjugated to endogenous SUMO. Wild-type HT-Dmp53 and the double-lysine KRKR mutant were transfected in S2 cells. Lysates were immunoprecipitated with a monoclonal antibody to the RGS-His tag, and revealed with an antibody to *Drosophila* SUMO (bottom right). Expression of HT-Dmp53 proteins was also analyzed in the immunoprecipitate (bottom left) and in total lysates (input). The antibody to *Drosophila* SUMO has a weak cross-reactivity to p53 (asterisk). Arrows indicate p53 modified with one or two SUMO molecules.

Drosophila p53 localizes to nuclear dots. The distribution of wild-type and non sumoylatable p53 was analyzed in transfected S2 cells. As shown in Figure 2.2A, transfected p53 is found throughout the nucleus with marked accumulation in dot-like structures. p53 forms nuclear dots in 70-80 percent of transfected cells, with most nuclei having 2 or 3 dots. This localization was not dependent on the adhesion substrate (Concanavalin A or poly-lysine) and did not change using N-terminally tagged or untagged p53 (not shown). The p53 K to R mutants form nuclear dots with similar frequency, shape, and size as the wild-type protein. When co-transfected with GFP-dSUMO, wild type p53 and single lysine mutants co-localize with SUMO in nuclear dots. However, only a subset of nuclear dots formed by the non-sumoylatable p53 KRKR mutant overlap with dots formed by GFP-dSUMO (Fig. 2.2 and Fig. 2.3).

Localization of wild type and non-sumoylatable p53 was also analyzed in transgenic flies, using the GAL4/UAS system. With a GMR-GAL4 driver, high levels of p53 are produced in the posterior of the developing eye imaginal disc, sufficient to induce ectopic apoptosis in the absence of DNA damage (Brodsky et al. 2000a; Ollmann et al. 2000; Brodsky et al. 2004). In the absence of GAL4 driver, p53 is expressed at low levels, insufficient to induce apoptosis (see Fig. 2.8). In both conditions, wild type p53 and p53 KRKR are found throughout the nucleus with a sub-nuclear domain of elevated staining, similar to that seen in cell culture (Fig. 2.2C-D). We used a polyclonal antibody to visualize endogenous SUMO in these cells (Long and Griffith 2000). Endogenous SUMO is not detected in cells expressing less p53, probably due to low levels of SUMO throughout the entire nucleoplasm. At the higher p53 expression levels, endogenous

SUMO accumulates in nuclear dots with wild type, but not with non-sumoylatable p53 (Fig 2.2C).

In contrast with cell culture results, p53 KRKR transgenic cells displayed lower overall levels of immuno-staining, suggesting reduced expression levels. This result was observed in four independent transformants per line (data not shown), and therefore is not a consequence of genomic insertion sites. No p53 immunostaining was detected in cells solely expressing endogenous levels of p53 (data not shown). Following exposure to ionizing radiation (IR), wild type p53 and p53 KRKR are still detected throughout the nucleus and in nuclear dots (Fig. 2.2D). Together, these results confirm that p53 accumulates in sub-nuclear structures in cultured cells and in normal developing tissues. The non-sumoylatable p53 KRKR mutant can also form nuclear dot-like structures, but has reduced capacity to recruit SUMO.

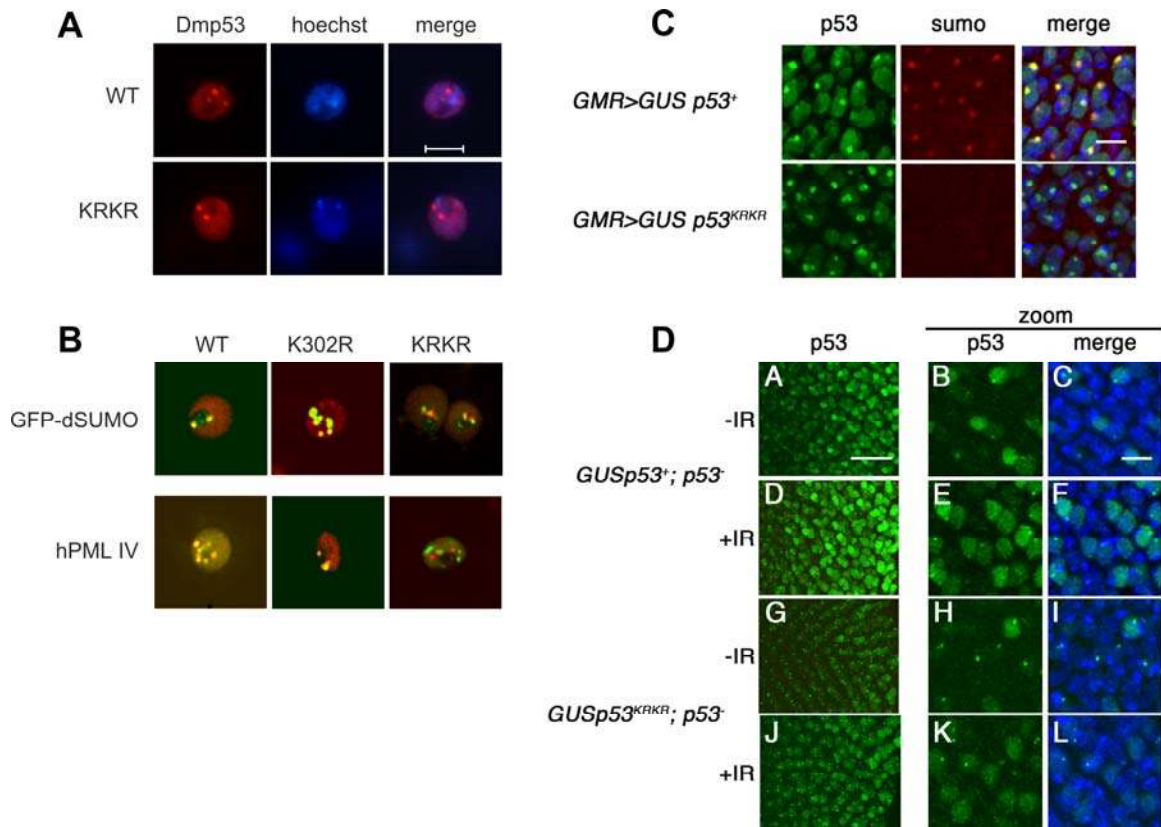


Figure 2.2. Nuclear localization of exogenous p53 in tissue culture and developing eye imaginal discs. (A) Wild-type and non sumoylatable p53 form nuclear dots in cultured cells. S2 cells were transfected with the indicated constructs, plated on Concanavalin A coated coverslips before fixation, and analyzed by confocal immunofluorescence using a monoclonal anti-Dmp53 antibody. Nuclei were visualized by Hoechst staining (scale bar 5mm). (B) p53 sumoylation mutants display differential localization with respect to GFP-dSUMO and human PML IV. Wild-type p53 and the indicated mutants were co-transfected with GFP-dSUMO or human PML IV in S2 cells. Cells were treated as above. Localization of p53 proteins (red) and GFP-dSUMO or PML

IV (green) was analyzed by confocal microscopy. Only merged images are shown, where yellow indicates co-localization. The complete set of single images for all the mutants are available in Figures 3.3 and 3.4. (C) *Drosophila* p53 (green), SUMO (red) and DAPI (blue) expression in the developing eye imaginal disc. High levels of p53 expression in the posterior of the developing eye imaginal disc were obtained using GMR-Gal4 to drive expression of *GUSp53* transgenes. Overexpressed wild type p53 accumulates endogenous SUMO in subnuclear domains. Overexpressed p53^{KRKR} also forms nuclear dots, but recruits much less SUMO (scale bar 5µm). (D) *Drosophila* p53 (green) and DAPI (blue) expression in irradiated developing eye imaginal discs. Overview (A) and high magnification (B-C) of moderately expressed wild type p53, forming nuclear dots in an untreated eye disc. Overview (D) and high magnification (E-F) of wild type p53 four hours after X-irradiation. Overview (G) and high magnification (H-I) of p53^{KRKR} forming dots in an untreated eye disc. Overview (J) and high magnification (K-L) of p53^{KRKR} four hours after X-irradiation.

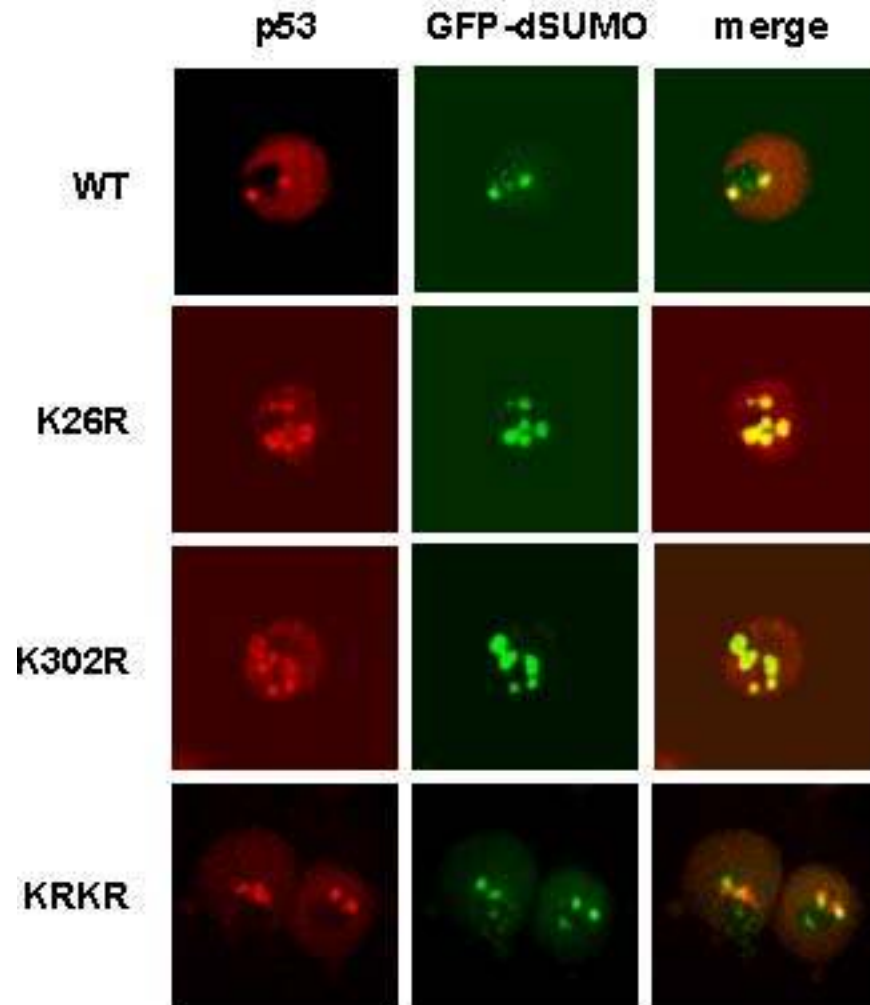


Figure 2.3. Dmp53 sumoylation mutants display differential localization with respect to GFP-dSUMO. Wild-type p53 and the indicated lysine mutants were co-transfected with GFP-dSUMO in S2 cells. Cells were plated on Concanavalin A coated coverslips two hours before fixation. Localization of the expressed proteins was analyzed by confocal microscopy. Images refer to single Z sections.

Sumoylation affects p53 localization to nuclear dots defined by human PML and *Drosophila* Daxx-like protein. We next asked if nuclear dots formed by wild type or non-sumoylatable p53 are in fact the same structures. In mammalian cells, p53 accumulates within PML nuclear bodies (NBs) under specific conditions (Fogal et al. 2000; Kwek et al. 2001; Melchior and Hengst 2002; Gostissa et al. 2003). Markers for such structures are PML and Sp100 (Lallemand-Breitenbach et al. 2001; Salomoni and Pandolfi 2002), but there are no *Drosophila* homologs of these proteins. However, transfected human PML IV forms nuclear dots that co-localize with SUMO in *Drosophila* cells (Lehembre et al. 2000). When co-transfected with hPML IV, wild type p53 and single lysine mutants co-localize with PML in nuclear dots. On the contrary, only a subset of nuclear dots formed by the non-sumoylatable p53 KRKR mutant overlap with those formed by PML (Fig. 2.2 and Fig. 2.4).

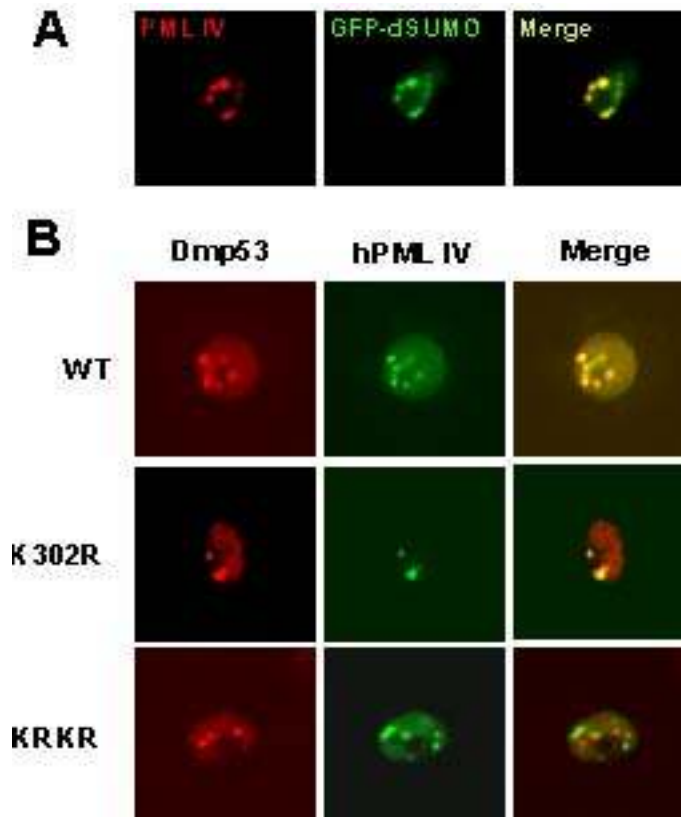


Figure 2.4. Analysis of the nuclear localization of Dmp53 mutants with respect to human PML IV. (A) Human PML IV co-localizes with GFP-dSUMO in nuclear dots. The indicated constructs were transfected in S2 cells. Cells were plated on Concanavalin A coated coverslips two hours before fixation. The expressed proteins were analyzed by confocal immunofluorescence. PML IV was visualized using the PGM-3 monoclonal antibody (Santa Cruz). (B) p53 sumoylation mutants display differential localization patterns with respect to human PML IV. Wild-type p53 and lysine mutants were co-transfected in S2 cells along with a plasmid expressing human PML IV. Localization of the expressed proteins was analyzed by confocal microscopy as above. p53 was visualized using a monoclonal antibody (Brodsky et al. 2000a), human PML IV was visualized using the H-238 polyclonal antibody (Santa Cruz).

Daxx, a transcriptional repressor and scaffolding protein, is also found in mammalian PML-NBs (Michaelson 2000; Salomoni and Khelifi 2006). The *Drosophila* homolog of Daxx, referred to as Daxx-like protein (DLP), has been described very recently (Bodai et al. 2007): it encodes a large peptide with similarity to Daxx in the C-terminus. We observed that DLP accumulates in nuclear dots when overexpressed in *Drosophila* S2 cells, and these dots co-localize with GFP-dSUMO and human PML IV (Fig. 2.5). A DLP deletion lacking the first 710 aminoacids, named DLP(ct), shows similar behavior, indicating that the region of Daxx similarity is sufficient for the observed localization (Fig. 2.5). We used GFP-DLP(ct) as a marker to analyze localization of p53 mutants; we counted the fraction of p53 nuclear dots co-localized with DLP(ct) in confocal images from independent co-transfection experiments (Fig. 2.6A). As summarized in Figure 2.6C, 85 to 90% of the dots formed by wild type p53 or single lysine mutants co-localize with DLP(ct). In contrast, only 45% of nuclear dots formed by the non-sumoylatable p53 KRKR co-localize with DLP(ct). Notably, the co-localization of the p53 KRKR mutant with DLP is restored to wild type levels when SUMO is fused to p53 KRKR to mimic constitutive K26 sumoylation (Fig. 2.6B). Together, these results indicate that sumoylation affects the recruitment of *Drosophila* p53 to specific nuclear domains.

Figure 2.5

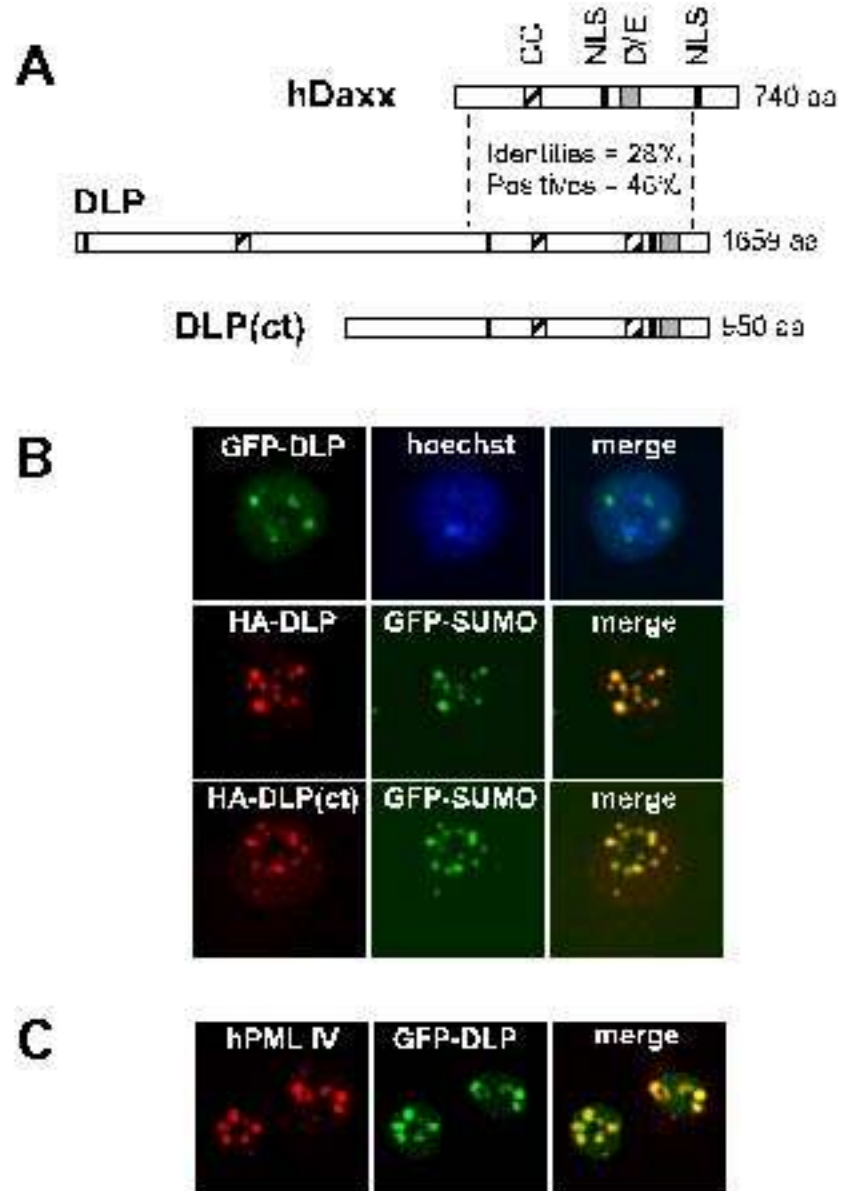


Figure 2.5. Daxx-like protein forms nuclear dots that co-localize with human PML

IV. (A) Schematic representation of DLP (Daxx-like protein) aligned with human Daxx. The region of similarity is indicated, together with some structural elements: CC, coiled coil; NLS, nuclear localization signal; D/E, acidic region. DLP(ct) is a N-terminal deletion of DLP, starting at methionine 710. (B) HA-DLP and HA-DLP(ct) form nuclear dots that co-localize with GFP-dSUMO. Constructs were transfected in S2 cells and analyzed by confocal immunofluorescence (scale bar 5 mm). (C) GFP-DLP co-localizes with human PML IV. GFP-DLP was co-transfected with human PML IV in S2 cells. Localization of the expressed proteins was analyzed by confocal microscopy. Human PML IV was visualized using the H-238 polyclonal antibody (Santa Cruz).

Figure 2.6

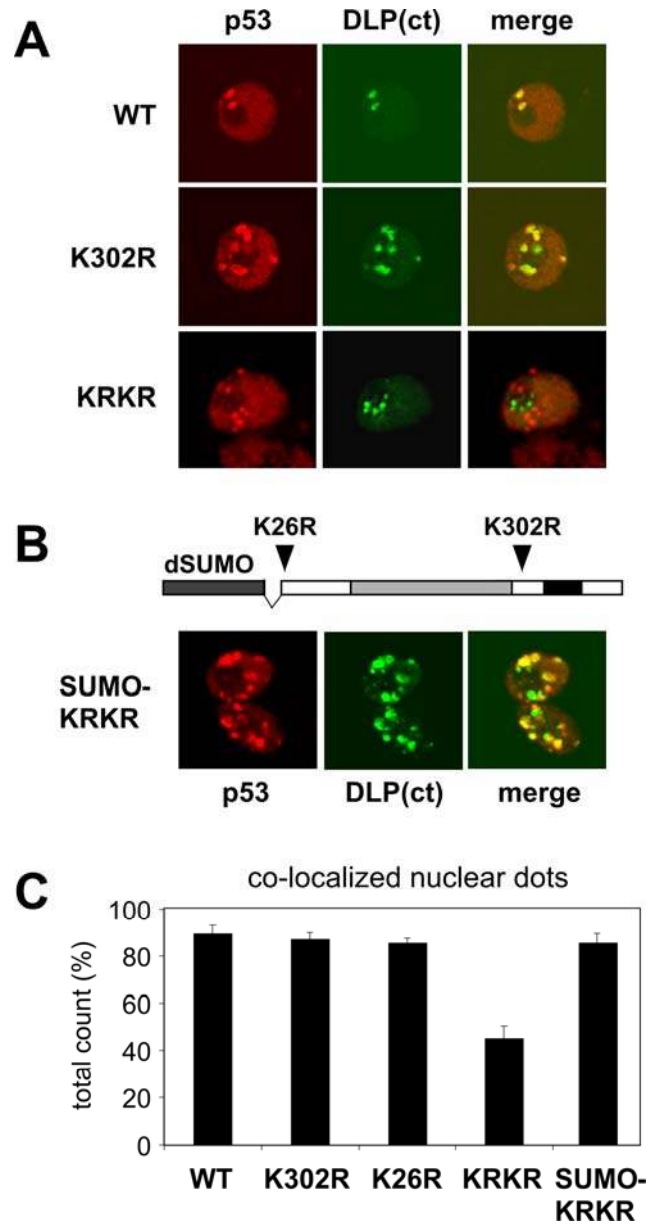


Figure 2.6. Sumoylation affects localization of p53 to nuclear dots marked by Daxx-like protein (DLP). (A) Mutation of the lysines affects p53 co-localization with DLP. Confocal analysis of the nuclear localization of wild-type p53 and lysine mutants with respect to GFP-DLP(ct) in transfected S2 cells. (B) Fusion to SUMO induces full co-localization of p53 KRKR with DLP. Confocal analysis of the nuclear localization of the SUMO-KRKR chimera with respect to GFP-DLP(ct). The structure of the SUMO-KRKR chimera is schematically drawn in the same panel: Drosophila SUMO (aminoacids 1 to 85) is fused to residue 18 of p53 KRKR. Images refer to a single Z section. (C) Quantification of p53 nuclear dots co-localized with GFP-DLP(ct) dots, assayed with the indicated constructs. More than 460 nuclear p53 dots were counted per mutant, in three independent experiments.

Non-sumoylatable p53 is less active than the wild-type protein in cultured cells.

Given the conflicting data on the functional relevance of sumoylation in mammalian p53, we asked whether sumoylation might affect the transcriptional activity of *Drosophila* p53. Initially, we used the pG13-LUC plasmid, a reporter responsive to mammalian p53 (Jin et al. 2000). We transfected this construct with increasing amounts of expression vectors encoding untagged versions of p53 mutants, and assayed for luciferase. With this reporter, equal expression levels of non-sumoylatable p53 KRKR mutant have significantly less transcriptional activity than wild-type p53 (Fig. 2.7). In contrast, mutants with substitution of either single lysine display a transactivation activity similar to wild-type p53. To confirm this behavior with a *Drosophila* promoter element, a luciferase reporter driven by a DNA damage responsive cis-regulatory sequence from the *reaper* locus, containing a p53 binding site (Rpr150 enhancer) (Brodsky et al. 2000a), was constructed and tested as above. The non-sumoylatable p53 KRKR mutant was also less active than wild-type p53 or single lysine mutants using this reporter (Fig. 2.7B).

To test if the reduced transcriptional activity of p53 KRKR is due to impaired sequence-specific DNA binding, we prepared lysates from transfected S2 cells and performed electrophoretic mobility shift assays (EMSA) using a double stranded DNA oligonucleotide containing the p53-binding element from the Rpr150 enhancer. As shown in Figure 2.7C-D, the p53 KRKR mutant binds efficiently to the oligonucleotide probe, indicating that mutation of both lysines does not prevent sequence-specific DNA binding.

Figure 2.7

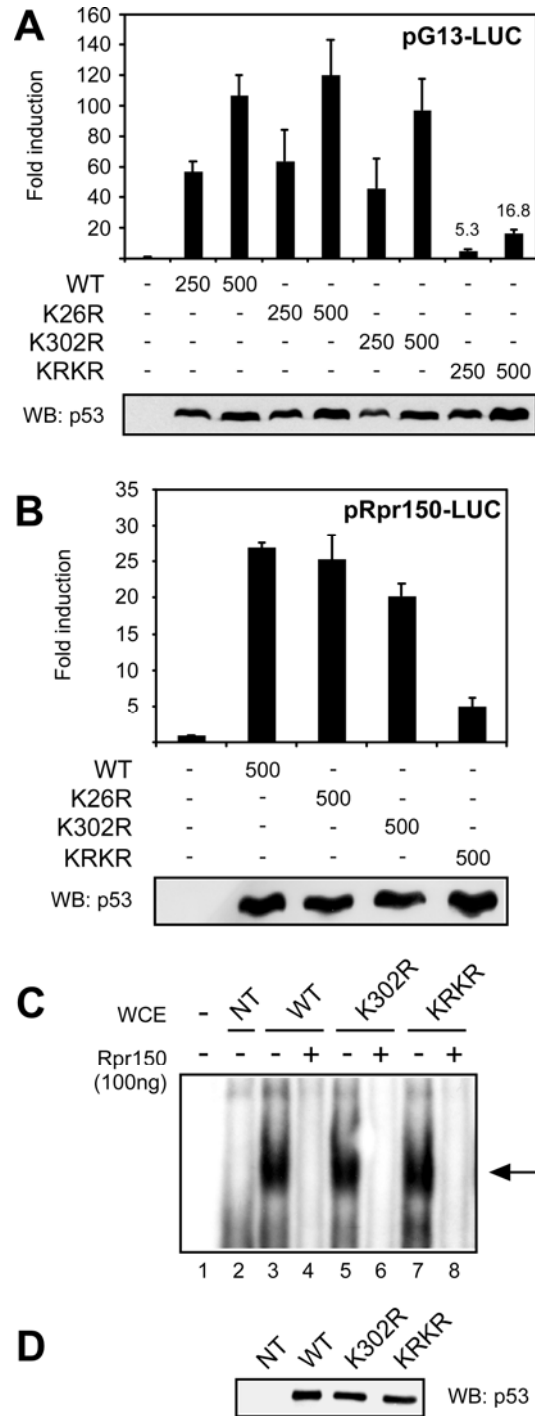


Figure 2.7. Mutation of both sumoylation sites affects transcriptional activity of p53 but not its DNA binding. (A) Transactivation of a human p53-responsive promoter. The pG13-LUC reporter plasmid was transfected in S2 cells together with increasing amounts of vector expressing wild type p53 or sumoylation mutants. A plasmid constitutively expressing beta-galactosidase was included as a control for transfection efficiency. p53 transcriptional activity was measured by luciferase assay, while the levels of expressed proteins were analyzed by immunoblotting of the same lysates (lower panel). Fold induction values of the p53 KRKR mutant are indicated. Error bars indicate s.e.m. (n=4). (B) Transactivation of a *Drosophila* p53-responsive promoter. The pRpr150-LUC reporter carrying the p53 binding site from the *Reaper* DNA-damage responsive enhancer was transfected and assayed as described above. Error bars indicate s.e.m. (n=3). (C) Electrophoretic mobility shift assay (EMSA). Wild-type p53 and lysine mutants were tested for sequence specific DNA binding by gel shift, using a double stranded oligonucleotide containing the p53-responsive element from the *Reaper* enhancer (Rpr150). Specificity of the binding was confirmed by competition with cold Rpr150 oligonucleotide. Lane 1, free probe. Lanes 2 to 9, whole cell lysates from S2 cells untransfected (NT) or transfected with the indicated p53 constructs. (D) Protein levels of transfected p53 mutants were assayed by immunoblotting of the lysates used for EMSA.

p53 sumoylation sites are essential for in vivo function. To determine if sumoylation affects p53 function *in vivo*, we compared the activity of wild type and non-sumoylatable p53 in the developing eye. We examined the ability of p53 to induce apoptosis under two conditions, when highly overexpressed and when activated following exposure to ionizing radiation (IR). Overexpression of p53 using GMR-Gal4 and GUS-p53 results in a rough, reduced eye phenotype, accompanied by a loss of pigmentation in the center of the eye (Ollmann et al. 2000; Brodsky et al. 2004)(Fig. 2.8A-B). Overexpression of p53 KRKR induces a similar rough eye, but with less loss of pigmentation (Fig. 2.8C), suggesting a difference in wild type and mutant p53 activity during eye development.

GMR-Gal4 induces target gene expression beginning in the morphogenetic furrow, which marks cells in the eye imaginal disc as they initiate synchronous cell cycle progression and differentiation. The furrow first forms in cells at the posterior of the disc and moves to increasingly anterior cells. As a result, cells near the furrow have just begun to express the transgene, while more posterior cells have expressed it for longer times. Overexpressed wild type p53 induces a high level of apoptosis in a band of cells immediately posterior to the furrow, as assayed by activated caspase and TUNEL staining (Fig. 2.8D, E, G, and data not shown). In eye discs overexpressing p53 KRKR, the band of apoptosis is initially weaker and extends further posterior from the furrow (Fig. 2.8F-G). In these experiments, the mean distance of apoptotic cells is increased from 38.0 (s.e.m.=3.0) for wild type p53 to 59.3 (s.e.m.=2.5) for p53^{KRKR} (p=0.036, two-tailed student's t-test). However, the total number of apoptotic cells is similar; the

average number of TUNEL positive cells is 568 (s.e.m.=55.5) for wild type p53 and is 474 (s.e.m.=37.4) for p53^{KRKR} (p=0.20). Since the distance from the morphogenetic furrow corresponds to the length of time cells have been overexpressing p53, these experiments indicate a delay in the induction of apoptosis by non-sumoylatable p53. This delay could reflect either lower levels of p53 KRKR expression, decreased transcriptional activity of p53 KRKR, or both.

The role of sumoylation in p53 function was also examined during DNA damage induced apoptosis. *Drosophila* p53 is required for the rapid induction of apoptosis by ionizing radiation (IR) (Brodsky et al. 2000a; Ollmann et al. 2000) (Fig. 2.8H, L, I, M, and P). In the absence of a GAL4 driver, the GUS vector expresses sufficient p53 in the posterior of the eye disc to fully restore IR-induced apoptosis in a p53-null background (Brodsky et al. 2004) (Fig. 2.8J, N, and P). In contrast, expression of p53 KRKR only weakly rescues IR-induced apoptosis (Fig. 2.8K, O, and P). Under these conditions, apoptosis is induced at the same time in all cells, and quantification of cleaved caspase-3 reveals a six-fold decrease in the level of IR-induced apoptosis in p53^{KRKR} transgenics (Fig. 5P, p=0.008, student's t-test). The decrease is greatest near the furrow, resulting in a change in the pattern of apoptotic cells. It is important to emphasize that the failure to fully rescue apoptosis is not due to insufficient expression levels, since both the wild type and mutant p53 transgenes are expressed at higher levels than endogenous p53 (endogenous p53 is not detected by immunofluorescence). These results confirm that the p53 KRKR mutant is less active than the wild-type protein and demonstrate that

sumoylation sites are critical for induction of apoptosis by p53 following DNA damage *in vivo*.

Figure 2.8

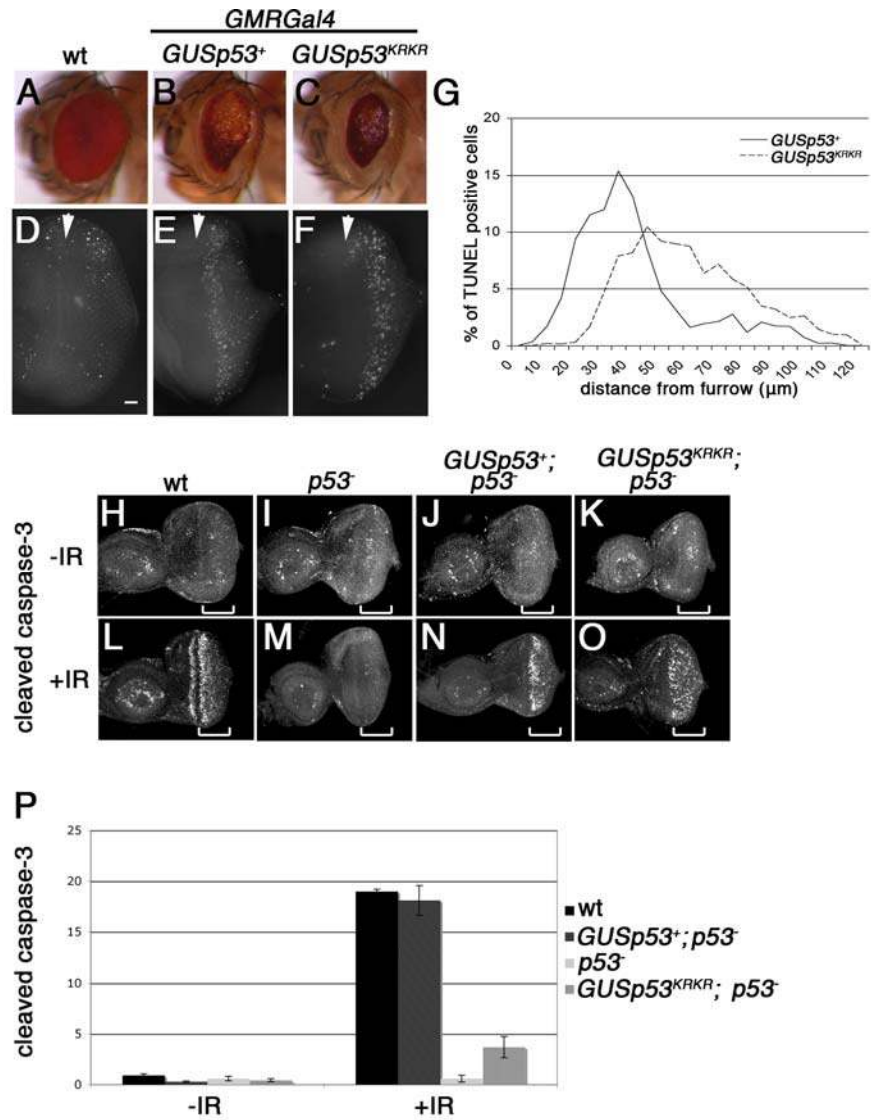


Figure 2.8. p53^{KRKR} does not induce apoptosis as efficiently as wild type p53, and is unable to fully rescue DNA damage-induced apoptosis. (A-F) High levels of p53 expression in the posterior of the developing eye imaginal disc were obtained using GMR-Gal4 to drive expression of GUS $p53$ transgenes. (A) Wild type adult eye. (B) Adult eye overexpressing wild type p53. (C) Adult eye overexpressing p53^{KRKR}. (D-F) TUNEL staining for apoptotic cells in eye imaginal discs. (D) Wild type eye imaginal disc. (E) Eye disc overexpressing wild type p53. (F) Eye disc overexpressing p53^{KRKR}. Scale bar: 20 μ m. (G) Distribution profiles of the distance of TUNEL positive cells from the furrow in p53+ expressing cells versus p53^{KRKR} expressing cells. All samples were normalized to calculate the mean percentage of apoptotic cells at a given distance from the furrow out of the total number of apoptotic cells in the disc. Distribution profiles were generated to plot percent of apoptotic cells at each distance from the furrow (n=5). (H-O) Cleaved caspase-3 staining of eye imaginal discs mock-treated, or four hours after X-irradiation. In the absence of a Gal4 driver, the Glass/multimer promoter of GUS $p53$ transgenes expresses levels of p53 that can rescue DNA damage induced apoptosis in a $p53$ mutant tissue, but are too low to induce apoptosis without an external stress. The transgene expression domain in the posterior of each eye disc is indicated with brackets. (H-K) Untreated eye discs. (L-O) Eye discs stained for cleaved caspase-3 four hours after X-irradiation. (P) Quantification of relative volumes of cleaved caspase-3 staining in the regions marked by brackets. See methods for details of caspase quantification. Bars indicate standard error of the mean (n=5). A two tailed *t-test* was used to determine the significance of the observed changes.

Discussion

In this work, we find that *Drosophila* p53 has two sites of sumoylation: one at the N-terminus and the other in the C-terminal region, before the oligomerization domain. Human p53, as well as p63 and p73, are sumoylated on a single residue at the extreme C-terminus (Feng et al. 2005; Watson and Irwin 2006). Therefore, the modification is conserved, but its position has changed during evolution. In mammalian p53 the last C-terminal amino acids are not required for oligomerization, and serve a regulatory function. In contrast, in *Drosophila* p53 the C-terminal 24 amino acids form an alpha-helix that interacts with the oligomerization domain and is required for tetramerization (Ou et al. 2007). Thus, both in mammals and *Drosophila*, C-terminal sumoylation of p53 occurs on a site where it should not interfere with oligomerization.

Because of very low expression levels, it is extremely difficult to detect endogenous p53 in *Drosophila* tissues or cell lines, even after DNA damage stimulation. Although we have not been able to examine endogenous p53, we have demonstrated that p53 is efficiently modified in cells in which SUMO and the SUMO ligating enzymes are present at physiological levels. Exploration of the signaling pathways that regulate p53 sumoylation *in vivo* will require the development of reagents and/or techniques to efficiently detect endogenous p53 protein in *Drosophila* cells.

p53 forms nuclear dots when over-expressed in *Drosophila* cells in culture and in developing eye imaginal discs. In tissue culture, dots formed by p53 co-localize with dots formed by human PML IV and *Drosophila* Daxx. It is tempting to speculate that such

structures may be related to mammalian PML nuclear bodies. However, an important caveat is that these observations rely on ectopic expression of transfected proteins, since no reagents are available to detect endogenous counterparts. The absence of an obvious PML homolog in *Drosophila* clearly indicates that these structures are not identical in insects and mammals; however, the recruitment of human PML IV to dots containing *Drosophila* SUMO, p53, and Daxx homologs does suggest that some aspects are conserved. We find that non-sumoylatable p53 also forms nuclear dots. However, it seems likely that nuclear dots formed by non-sumoylatable p53 are qualitatively different from those formed by the wild-type protein, as suggested by the reduced co-localization with dots marked by GFP-SUMO, hPML IV and DLP. Thus, the change in localization to specific sub-nuclear domains correlates with the reduced activity of the p53 KRKR mutant.

Our experiments demonstrate that sumoylation sites are important for the activity of *Drosophila* p53 both in tissue culture and *in vivo*. However, the sumoylation-deficient p53 KRKR mutant is not completely inactive; it retains sequence-specific DNA binding, and moderately transactivates both reporter constructs tested (Fig. 2.7). p53 KRKR also retains some residual ability to induce apoptosis in irradiated imaginal discs, indicating that sumoylation sites are not absolutely required for p53 activation by DNA damage (Fig. 2.8), but are essential for optimal activity.

It is important to note that single mutation of either sumoylation site had no significant effect on the activity of p53. This implies that modification of a specific lysine is not critical; rather, it is important that SUMO can be attached to the protein. This

observation indicates that sumoylation does not simply function to compete with another modification of the same residue (i.e. ubiquitination or acetylation). Our observation that sumoylation is not required for DNA binding suggests that this modification may mediate recruitment of additional factors needed for p53 dependent transcription of target genes. Alternatively, sumoylation may indirectly control p53 modification on other residues via interaction with specific modifying enzymes.

Sumoylation affects turnover of human p63 α and p73 α (Watson and Irwin 2006), but we see no difference in the expression levels of transfected wild-type or mutant p53 in S2 cells, where p53 KRKR has clearly reduced transcriptional activity (Fig.2.1, Fig. 2.7 and data not shown). In contrast, in developing eye discs, p53 KRKR seems to be expressed at lower levels than wild type. This difference may contribute to the difference in apoptosis induced by strong p53 overexpression. However, p53 KRKR cannot rescue DNA damage-induced apoptosis in *p53* mutant animals, despite being expressed at much higher levels than endogenous p53 in wild-type animals (see Fig. 2.2 and Fig. 2.8. Endogenous p53 was not detectable in wild-type cells). This observation demonstrates that sumoylation sites are critical for induction of apoptosis by p53 *in vivo*, regardless of the difference in expression levels detected between exogenous wild-type and KRKR p53 proteins in eye discs.

Our results in *Drosophila* are consistent with studies in mammalian cells reporting that sumoylation promotes p53 function (Gostissa et al. 1999; Rodriguez et al. 1999b; Li et al. 2006), but are in contrast with knock-in mouse models demonstrating that C-terminal lysines are not crucial for p53 function *in vivo* (Feng et al. 2005; Krummel et

al. 2005; Toledo and Wahl 2006). There are several possible explanations for this discrepancy. First, since knock-in p53 models had mutations in all C-terminal lysines, it is possible that loss of other modification sites masks a specific requirement for sumoylation. Second, human p53 might be sumoylated at additional non-canonical residues (Kwek et al. 2001; Jakobs et al. 2007); a weak secondary site may compensate for loss of the primary sumoylation site. Third, interaction with sumoylated proteins may be sufficient to substitute for direct sumoylation of mammalian p53; these interacting partners may not be present in *Drosophila* (e.g. PML)(Fogal et al. 2000). Finally, specific features of the molecular regulation of *Drosophila* p53 may account for a more stringent requirement for sumoylation.

In conclusion, our data demonstrate that SUMO attachment is a modification of p53 that is evolutionarily conserved from insects to mammals. Specific requirements for this modification may have changed with the emergence of three p53 paralogs in vertebrates, but sumoylation sites are clearly important for function of the single p53 protein in *Drosophila*. Our results support the general hypothesis that sumoylation has an important role in regulation of metazoan p53 and p53-related proteins.

Materials and Methods

Plasmids. The cDNA for *Drosophila* p53 was picked from the *Drosophila* Gene Collection (DGC1.0). Mutants K26R, K302R and KRKR were generated by PCR-based mutagenesis. The cDNAs for *Drosophila* SUMO and DLP(ct) were retrieved from

DGC1.0, while full length DLP was obtained from the Drosophila Genomics Resource Center (DGRC). Coding regions were amplified by PCR and inserted in pAc5.1 vectors (Invitrogen) modified for expression of N-terminally RGS-His-, HA- or GFP- tagged proteins. In the SUMO-KRKR chimera, *Drosophila* SUMO (aa 1 to 85) is fused to residue 18 of the p53 KRKR mutant. Expression of the fusion protein at the expected molecular weight was verified by immunoblotting (not shown). For luciferase assays, untagged wild-type p53 and K to R mutants were cloned in pAc5.1 vectors. All constructs involving PCR were fully sequenced. pRpr150-LUC was constructed inserting the 150 bp EcoRI-XhoI fragment from the pH150-LacZ reporter (Brodsky et al. 2000a) into the EcoRI-XhoI sites of the pGL2-promoter vector (Promega).

Cell culture, transfection and luciferase assays. S2 cells were cultured at 26°C in Schneider's *Drosophila* medium (Invitrogen) with 10% complemented fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 mg/ml). Transfections were performed by calcium phosphate co-precipitation. For luciferase assays, S2 cells in 3 cm petri dishes were transfected with 500 ng of the reporter, and 250 ng or 500 ng of p53 expression plasmids. In all samples, 100 ng of pPacLacZ were included for normalization of transfection efficiency. After 36 hours, cells were lysed and assayed for Luciferase and beta-galactosidase activity. Fold induction is the ratio of luciferase over beta-galactosidase, normalized to the activity of the reporter co-transfected with empty vector. Expression levels of transfected proteins were verified by immunoblotting of the same

lysates; gel loading was normalized for transfection efficiency using beta-galactosidase levels.

Western blotting, immuno-precipitation and immunofluorescence.

Immunoblotting was performed in standard conditions. For immunoprecipitations, S2 cells seeded in 6 cm Petri dishes were collected 24 hours after transfection and lysed in RIPA buffer (300mM NaCl) containing 10 mM N-ethylmaleimide, 1 mM PMSF, and protease inhibitors. Clarified lysates were incubated at 4°C with anti RGS-His primary antibody cross-linked to Protein G-Sepharose (GE Healthcare). For immunofluorescence, 36 hours after transfection S2 cells were plated on glass coverslips coated with 0.5 mg/ml Concanavalin A (Sigma) or 0.5 mg/ml poly-lysine (Sigma). After 2 hours, cells were washed with PBS and fixed in 4% paraformaldehyde at RT for 20 min. Cells were permeabilized in PBS plus 0.1% Triton X-100. Images were captured using a laser-scanning microscope (Zeiss Axiocam 100M). The following primary antibodies were used: mouse anti-RGS-His (Qiagen), rabbit anti-GFP (self produced), rabbit anti-SUMO (Long and Griffith 2000), mouse anti-HA (mAb 12CA5), mouse anti-*Drosophila* p53 (Brodsky et al. 2000a).

Electrophoretic mobility shift assay. For EMSA, approximately 5×10^6 S2 cells seeded in 6 cm petri dishes were transfected with p53 expression plasmids and harvested 48 hr after transfection in lysis buffer (10mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% NP40, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.5 mM PMSF, and protease

inhibitors). After 20 minutes on ice, extracts were centrifuged at 16000xg for 20 min at 4°C to remove cell debris. Protein concentration in supernatants was determined using Bio-Rad protein assay. Expression levels of transfected proteins were verified by immunoblotting of the same lysates. A 26-mer DNA oligonucleotide containing the cis-acting p53 responsive sequence from the *Reaper* enhancer (5'-ACCTGACATGTTTGAACAAGTCGAGC -3') was end-labeled with ³²P and annealed to the complementary strand. For binding reactions, 30 µg of whole cell extract were added to gel shift buffer (20 mM HEPES [pH 8], 25 mM KCl, 0.1 mM EDTA, 2 mM MgCl₂, 0.5 mM DTT, 0.025% NP-40, 2 mM spermidine, 10% glycerol, 0.1 mg/ml acetylated BSA, 120 ng double-stranded poly(d[I-C])) containing the labeled oligonucleotide in a final volume of 30 µl. Competition was done adding 500 ng of unlabeled double stranded oligonucleotide. Reactions were incubated for 30 min at RT, and electrophoresed on a non-denaturing 4% polyacrylamide gel before autoradiography.

Transgenes and genetics. Flies were raised at 25°C. Wild type *p53* and *p53*^{KRRK} were expressed using the GUS vector which contains both the UAS promoter for inducible expression by Gal4, and Glass binding sites from GMR for low to moderate expression in the developing eye (Brodsky et al. 2000a; Brodsky et al. 2004). Cloning of wild type p53 was previously described (Brodsky et al. 2004), while GUS*p53*^{KRRK} was constructed using Gateway cloning. For high levels of p53 expression, GMRGal4 animals were crossed to four independent lines of GUS*p53*⁺ and GUS*p53*^{KRRK}. For lower

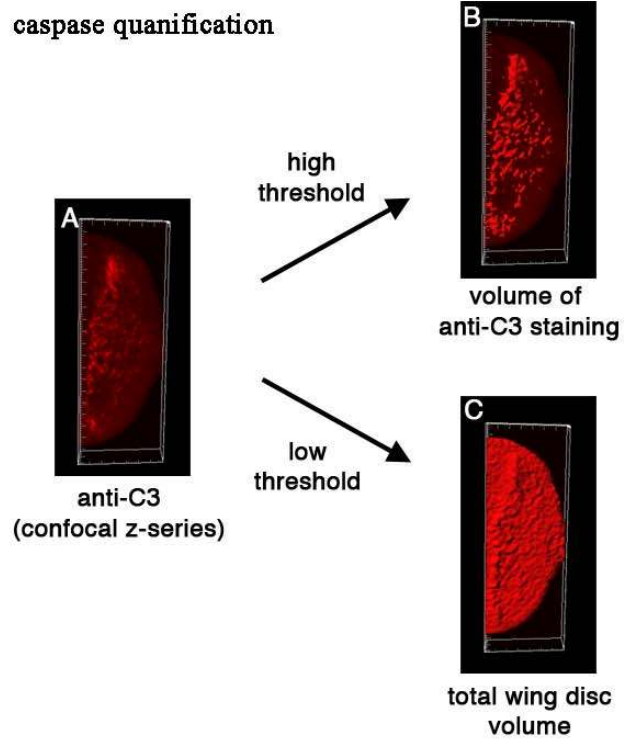
levels of expression and rescue of damage-induced apoptosis, $p53^-$ animals were crossed to $GUSp53^+$; $p53^-$ and $GUSp53^{KRKR}$; $p53^-$ flies.

Irradiation and immuno-histochemistry. Climbing third instar larvae were irradiated with 4000 rads using a faxitron X-ray cabinet or mock treated. Four hours following irradiation, eye discs were dissected and stained with antibodies as previously described (Brodsky et al. 2000a). Discs were incubated with primary antibodies in PBTN (PBS, 0.3% triton, 5% normal goat serum) overnight at 4°C, and with secondary antibodies in PBTN for two hours at room temperature. The primary antibodies used were rabbit anti-cleaved caspase-3 (1:100, Cell Signaling), rabbit anti-SUMO (1:1000, gift from L.C. Griffith) (Long and Griffith 2000), and mouse anti-*Drosophila* p53 (1:10) (Brodsky et al. 2000a). The secondary antibodies used were donkey anti-mouse Alexa 488 and donkey anti-rabbit Alexa 555 (1:2000, Molecular Probes). TUNEL staining was performed using ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon). Eye imaginal discs were fixed in 4% formaldehyde, washed 5 times with PBTw (PBS+ 0.3% Tween-20), and post fixed with cold ethanol/PBS (2:1). Following rehydration and washing, discs were treated with TdT mix for 1hr at 37°C. After the reaction was stopped, discs were incubated with Fluorescein-conjugated anti-dig antibody for 30 minutes and mounted with Vectashield.

Confocal microscopy and quantification of TUNEL and active caspase staining. Localization of p53 and SUMO was visualized using a Leica SP2 AOBS confocal

microscope with a 63X objective. For an overall view of the GMR region, a Z-series was taken through the eye disc at intervals of 284 nm, at a zoom of 1.75. For a higher magnification view, a Z-series was taken through the eye disc at intervals of 122 nm, at zoom of 4. To quantify the amount of cleaved caspase-3 in the eye disc, a Z-series was taken through the entire eye disc at intervals of 1.42 μm with a 20x objective. A 3-D reconstruction of each eye disc was generated using Imaris 5.0 image analysis software (Bitplane AG). Only the posterior region of the eye disc in which the p53 transgene is expressed was analyzed. The volume positive for cleaved caspase-3 was determined using a high intensity threshold, while the total disc volume was determined using a low intensity threshold (Fig. 2.9). The caspase-positive index for the posterior of the eye disc was calculated by dividing the cleaved caspase-3 volume by the total volume. To quantify the number and location of TUNEL positive cells in the eye disc, a Z-series was taken through the entire eye using a Zeiss Axioplan2 microscope and an Hamamatsu ORCA-ER camera with a 20x objective. Images were deconvolved using an inverse filter algorithm in the Zeiss Axiovision 4.5 image analysis software. Imaris 5.0 image analysis software was used to create a 3-D reconstruction of the TUNEL staining in the eye disc. Individual positive cells were marked using the “spot” function, which identifies local maxima of signal intensity (Fig. 2.9). The distance of TUNEL positive cells from the morphogenetic furrow was determined by subtracting the position of the furrow from the position of each cell.

Figure 2.9



TUNEL analysis

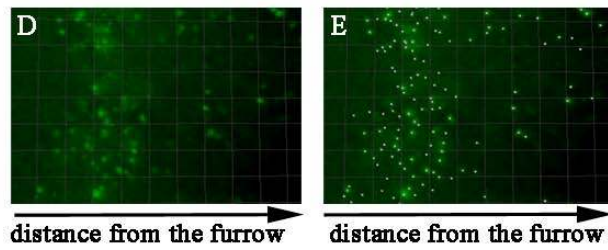


Figure 2.9. Quantification of apoptosis in developing eye discs. In all panels, anterior is to the left (on the X-axis), dorsal is at the top (on the Y-axis) and the disc is viewed looking down on the apical surface (along the Z-axis). (A-C) Activated caspase-3 quantification assay. (A) An X-Y view of a confocal Z-series from an eye imaginal disc treated with ionizing radiation and stained with anti-cleaved caspase-3. Eye imaginal disc images were cropped at morphogenic furrow. (B) Isosurface of objects with signal above a high intensity threshold (only anti-cleaved caspase-3 stained cells). (C) Isosurface of object with signal above a low intensity threshold (entire disc). Percent volume was calculated by dividing the volume of isosurfaces at high intensity threshold by the volume of the isosurface at low intensity threshold, multiplied by 100. For each sample, 5 eye imaginal discs were analyzed. (D-E) TUNEL analysis. Eye imaginal discs were cropped at morphogenic furrow. A small area in the middle of the eye disc (120 μ m wide by 275 μ m long) was analyzed. (D) An X-Y view of a confocal Z-series of eye imaginal disc overexpressing p53+. (E) TUNEL positive cells were marked using the spot function. Distribution profiles of TUNEL positive cells were constructed for 5 imaginal wing discs of each genotype and averaged together.

CHAPTER III

p53-INDEPENDENT APOPTOSIS LIMITS DNA DAMAGE-INDUCED

ANEUPLOIDY

Foreword

The work presented in this chapter has been submitted to Genetics. Laura McNamee and Michael Brodsky are the authors for the data presented.

Introduction

Eukaryotic cells employ diverse mechanisms to preserve the structure and function of their genome following chromosome damage. Unicellular organisms rely on multiple DNA repair systems and cell cycle arrest to prevent propagation of genome damage, while multi-cellular organisms additionally activate programmed cell death pathways to eliminate cells following damage (Sancar et al. 2004; Roos and Kaina 2006). In response to double strand DNA breaks (DSBs), the MRN complex (Mre11, Rad50 and Nbs1) process the damage and activate the related ATM and ATR kinases. ATM and ATR phosphorylate many substrates including repair proteins and the downstream kinases Chk1 and Chk2, which regulate cell cycle arrest and apoptosis. The p53 transcription factor plays an evolutionarily conserved role connecting the DNA damage signaling pathway to the core apoptotic machinery (Murray-Zmijewski et al. 2006).

Direct phosphorylation of mammalian p53 by ATM and Chk2 activates many target genes including p21, which regulates cell cycle arrest, and pro-apoptotic Bcl-2 family members (Roos and Kaina 2006). The p53 homologs p63 and p73 also contribute to p53-dependent apoptosis by helping p53 bind NOXA and bax promoters in some, but not all cell types (Flores et al. 2002; Senoo et al. 2004).

Approximately 50% of all human cancer cells lack functional p53, therefore reducing their susceptibility to induction of apoptosis by therapeutic agents that cause DNA damage. One mechanism to activate p53-independent apoptosis is the activation of p73 by the c-abl, Chk1 and Chk2 kinases following DNA damage (Yuan et al. 1999; Irwin et al. 2003; Urist et al. 2004; Ozaki and Nakagawara 2005)}. Although Chk1 and Chk2 are implicated in activation of apoptosis via p73 and E2F1 (Roos and Kaina 2006), other studies suggest that Chk1-mediated cell cycle arrest acts to promote survival following damage of p53 mutant cells by preventing cell cycle progression in the presence of unrepaired DNA damage. In one recent study ATM, ATR, Chk1 and p38MAPK/MK2 were associated with cell cycle delay and inhibition of this response resulting in caspase-3 activation and mitotic catastrophe (Reinhardt et al. 2007). In another study using zebrafish embryos and mammalian cells that lack both p53 and Chk1 function, ATM and ATR are required to activate an unusual apoptotic response requiring caspase-2, but not caspase-9 or caspase-3 (Sidi et al. 2008).

In *Drosophila*, G2 arrest following IR requires the ATR, ATRIP and Chk1 homologs (Mei-41, Mus304 and Grp, respectively) (Brodsky et al. 2000b; Brodsky et al. 2004; de Vries et al. 2005), while induction of apoptosis requires ATM, Chk2 (Tefu and

Mnk) and p53 (Brodsky et al. 2000a; Ollmann et al. 2000; Peters et al. 2002; Brodsky et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). p53 activates expression of several pro-apoptotic genes following IR, including *reaper (rpr)*, *hid*, and *sickle (skl)* (Brodsky et al. 2000a; Sogame et al. 2003; Brodsky et al. 2004). *hid* is essential for the rapid induction of apoptosis and *rpr* plays a smaller role (Brodsky et al. 2004; Moon et al. 2008). *rpr* and *hid* encode proteins that induce apoptosis by directly binding and inhibiting DIAP1 (*Drosophila* Inhibitor of Apoptosis Protein 1), leading to activation of an initiator caspase, Dronc, and two effector caspases, Drice and Dcp-1 (Chew et al. 2004; Daish et al. 2004; Waldhuber et al. 2005; Kondo et al. 2006; Xu et al. 2006). In addition, Hid and Rpr activity at mitochondria induces changes in mitochondrial ultrastructure and is required for optimal cell death after DNA damage (Holley et al. 2002; Olson et al. 2003; Abdelwahid et al. 2007; Goyal et al. 2007).

The JNK signaling pathway helps regulate apoptosis in response to DNA damage and in response to other cellular stresses in *Drosophila*. Following IR, JNK signaling is activated in a p53-dependent manner and inhibition of JNK signaling reduces caspase activation (McEwen and Peifer 2005). Following UV irradiation, JNK signaling is required for apoptosis, but p53 is not and instead appears to help repair UV damage (Jassim et al. 2003; Luo et al. 2007). JNK-dependent apoptosis can also act to eliminate cells with inappropriate gene expression during development. For example, the JNK pathway is activated in the developing *Drosophila* wing in response to abnormal activity of the morphogens Dpp or Wg (Adachi-Yamada et al. 1999; Adachi-Yamada and O'Connor 2002). In another example, clones of cells heterozygous for mutations in

ribosomal protein genes (called *Minutes*) are eliminated by JNK-dependent apoptosis (Morata and Ripoll 1975; Moreno et al. 2002a). Similar to the response to IR, activation of the JNK pathway by UV or cell competition activates the pro-apoptotic gene, *hid* (de la Cova et al. 2004; Carter et al. 2007). However, IR is the only activator of JNK signaling known to require *Drosophila* p53.

Although rapid induction of apoptosis following IR requires *Drosophila* p53, there is also evidence for a p53-independent response to chromosome damage. The high levels of spontaneous apoptosis associated with loss of telomere protection are only partly suppressed by loss of p53 (Oikemus et al. 2004; Oikemus et al. 2006). Similarly, while the early apoptotic response to IR is p53-dependent, lower levels of p53-independent apoptosis are observed at later time points (Wichmann et al. 2006). In both cases, p53-independent apoptosis is associated with caspase activation, but the signaling pathways that act upstream of caspases have not been described. Additional p53 family members cannot contribute to this reduced or delayed apoptotic response since *Drosophila* only has a single p53 homolog.

Here, we examine the regulation and function of p53-independent apoptosis following IR. We show that p53-independent apoptosis requires *hid*, which is a target of IR-induced JNK signaling. Mutations in *grp* or *puc* sensitize *p53* mutant cells to IR-induced apoptosis, suggesting that cell cycle progression and JNK signaling are critical for this response. We find that this p53-independent apoptosis acts to maintain genomic stability by reducing the number of IR-induced aneuploid adult cells exhibiting the *Minute* phenotype. Based on the results from this study and others, we propose a model

in which some cells undergoing p53-independent apoptosis are eliminated by this mechanism.

Results

p53-independent apoptosis requires the pro-apoptotic genes *hid* and *dronc*. Apoptosis was examined following irradiation of third instar *Drosophila* imaginal wing discs. For all experiments in this study, at least 5 discs were examined for every genotype and time point shown. Untreated wild type or *p53* mutant wing discs exhibit very low levels of spontaneous apoptosis. Four hours after irradiation with 4000 rads of X-rays, there is a robust induction of apoptosis, as assayed by acridine orange and cleaved caspase-3 staining in wild type discs (anti-C3)(Fig. 3.1A, Fig. 3.2A, and Fig. 3.3A). *p53* or *mnk* mutant discs show no response at 4 hours, but a significant increase between 16 and 24 hours (Fig. 3.1B and F; Fig. 3.3B-C), confirming previously described results (Wichmann et al. 2006). This study also indicated that one or more of the pro-apoptotic genes in the genomic region defined by the *Df(3L)H99* deletion is likely to contribute to p53-independent apoptosis. We tested the effect of loss-of-function mutations in one of these genes, *hid*. *hid* single mutant wing discs fail to induce apoptosis at early time points (4 to 8 hours) and have reduced apoptosis compared to wild type at later time points (16 to 24 hours; Fig. 3.1A and C; Fig. 3.3A and F), confirming that *hid*, a p53 target gene, is required for p53-dependent apoptosis at early time points (Brodsky et al. 2004). At 24

hours following IR, *hid*, *p53* double mutant discs exhibit a significant decrease in apoptosis (Fig. 3.1B-D). Quantification of anti-C3 staining in a *hid*, *p53* mutant showed a 7 and 11-fold reduction in the anti-C3 staining compared to a *p53* or *hid* single mutant discs, respectively, demonstrating that *hid* is required for p53-independent apoptosis (Fig. 3.1E-F).

The requirement for *hid* function and induction of cleaved caspase-3 staining suggests that the caspase proteins acting in the core apoptotic machinery in *Drosophila* would regulate p53-independent apoptosis. Wing discs mutant for the *Drosophila* caspase-9 homolog *dronc* suppress most or all IR-induced acridine orange and anti-C3 staining at both early and late time points (Fig. 3.2B and Fig. 3.3D), indicating that *dronc* is required for both p53-dependent and p53-independent apoptosis. Similarly, in discs expressing the anti-apoptotic baculovirus protein p35, which binds and inhibits the partially redundant effector caspases, Dcp-1 and Drice (Xue and Horvitz 1995; Yoo et al. 2002; Kondo et al. 2006; Xu et al. 2006; Baum et al. 2007; Lannan et al. 2007), no acridine orange staining was observed at any time point (Fig. 3.3E). Because p35 inhibits effector caspases following proteolytic cleavage, cleaved caspase-3 staining should still be observed (Yoo et al. 2002). To compare the pattern of anti-C3 staining between cells that do or do not express p35, p35 was specifically expressed in the posterior half of wild type or *p53* mutant wing discs. Following irradiation of these discs, anti-C3 staining is clearly induced in both halves, but the staining is more diffuse and slightly delayed in the p35-expressing region (Fig. 3.2C-D).

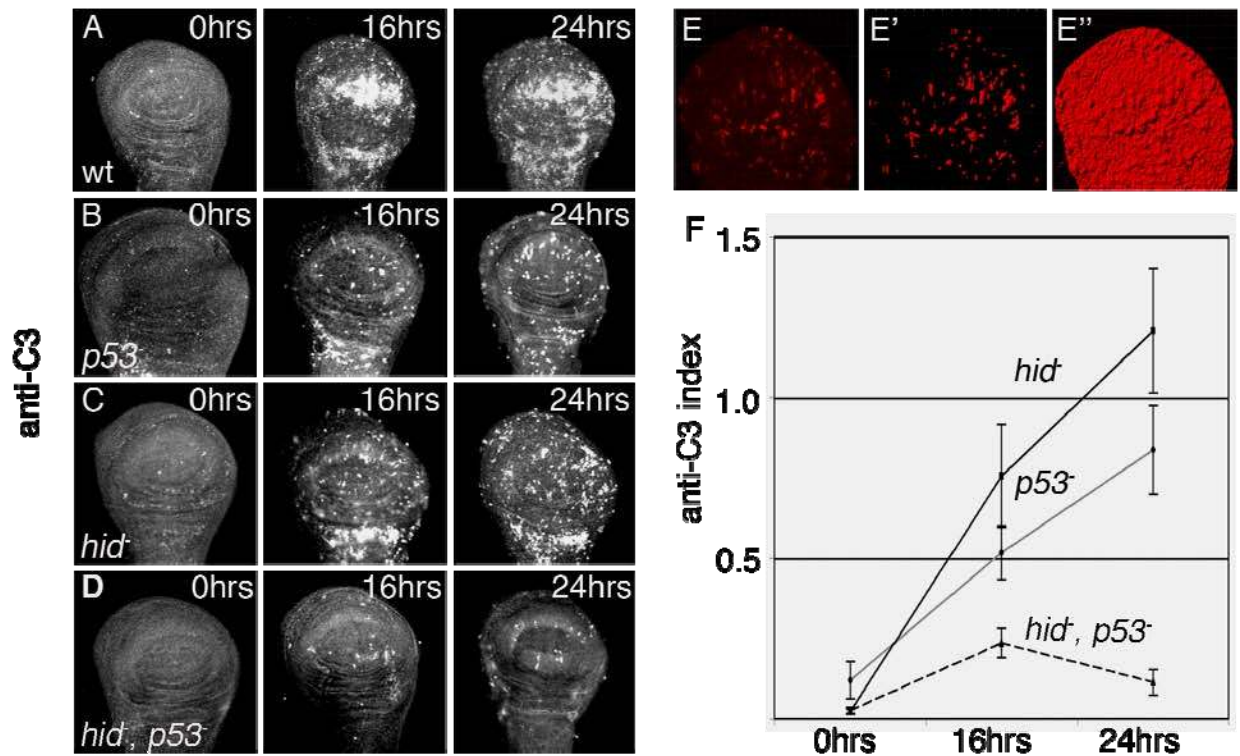


Figure 3.1. *hid* is required for p53-independent apoptosis. (A-D) Apoptosis was assayed in wild type (wt), *p53*, *hid*, and *p53*, *hid* mutant imaginal wing discs at different time points following ionization radiation (IR) by staining with an antibody to cleaved caspase 3 (anti-C3). (A) A large induction of apoptosis is observed from 4 to 16 hours (hrs) following DNA damage in wt wing discs. (B-D) Apoptosis is delayed and reduced in *p53* mutant wing discs compared to wild type. Apoptosis is induced in *p53* and *hid* single mutant wing discs, but not in *p53*, *hid* double mutant wing discs at 16 and 24 hours following IR. (E-E'') Quantification of anti-C3 staining (see methods) (E) A confocal z-series of a imaginal wing disc stained with anti-C3. (E') A high intensity threshold was used to create an isosurface for anti-C3 stained regions. (E'') A low intensity threshold

was used to create an isosurface for the entire disc. **(F)** The percent volume of anti-C3 staining was quantified at 0, 16, and 24 hours after treatment with IR. Anti-C3 staining does not increase in *hid, p53* double mutant discs. For all discs and data points shown, at least five discs were analyzed. Error bars indicate the standard error of the mean.

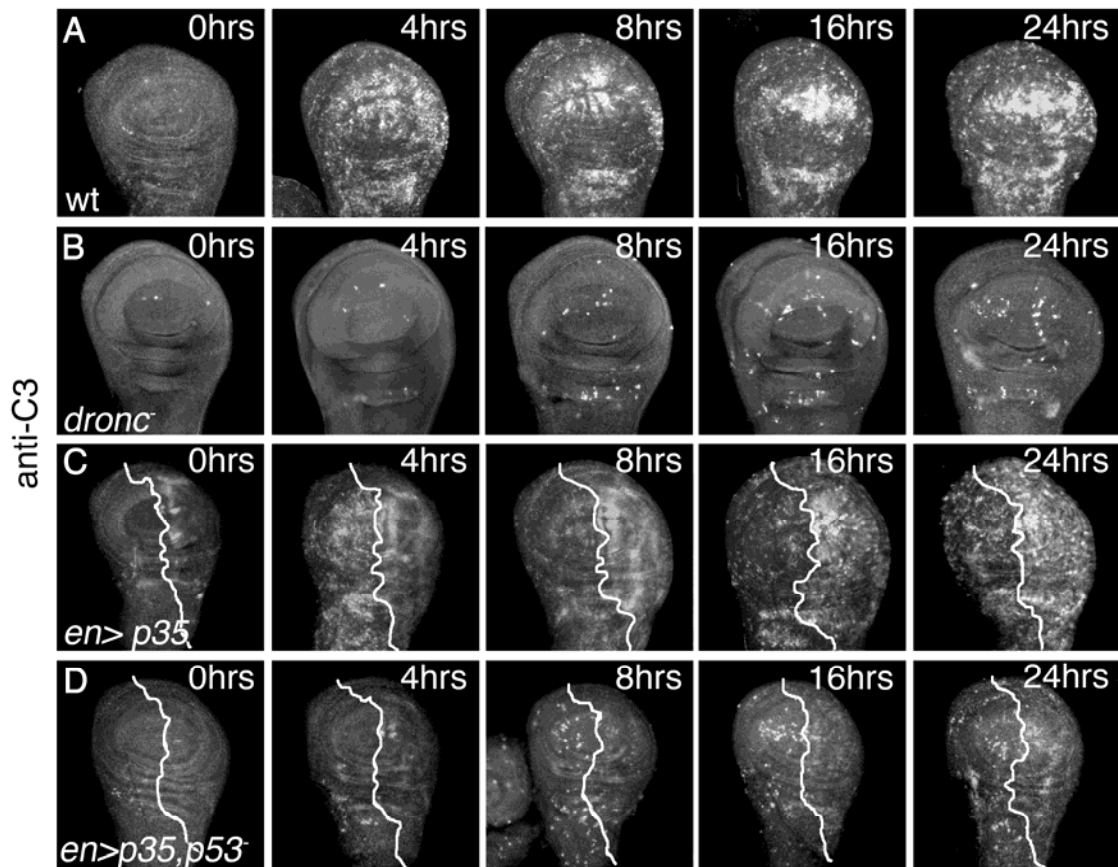


Figure 3.2. Basal and apical caspases are required for p53-independent apoptosis as detected by anti-C3. (A) p53-dependent apoptosis is observed 4 hours following IR. (B) Anti-C3 staining is not induced in wings discs mutant for the apical caspase *dronc*. (C and D) Expression of the baculovirus protein p35 (under control of the posterior-specific driver *en-gal4*) alters the timing and intensity of anti-C3 staining by blocking the activity of effector caspases following cleavage by apical caspases. In an otherwise wild type disc, the pattern of anti-C3 staining is initially altered following IR treatment and continues to accumulate over time. In a *p53* mutant wing disc, more diffuse anti-C3 staining accumulates by 24 hours following IR treatment.

Figure 3.3

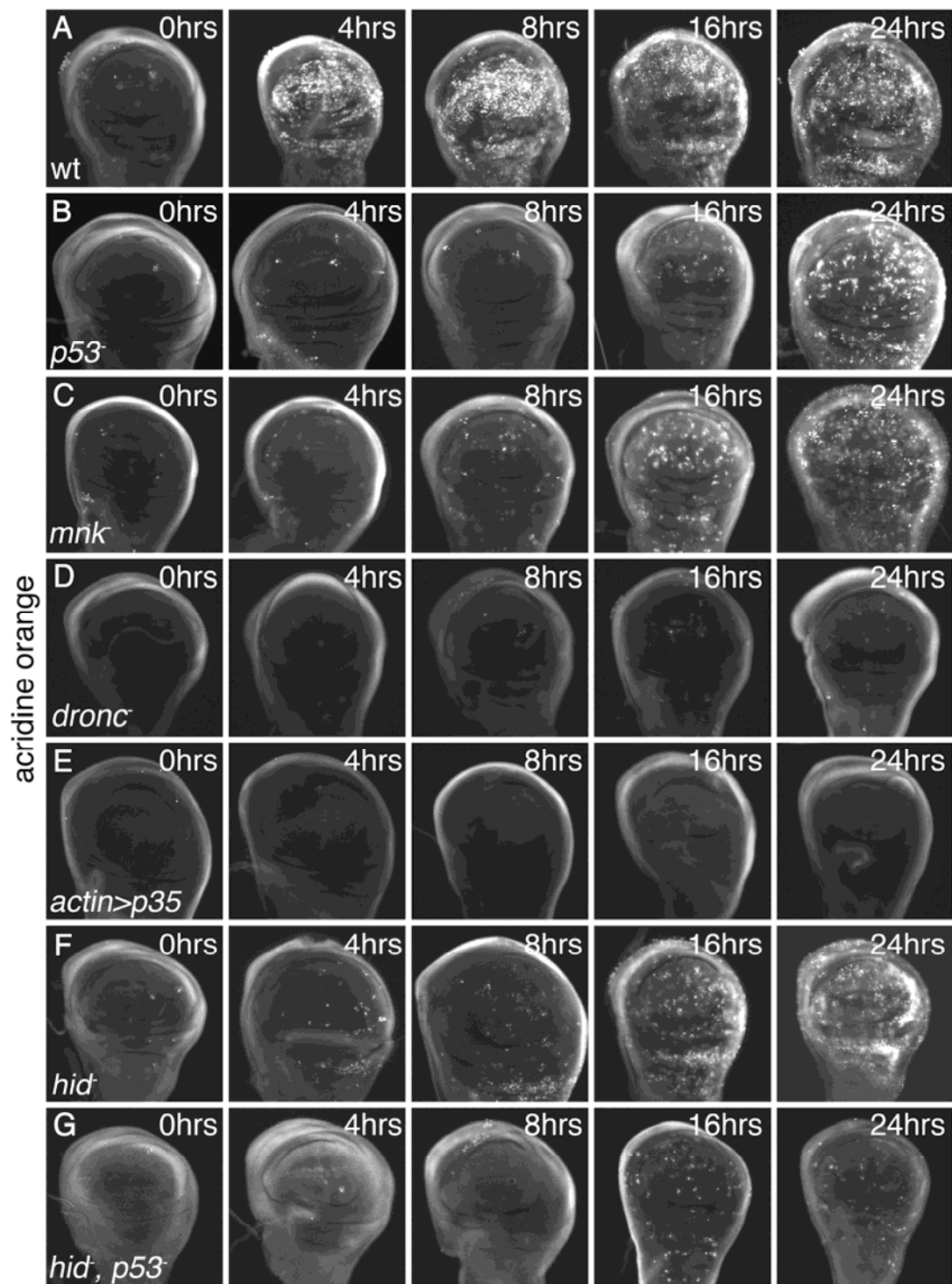


Figure 3.3. Basal and apical caspases and Hid are required for p53-independent apoptosis as detected by acridine orange. (A) p53-dependent apoptosis is observed four hours following IR. (B and C) p53- and Mnk-independent apoptosis begins between 8 and 16 hours following IR. (D and E) Acridine orange staining is not induced in wings discs mutant for the apical caspase *dronc* or expressing the baculovirus protein p35 (under control of the ubiquitous actin-Gal4 driver), which inhibits the activity of the effector caspases Dcp-1 and Drice. (F and G) The pro-apoptotic gene *hid* is required for p53-dependent apoptosis at early time points and for most p53-independent apoptosis at late time points.

Discs expressing p35 were also used to examine if Hid protein is induced during p53-independent apoptosis. p35 expression allows cells with induced *hid* gene expression to accumulate Hid protein without undergoing cell death. Induction of Hid expression is observed at 16 and 24 hours following irradiation of *p53* mutant cells expressing p35 (Fig. 3.4A, posterior cells). Overall, our analysis of *hid* and caspase function during p53-independent apoptosis indicates that this response to DNA damage utilizes the conventional *Drosophila* apoptotic machinery.

Regulation of p53-independent apoptosis by the JNK pathway. Since *hid* is a target of the JNK pathway in response to some other cellular stresses, the role of JNK signaling on Hid induction was examined. *puc* is a JNK target gene that encodes a JNK phosphatase; it normally acts in a negative feedback loop to limit JNK signaling and can be overexpressed to block JNK activity (Martin-Blanco et al. 1998; Moreno et al. 2002a; Kanda and Miura 2004; McEwen and Peifer 2005). Overexpression of *puc* reduced the induction of Hid in irradiated *p53* mutant discs (compare posterior compartments in Fig. 3.4A-B). *puc* overexpression in the posterior compartment of *p53* mutant discs also reduces anti-C3 staining following IR compared to the anterior compartment (Fig. 3.4C-D); the ratio of posterior to anterior anti-C3 staining is 1.4 in *p53* mutant discs and is reduced to 0.3 in *p53* mutant discs overexpressing *puc* in the posterior. These results indicate that JNK regulates induction of Hid following and is required for full induction of p53-independent apoptosis. It is not clear whether the remaining induction of apoptosis

in these discs is due to incomplete inhibition of JNK activity or some JNK-independent apoptosis.

To confirm that Hid is expressed in cells with elevated JNK activity during p53-independent apoptosis, expression of a *puc-lacZ* reporter, *puc^{A29}*, was examined in irradiated discs (Martin-Blanco et al. 1998; Kanda and Miura 2004). *p53* mutant cells with cytoplasmic expression of Hid 24 hours following irradiation also exhibit nuclear lacZ staining (Fig. 3.4E). We have confirmed the results of a previous study (McEwen and Peifer 2005) that induction of *puc-lacZ* 4 hours following IR is p53-dependent (data not shown), but find that *p53* mutant discs upregulate *puc-lacZ* 16-24 hours following irradiation (Fig. 3.5A). These results indicate that JNK activity is both induced and required during p53-independent apoptosis.

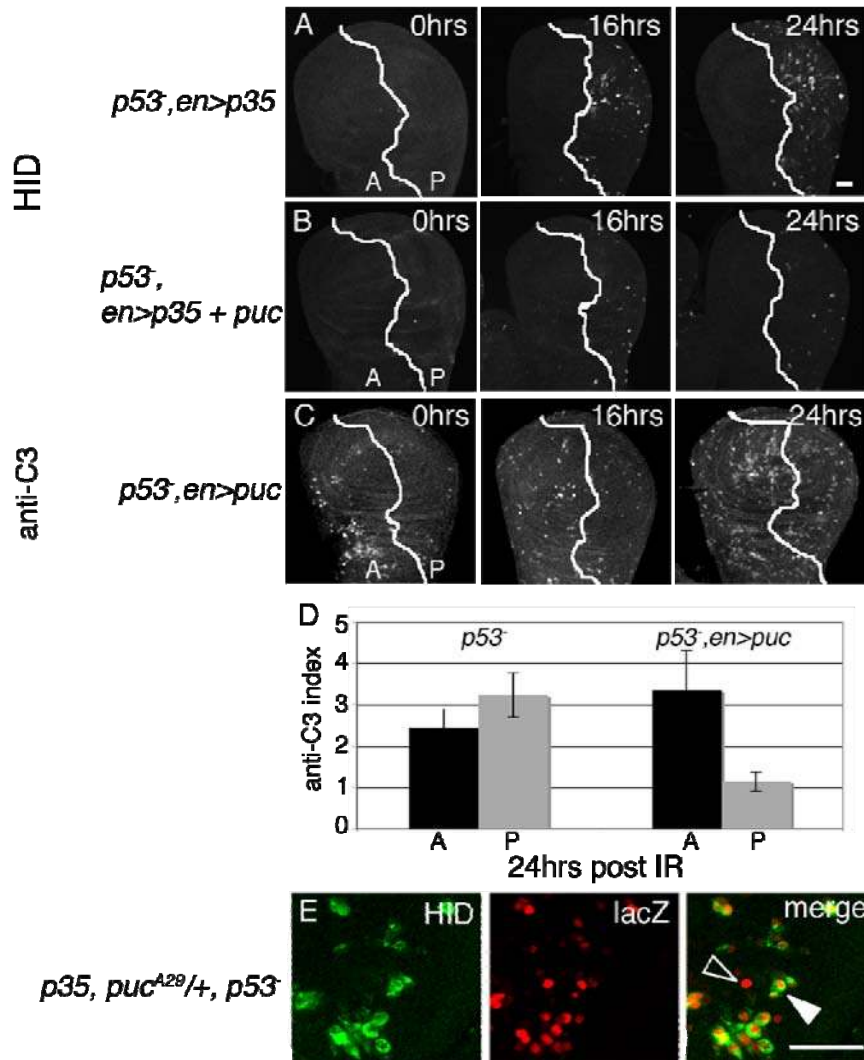


Figure 3.4. The JNK pathway regulates p53-independent induction of Hid and apoptosis following IR. (A) HID protein levels are increased following irradiation of *p53* mutant wing discs cells. The white line marks the anterior (left) /posterior (right) boundary. The baculovirus anti-apoptotic protein p35 is expressed in posterior cells using UAS-p35 and an *engrailed*-Gal4 driver. Scale bars = 50 μm. (B) Induction of HID protein by irradiation is blocked in *p53* mutant cells that express the JNK inhibitor *puc*.

(C) *puc* expression reduces induction of p53-independent apoptosis following IR. Apoptosis is assayed by anti-C3 staining. (D) Quantification of anti-C3 staining in *p53*- cells compared to *p53*- cells overexpressing *puc* in the posterior. For each genotype, levels of anti-C3 staining were quantified in the anterior (A) and posterior (P) in five discs. (E) At higher magnification, irradiation-induced HID (green) and Puc-lacZ (red) are expressed in overlapping cells in the posterior of *puclacZ/+*, *p53* mutant wings discs expressing p35. Most stained cells express both Puc-lacZ and Hid (filled arrowhead). Some cells express Puc-lacZ, but not Hid. Scale bars = 20 μ m

puc gene dosage is rate limiting for JNK activity; cells heterozygous for *puc* exhibit hyperactivation of the JNK pathway (Martin-Blanco et al. 1998). If the level of JNK activation determines the amount of p53-independent apoptosis, then *p53* mutant discs heterozygous for *puc* mutations should exhibit increased apoptosis. At 16 and 24 hours after IR, there is a 3 and 4-fold increase in anti-C3 staining in *p53* mutant discs that are also heterozygous for *puc* compared to *p53* single mutant discs (Fig. 3.5C-D, and G). Thus, the JNK pathway is also limiting for the full induction of p53-independent apoptosis following IR.

Drosophila Chk1 is a negative regulator of p53-independent apoptosis. Unlike p53-dependent apoptosis, the induction of p53-independent apoptosis following irradiation correlates with cell cycle progression. IR-induced G2 arrest in the wing disc lasts up to 8 hours and requires the *Drosophila* *Chk1* kinase homolog *grp* (Fogarty et al. 1997; Brodsky et al. 2000a), even in the absence of p53 function (Fig. 3.6C- D). While IR-induced apoptosis in wild type discs begins during G2 arrest (within 3-4 hours), apoptosis in *p53* mutant discs occurs following resumption of cell cycle progression. IR-induced apoptosis is significantly increased in *grp; p53* double mutant discs at 12 and 16 hours compared to *p53* single mutant discs (Fig. 3.5C, E, and H). Similarly, apoptosis is increased in *grp, mnk* double mutant wing discs compared to *mnk* single mutant discs (Fig. 3.7A, B, and E). Previous studies have established that, unlike several other cell cycle checkpoint genes, mutations in *mnk* and *grp* do not affect the frequency of chromosome breaks following IR (Jaklevic and Su 2004; Oikemus et al. 2006), indicating

that the increased apoptosis is due to a defect in cell cycle arrest, not simply an increase in unrepaired DNA breaks.

In cultured mammalian cells that lack both p53 and Chk1-dependent cell cycle arrest, DNA damage can induce a type of mitotic catastrophe in which the mitotic marker phosphohistone-3 and cleaved caspase-3 are present simultaneously in the dying cells (Reinhardt et al. 2007). However, in irradiated *p53* mutant discs, this analysis reveals two separate cell populations with the characteristic apical localization of mitotic cells marked with anti-phosphohistone-H3 and basal localization of apoptotic cells marked with anti-cleaved caspase-3 (Fig. 3.6E-F). These results indicate that apoptosis is not activated in cells that are actively progressing through mitosis.

Since the JNK and cell cycle arrest pathways both help regulate p53-independent apoptosis, the effect of *grp* function on induction of the JNK reporter *puc-lacZ* was examined. *puc-lacZ* expression is observed starting 16 hours after IR in *p53* mutant discs, but is present at higher levels beginning at 12 hours in *grp; p53* double mutant discs (Fig. 3.5A-B). The effect of both inactivating cell cycle arrest and reducing the level of negative feedback signaling by *puc* was examined in a *grp; puc/+; p53* triple mutant discs, which have a higher level of apoptosis than either *grp; p53* or *puc/+; p53* double mutant discs (Fig. 3.5D-F and I). Thus, while inactivation of cell cycle delay accelerates the formation of cells with increased JNK signaling, decreasing the negative feedback signal from *puc* can further increase the number of cells that undergo p53-independent apoptosis following IR.

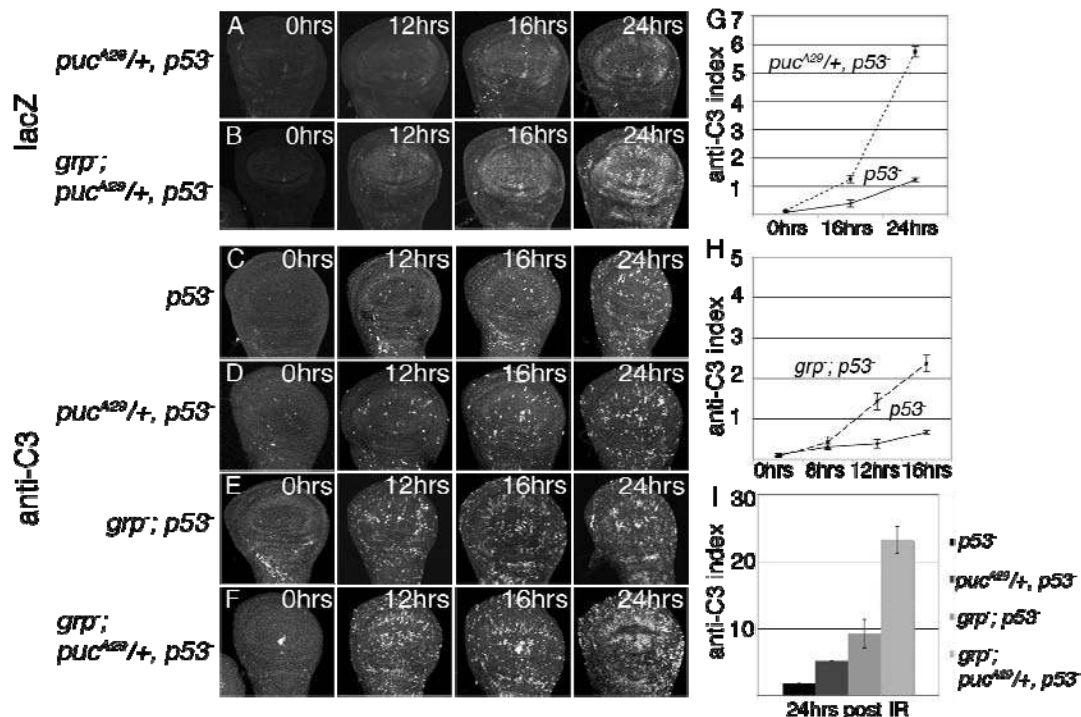


Figure 3.5. *grp* and *puc* limit *p53*-independent apoptosis following IR. (A and B)

lacZ expression is increased following irradiation of *puc^{A29}/+, p53* mutant wing discs.

puc^{A29} is an enhancer trap line in which the *lacZ* reporter is inserted in the *puc* locus.

Induction of *lacZ* is stronger and faster in *grp; puc^{A29}/+, p53* mutant discs, which are defective for cell cycle arrest following DNA damage. (C-F) Apoptosis was assayed using anti-C3. (C-E) *grp; p53* and *puc/+ , p53* mutant discs have increased apoptosis compared to *p53* single mutant discs. (F) Triple mutant *grp; puclacZ/+ , p53* mutant discs

have increased apoptosis compared to either *puc/+ , p53* or *grp; p53* double mutant discs. (G) Quantification of anti-C3 staining in *puc/+ , p53* compared to *p53* mutant discs. (H) Quantification of anti-C3 staining in *p53* and *grp; p53* mutant discs. (I) Quantification of anti-C3 staining in *p53* single mutant discs, *puc/+ , p53* or *grp; p53* double mutant discs and *grp; puclacZ/+ , p53* triple mutant discs. Apoptosis was examined 24 hours following

IR treatment. For each disc and data point, at least five wing discs were examined. Error bars indicate the standard error of the mean.

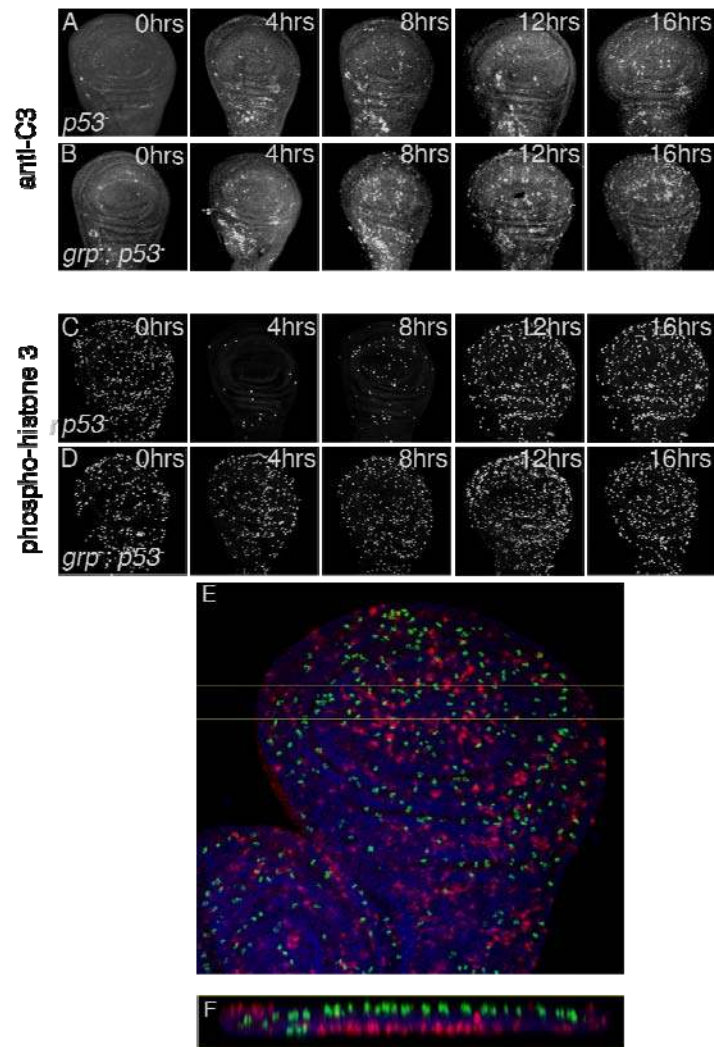


Figure 3.6. The checkpoint gene, *grp*, suppresses *p53*-independent apoptosis following irradiation. (A and B) Apoptosis was assayed in *p53* and *grp; p53* mutant imaginal wing discs at different time points following IR by staining with anti-C3. *grp; p53* double mutant discs have increased apoptosis compared to *p53* single mutant discs. (C and D) Developing wing discs were stained after treatment with IR with anti-phospho-Ser10 histone-3 (PH3) to detect mitotic cells. Mitotic entry is largely blocked at 4 and 8 hours following IR in *p53* single mutant wing discs, but not in *grp; p53* double

mutant discs. (E) X-Y view of a confocal z-series of a *grp; p53* mutant wing disc, 16hrs after IR, stained with anti-C3 (red), PH3 (green), and DAPI (blue). (F) X-Z cross-section of same wing disc viewed at position marked with yellow lines. Little or no overlap of apoptotic and mitotic cells is observed.

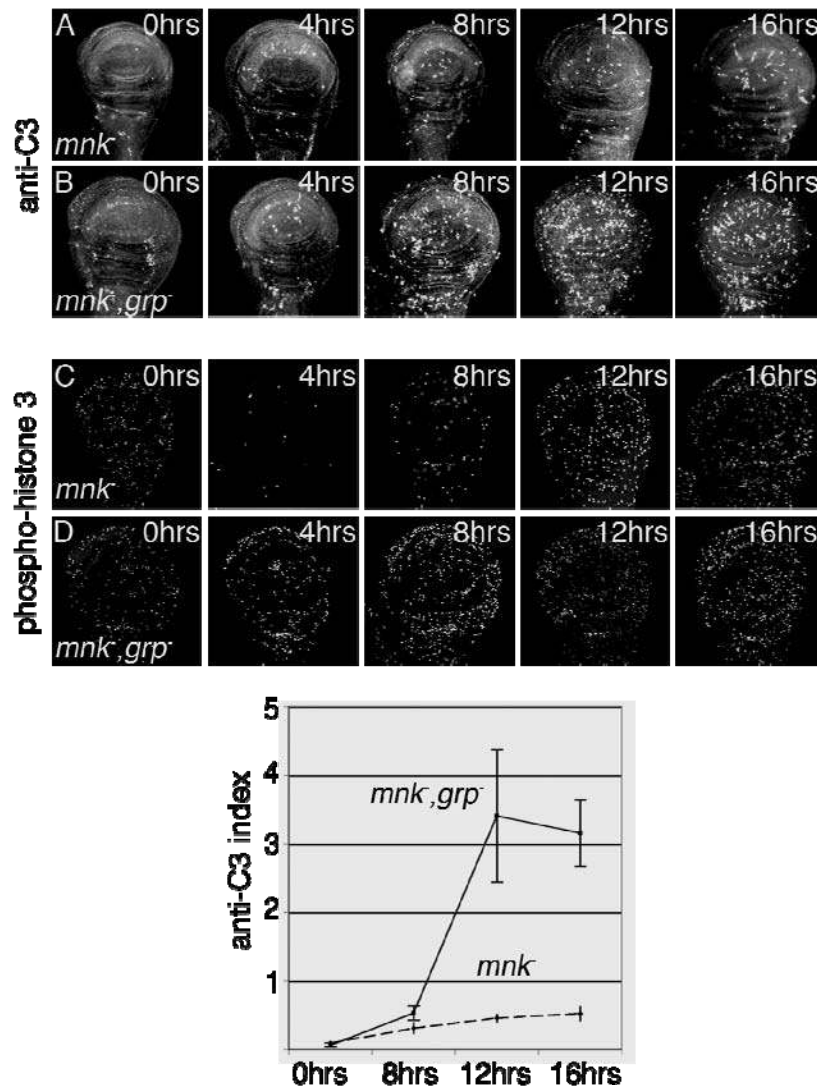


Figure 3.7. The checkpoint gene, *grp*, suppress Mnk-independent apoptosis following irradiation. (A and B) Apoptosis was assayed in *grp; mnk* and *mnk* mutant imaginal wing discs at different time points following ionization radiation (IR) by staining with anti-C3. *grp; mnk* double mutant discs have increased apoptosis compared to *mnk* single mutants. **(C and D)** Developing wing discs were stained after treatment with IR with anti-phospho-Ser10 histone-3 (PH3) to detect mitotic cells. Mitotic entry is

largely blocked at 4 and 8 hours following IR in *mnk* single mutant wing discs, but not in *grp; mnk* double mutant discs. (E) The percent volume of anti-C3 staining was quantified at 0, 8, 12, and 16hrs after treatment with IR. Double mutant discs lacking the cell cycle checkpoint gene *grp* have elevated irradiation-induced staining compared to single mutants. n = 5. Error bars indicate the standard error of the mean.

p53-independent apoptosis reduces the number aneuploid cells recovered following IR. Having established that JNK-dependent induction of HID regulates p53-independent apoptosis, the role of p53-dependent and p53-independent apoptosis in maintaining genomic integrity was examined using an assay based on haploinsufficiency of ribosomal protein genes. A previous study used loss of heterozygosity (LOH) at individual loci to characterize the genetic consequences of IR or mutation of DNA repair loci; most LOH was accompanied by loss of multiple genetic markers and by the *Minute* phenotype, due to haploinsufficiency of ribosomal protein genes (Baker et al. 1978). These results indicate that LOH was largely due to induction of segmental aneuploidy (loss of large chromosomal regions) rather than induction of point mutations. This conclusion is consistent with cytological data showing that a high frequency of cells treated with IR enter M-phase with chromosome rearrangements (Gatti et al. 1974) that can give rise to aneuploidy following completion of mitosis. To probe genetic loss across the entire genome, we score the frequency of *Minute* cells following IR. *Minute* genes make up approximately 65 different loci present throughout the euchromatic genome (Marygold et al. 2007), meaning that for most large chromosome regions, loss of one copy is likely to result in the *Minute* phenotype. Previous studies have confirmed that diverse methods of inducing chromosome damage induce this phenotype, including mutations in telomere protection genes, (Oikemus et al. 2004; Oikemus et al. 2006), mutations in DNA damage response and repair genes (Brodsky et al. 2000), telomeric loss due to dicentric chromosomes (Ahmad and Golic 1999), and induction of DNA breaks by P-element mobilization (Engels et al. 1987). Thus, it is likely that the induction of *Minute* bristles

following IR is due to chromosome damage rather than other types of cellular damage. It is possible that a small percentage of defective bristles are not due to induction of aneuploidy; these events would probably reflect other types of genetic damage.

Minute cells were scored in adults following X-ray treatment during larval development. If IR-induced apoptosis eliminates aneuploid cells, then blocking this apoptosis should increase the number of *Minute* cells that survive to adulthood. Because the X-ray dose used in our other experiments (4000 rads) is pupal lethal, a lower dose (1000) was used. Following irradiation of wild type larvae, approximately two percent of bristles exhibit the *Minute* phenotype (Fig. 3.8A and C). The bristle phenotype observed after IR is often more extreme than observed with the viable *Minute* mutations, suggesting the loss of very strong or haplo-lethal *Minutes* (Lindsley et al. 1972; Marygold et al. 2007). Other common bristle phenotypes associated with recessive or gain-of-function mutations in non-*Minute* loci, such as thicker or misshapen bristles, were not observed. No difference in the number of defective bristles was seen in *p53* mutant compared to wild type animals, indicating that p53-dependent apoptosis is not required to eliminate *Minute* cells following IR (Fig. 3.8C). However, when p53-independent apoptosis is also blocked, the frequency of IR-induced *Minute* bristles increased from two to five percent of all cells (Fig. 3.8B-C). These results indicate that in the absence of apoptosis, irradiation results in approximately five percent of all bristle precursor cells becoming aneuploid cells that will exhibit the *Minute* phenotype. p53-independent apoptosis acts to eliminate the majority (3/5ths) of these cells. If bristle precursor cells are representative of the approximately 50,000 cells in the developing wing disc, roughly

2500 (5% of 50,000) *Minute* cells would be induced by IR in one disc and roughly 1500 (3%) would be eliminated by apoptosis in wild type, but not *p53*, *hid* mutant wing discs.

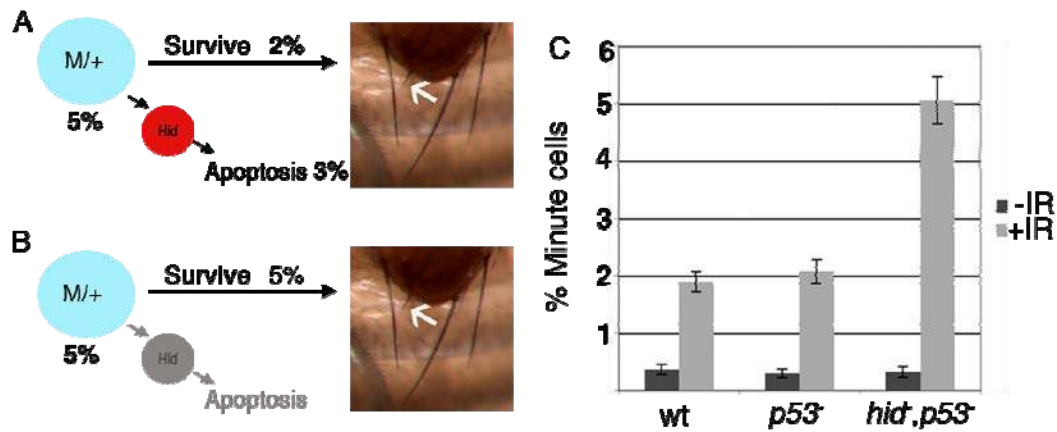


Figure 3.8. p53-independent apoptosis limits the accumulation of *Minute* cells following irradiation. (A-B) Aneuploid cells with the *Minute* phenotype are induced following irradiation of the developing wing. Some of these cells may be eliminated by *hid*-dependent apoptosis. Others survive and differentiate into adult cell types. Adult bristles form a stereotypic pattern on the notum of an untreated, wild type animal. Following irradiation, some adult bristles exhibit the *Minute* phenotype, shorter and thinner bristles. An example is indicated with an arrow. (C) The frequency of *Minute* bristles induced by IR is higher in *hid*, *p53* double mutant animals compared to wild type or *p53* single mutant animals. One hundred twenty-five animals were scored for each genotype in five independent experiments. Error bars indicate standard error of the mean.

Altered *brk* expression following IR. The observations that IR induces the formation of chromosome rearrangements in dividing larval cells (Gatti et al. 1974) and of adult aneuploid cells with the *Minute* phenotype (Fig. 3.8) suggests that the *Minute* phenotype may first appear in the irradiated larval tissue. Phenotypes associated with *Minute* cell clones induced in the developing wing include apoptosis (activated caspase-3), JNK pathway activation (assayed by *puc-lacZ*) and ectopic expression of the transcriptional repressor *brinker* (*brk*) due to reduced signaling by the *dpp* pathway (Moreno et al. 2002a; Tyler et al. 2007). As described above, we observe increased *puc-lacZ* and apoptosis following IR, but the JNK pathway is known to be activated by multiple cellular stresses. Because ectopic *brk* expression is sufficient to activate JNK-dependent apoptosis (Martin et al. 2004), this transcriptional change could contribute to the elimination of *Minute* cells (Moreno et al. 2002a). *brk* expression is normally repressed in the center of untreated wing discs and gradually increases toward the anterior and posterior edges of the disc (*brk-lacZ* in Fig. 3.9). At 16 and 24 hours following IR, individual cells in the center of the disc ectopically express *brk* and expression in the lateral regions becomes more irregular (Fig. 3.9A) and inhibition of apoptosis by p35 expression increases this effect. An average of 9 (s.e.m. = 2.6, n = 5) cells ectopically express high levels of *brk* in the center of the wing discs expressing p35, while an average 2.6 (s.e.m. = 1.7, n = 5) cells ectopically express *brk* without p35. Ectopic *brk* expression following IR was observed in both wild type and *p53* mutant discs, revealing that induction of the *Minute* mutant phenotype by IR can occur in parallel to p53-dependent apoptosis and is not a compensatory response to the absence of p53

(Fig. 3.9B-D). Although p53-dependent apoptosis is induced within 4 hour after IR treatment, ectopic expression of *brk* is not observed at this time point, even in wild type discs (data not shown), indicating that altered *brk* expression is not a secondary consequence of induced apoptosis. In contrast, *engrailed*, another gene with restricted expression in the developing wing disc, is not ectopically expressed following IR (data not shown), indicating that that ectopic expression of *brk* is not due to a genome-wide mis-regulation of genes with restricted expression patterns in the developing wing.

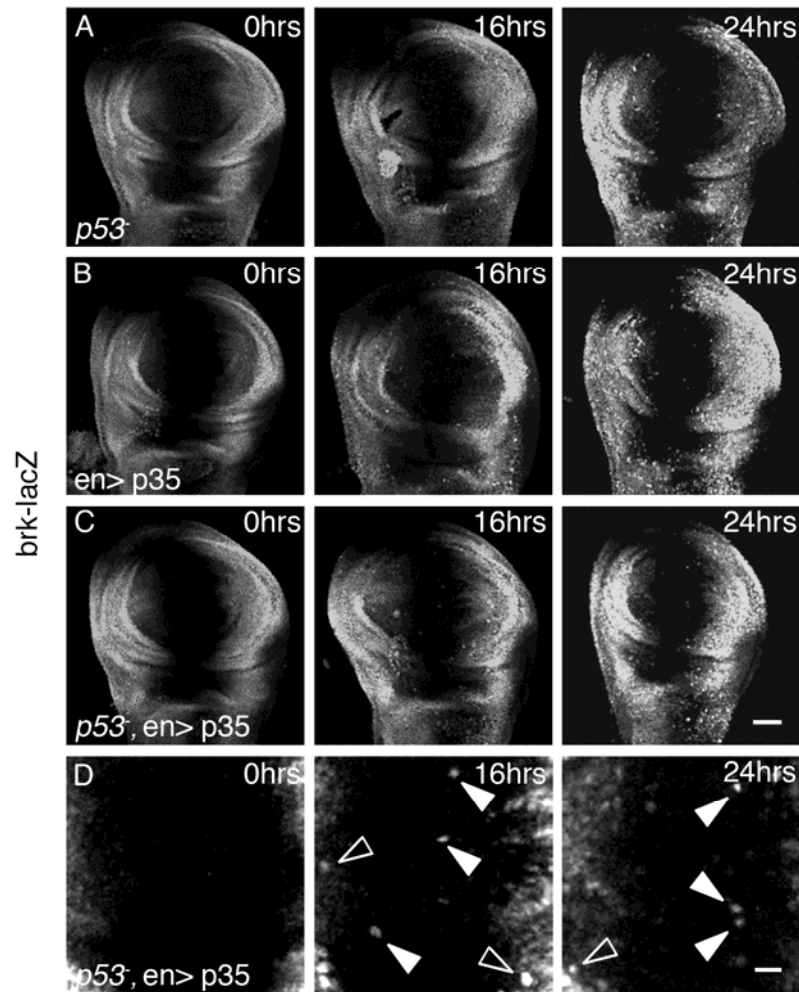


Figure 3.9. *brk* is ectopically expressed following irradiation. (A-D) A *lacZ* enhancer trap reporter inserted at the *brk* locus is used to monitor *brk* expression. Without IR, *brk* expressed at higher levels in the anterior (left) and posterior (right) regions of the developing wing and lower levels in the central region. (A-C) Following IR, altered *lacZ* expression is observed in *brk-lacZ/+; p53-* discs, *brk-lacZ/+;p53-* discs expressing p35 and *brk-lacZ/+* discs expressing p35. (A) Without p35, the *lacZ* expression pattern is uneven in the lateral regions of the disc. (B-C) With p35, ectopic expression of *brk* is

also observed in individual cells in the medial region of the developing disc near the anterior-posterior boundary. Scale bar = 50 μ m. **(D)** Higher magnification of the medial region of the wing disc. Imaginal discs treated with IR contain cells ectopically expressing *brk*. Medial disc cells that clearly express ectopic *brk* were counted. Filled arrows mark cells in the medial region that were scored as positive for ectopic *brk* expression. Outlined arrows mark medial cells with inappropriate levels of *lacZ* expression that were not scored because they are within or adjacent to the normal *brk* expression domain. Scale bars = 20 μ m. For each genotype 5 wing discs were scored.

Discussion

While p53 plays a conserved role in metazoans connecting the DNA damage response pathway to the core apoptotic machinery, delayed or reduced levels of apoptosis following chromosome damage are still observed in the absence of p53 function. Here, we show that p53-independent apoptosis in *Drosophila* requires the pro-apoptotic gene *hid*, the apical caspase, *Dronc* and effector caspases inhibited by the baculovirus protein p35. The JNK pathway is required and limiting for this response and acts upstream of HID protein expression. The cycle checkpoint gene *grp* and the JNK phosphatase *puckered* are both negative regulators of this response and mutations in these genes strongly sensitize *p53* mutant cells to IR. Induction of p53-independent apoptosis is required to limit accumulation of aneuploid cells following IR. Genetic data indicate that canonical DNA damage response pathways are not required for p53-independent apoptosis. We propose an alternative mechanism (Figure 3.10) in which either incorrect repair of chromosome breaks or loss of telomere protection generates cells with segmental aneuploidy and the haploinsufficiency of genes required for cell survival lead to apoptosis.

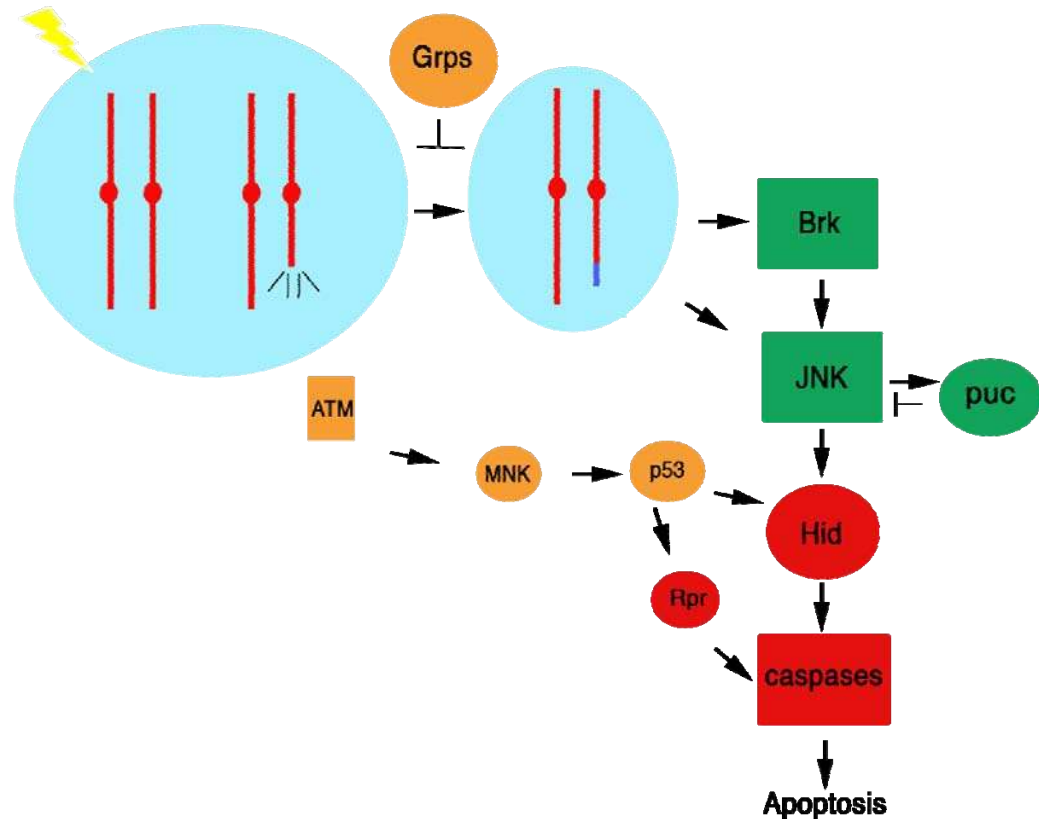


Figure 3.10. Model for p53-dependent and p53-independent apoptosis. In response to DNA damage, cells sense DNA breaks and activate repair, cell cycle delay and apoptosis. DNA damage detection by the ATM and ATR kinases activate p53 to induce apoptosis (orange boxes). p53 regulates the proapoptotic genes *hid* and *rpr*, resulting in caspase activation (red boxes). In parallel, *grp* mediates a cell cycle arrest allowing time for DNA repair. Incorrect repair of chromosome breaks followed by progression through mitosis will result in cells with reduced copy number of large chromosome regions (segmental aneuploidy). Haploinsufficiency of genes in these regions can induce apoptosis by ectopic *brk* expression, JNK pathway activation, and induction of *hid* expression (green boxes).

Several observations indicate that apoptosis can be induced following DNA damage or loss of telomere function without the activity of the central components of the well-characterized ATM/ATR/MRN DNA damage signaling pathways. In *Drosophila*, these pathways play critical roles in preventing telomere fusion. Loss of one or more components of these pathways leads to high levels of apoptosis, likely due to loss of telomere protection and frequent chromosome breaks (Sibon et al. 1999; Ciapponi et al. 2004; Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004; Ciapponi et al. 2006; Oikemus et al. 2006). In both *tefu* and *nbs* mutants, a significant percentage of this apoptosis is suppressed by mutations in *p53* or *mnk* (Oikemus et al. 2004; Oikemus et al. 2006), but the remaining cell death reveals an *mnk*- and *p53*-independent mechanism. It is possible that the components of the DNA damage signaling pathway mediate this response through other effector molecules. However, all combinations of double mutants in *tefu*, *mus304*, and *nbs* exhibit high levels of apoptosis, suggesting that the activation of apoptosis is independent of these molecules. In genetic backgrounds with normal telomere protection, IR can be used to induce chromosome breaks resulting in a rapid increase in apoptosis, which requires *mnk* and *p53* (Brodsky et al. 2000a; Jin et al. 2000; Ollmann et al. 2000; Xu et al. 2001; Sogame et al. 2003; Brodsky et al. 2004; Oikemus et al. 2004) and a delayed response to IR in the absence of *p53* or *mnk* (Wichmann et al. 2006)(this study). These results do not absolutely rule out a role for other, untested components of DNA damage response pathway in *p53*-independent apoptosis. However, it seems likely that the signal that activates this pathway, unprotected chromosome ends, has mostly been removed prior to the first appearance of *p53*-dependent apoptosis. The

activation of ATM and ATR kinases following IR (as measured by phospho-H2Av staining) largely subsides within a few hours of IR in embryos (Kusch et al. 2004) and discs (unpublished results), before we observe p53-independent apoptosis, JNK target gene expression, or induction of HID.

Not only are core components of the DNA damage response pathway not required for apoptosis following IR or telomere fusion, mutation of some of these genes can increase apoptosis. Many of these genes are required for repair of IR-induced breaks, which could provide a simple mechanism for increased apoptosis following IR (Hari et al. 1995; Brodsky et al. 2000b; Oikemus et al. 2004; Wichmann et al. 2006). Unlike some of the upstream components of the signaling response, *grp* is required for cell cycle arrest following IR, but cells mutant for *grp* or for *grp* and *mnk* do not show a decreased rate of chromosome break repair (Brodsky et al. 2000b; Jaklevic and Su 2004; Oikemus et al. 2006)(this study). *grp*, *p53* and *mnk*, *p53* double mutant discs exhibit accelerated activation of p53- and Mnk-independent apoptosis. The correlation of cell cycle progression following IR of single and double mutant discs with accelerated p53-independent apoptosis suggests that cell cycle delay is the critical role of *grp* in limiting p53-independent apoptosis. While it remains possible that *grp* regulates responses independent of its role in cell cycle progression, such responses have yet to be described in *Drosophila*.

grp function also regulates induction of JNK signaling. Following IR, expression of the JNK reporter gene *puc* is rapidly induced in a p53-dependent manner (McEwen and Peifer 2005). Similar to induction of apoptosis, we find that p53-

independent induction of *puc* by IR occurs after resumption of cell cycle progression and is accelerated by mutations in *grp*. Inhibition of JNK signaling by overexpression of *puc* substantially reduces p53-independent induction of HID protein and apoptosis, indicating that the majority of this response is JNK-dependent. The remaining apoptosis may reflect either incomplete inhibition of JNK signaling by *puc* overexpression or may suggest that an alternative pathway acts in parallel to JNK to promote a lower level of p53-independent apoptosis. Together, our data and previous studies supports the following sequence of events in *p53* mutant discs following IR: activation of the DNA damage response pathway by chromosome breaks leads to cell cycle arrest and DNA repair; following completion of DNA repair, a subset of cells that reenter the cell cycle activate the JNK pathway leading to apoptosis due to increased expression of *hid*.

The haploinsufficient phenotype of *Minute* genes was used to test the role of p53-dependent and p53-independent apoptosis on genomic stability. Because *Minute* genes have a common phenotype and are spread throughout the genome, this method provides a convenient functional assay for the most frequent source of genetic loss following IR, segmental aneuploidy. Using this assay, we examined the effect of blocking IR-induced apoptosis during development on the accumulation of aneuploid cells in the adult. The induction of p53-dependent apoptosis is often assumed to help preserve genome stability by eliminating cells with severe chromosome damage. Under our experimental conditions, we did not find evidence for this model, despite the very high levels of p53-dependent apoptosis that are induced following IR. It is possible that the importance of p53-dependent apoptosis in genome stability depends on the source of chromosome

damage. For example, repair of DNA breaks following IR can lead to restoration of normal chromosome structure or to chromosome rearrangements that lead to aneuploidy; induction of apoptosis by the canonical DNA damage response pathway will not be able to distinguish between these possible outcomes. However, nearly all breaks formed following loss of telomere protection are likely to lead to aneuploidy; any reduction in the number of cells with these breaks is likely to reduce the number of aneuploid cells.

In contrast to the effect of only blocking p53-dependent apoptosis, there is a significant increase in the number of aneuploid cells recovered when p53-independent apoptosis is also blocked. In our experiments, approximately two percent of cells are *Minute* following IR treatment of wild type or *p53* mutant cells, while this number increases to five percent for cells mutant for both *p53* and *hid*. The simplest interpretation of these results is that five percent of bristle precursor cells become aneuploid following IR and that p53-independent apoptosis eliminates two-thirds of these cells. However, the number of adult *Minute* bristles may not precisely correlate with number of aneuploid cells produced and eliminated if the cell division rates of these precursors is altered. Regardless, our results clearly demonstrate that p53-independent apoptosis reduced the eventual number of aneuploid bristle cells by two-thirds. While our analysis was performed in bristle cells, there is no particular reason to suspect that these cells are not representative of most *Drosophila* cell types; bristles were chosen for this analysis solely because the *Minute* phenotype can be used in these cells to score segmental aneuploidy.

A key unresolved question in this study is what signal activates p53-independent JNK activity and apoptosis. One attractive possibility is that aneuploidy itself is the

signal. Our analysis of adult *Minute* bristles suggests that a significant number of aneuploid cells generated following IR are eliminated by apoptosis. Unlike the small bristle cell phenotype in adults, a completely specific marker is not available to demonstrate that aneuploid cells are present in developing wing disc during p53-independent apoptosis. However, two changes in gene expression patterns associated with *Minute* cells in the wing disc, expression of the JNK target gene *puc* and ectopic expression of *brk* (Moreno et al. 2002a), are both observed following IR. Furthermore, previous cytological data has demonstrated that chromosome rearrangements likely to produce aneuploid cells following completion of mitosis are readily generated following similar doses of IR (Gatti et al. 1974). The correlation of p53-independent apoptosis and expression of *puc* and *brk* with cell cycle progression in discs with or without *grp* mutants is also consistent with a role for aneuploidy in p53-independent apoptosis. Two additional observations supported the hypothesis that aneuploidy induces p53-independent apoptosis. Previous studies have shown that haploinsufficiency of *Minute* genes is sufficient to induce apoptosis (Moreno et al. 2002a; Coelho et al. 2005). Importantly, induction of spontaneous apoptosis in *Minute* heterozygous animals does not require p53 (Figure 3.11), indicating that this mechanism would eventually be activated in aneuploid cells induced by IR, even in cells lacking p53 function. Nonetheless, it remains formally possible that some additional response to unrepaired chromosome damage could kill these cells before apoptosis due to aneuploidy could be activated. However, a recent study using targeted formation of dicentric chromosomes to induce p53-dependent and p53-independent apoptosis argues against this possibility; p53-

independent apoptosis is only observed if the resulting chromosome aberrations result in aneuploidy (Titen and Golic, submitted to Genetics). Thus, formation of chromosome breaks alone is insufficient to induce p53-independent apoptosis, indicating that generation of aneuploidy is required for this response.

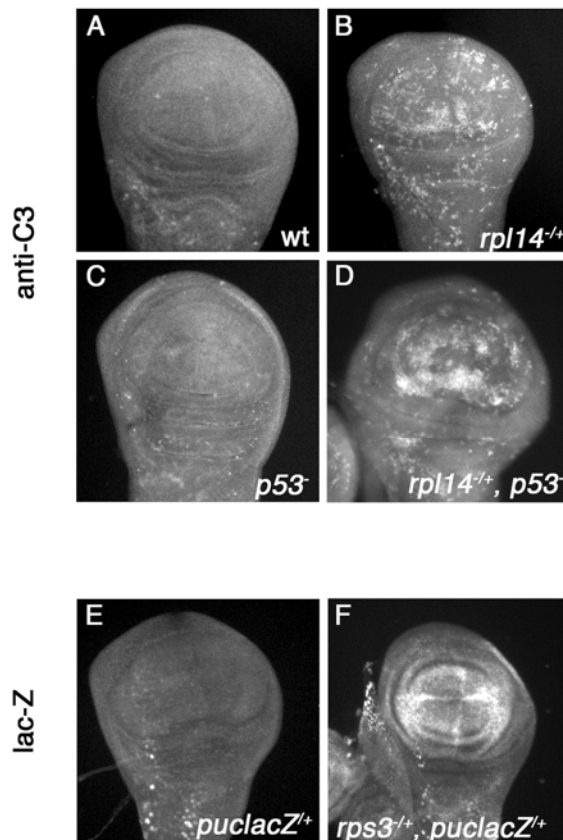


Figure 3.11. *Minute* imaginal wing discs exhibit increased levels of JNK-signaling and p53-independent apoptosis. (A-D) Imaginal wing discs stained with anti-C3. (A and C) wt and *p53*⁻ imaginal wing disc show no spontaneous apoptosis. (B and D) *rpl14*^{-/-} imaginal wing discs exhibit increased levels of spontaneous apoptosis that is not suppressed in a *p53*⁻ background. (E-F) Levels of lacZ reporter in *puc* locus are increased in *rps3*^{-/-} mutant.

Haploinsufficiency of *Minute* genes should be sufficient to induce apoptosis in aneuploid cells. *Minute* heterozygous flies show increased apoptosis and JNK activation in imaginal tissues, suggesting that these cells are normally under cellular stress (Coelho et al. 2005)(Figure 3.11). In addition to the spontaneous apoptosis observed in heterozygous *Minute* tissues, clonal populations of *Minutes* are actively eliminated by their surrounding wild type neighbors, a phenomenon called cell competition (Morata and Ripoll 1975; Moreno et al. 2002a; Li and Baker 2007; Tyler et al. 2007). These clones ectopically express *Brk*, which is sufficient to induce JNK-dependent apoptosis (Adachi-Yamada and O'Connor 2002; Moreno et al. 2002a). Aberrant expression of *brk* following IR should be sufficient to induce some apoptosis in response to aneuploidy. Extensive surveys of *Drosophila* chromosome aberrations indicate that *Minute* genes represent the primary source of loci that are haploinsufficient for normal growth and that all *Minute* loci are likely to encode either ribosomal proteins or translation initiation factors (Lindsley et al. 1972; Marygold et al. 2007). These observations do not rule out the possibility that the cumulative effect of reduced dosage for many genes in aneuploid cells could also disrupt normal gene expression and cell survival, but do indicate that induction of the *Minute* phenotype would be sufficient to induce apoptosis in aneuploid cells generated following chromosome damage.

Haploinsufficiency of ribosomal genes has the potential to contribute to damage-induced apoptosis in vertebrates as well. In mice heterozygous for mutations in ribosomal protein genes, there is evidence for induction of both p53-dependent and p53-independent signaling associated with increased apoptosis and reduced growth (Danilova et al. 2008;

McGowan et al. 2008) (Panic et al. 2006). Diamond Blackfan anemia (DBA) is linked to haploinsufficiency of at least four different human ribosomal protein genes (Draptchinskaia et al. 1999; Gazda et al. 2006; Gazda and Sieff 2006; Cmejla et al. 2007; Choesmel et al. 2008) and causes a congenital hypoplastic anemia in which patients exhibit low numbers of erythroid precursors in the bone marrow and increased apoptosis. (Flygare and Karlsson 2007). As in *Drosophila*, mammalian ribosomal protein genes are widely distributed throughout the genome. Thus, genetic or environmental changes resulting in aneuploidy should frequently induce the cellular responses associated with ribosomal protein gene haploinsufficiency.

In summary, we have identified a p53-independent pathway that limits the formation of aneuploid cells through JNK signaling and Hid-dependent apoptosis. We hypothesize that rather than detect damaged chromosomes directly, this mechanism is activated by the reduced dosage of critical genes, such as ribosomal protein genes, in aneuploid cells. Our data demonstrates that loss of this response results in an increase in visibly defective bristle cells following DNA damage. Thus, in *Drosophila*, this mechanism helps eliminate cells that should reduce the fitness of the adult animal. Although other cell types are not as dramatically altered, it is possible that many other cell types would exhibit less robust function due to reduced ribosomal protein synthesis. In humans and other animals at risk for malignancies, this mechanism could also act as an aneuploidy sensor and help reduce the proliferation of cells with chromosome aberrations.

Materials and Methods

Drosophila melanogaster genetics. All flies were raised at 25° C. Genes and alleles are described in www.flybase.org. *w¹¹¹⁸* was used as the wild type strain. The following mutant genotypes were used:

p53^l

mnk^{P6}

grps^{fs1}; p53^l

grps^{fs1}, mnk^{P6}.

actin-Gal4/+; UASp35/+

en-Gal4/+; UASp35

en-Gal4/+; UASp35, p53^l/+, p53^l

en-Gal4/+; UASpuc, p53^l/+, p53^l

en-Gal4/+; UASpuc, UASp35, p53^l/+,+, p53^l

en-Gal4/+; puc^{A29}lacZ, p53^l/UASp35, p53^l

puc^{A29}-lacZ/+; p53^l

grps^{fs1}; puc^{A29}-lacZ/+; p53^l

brk³⁸⁻²⁰-lacZ/+; p53^l

brk³⁸⁻²⁰-lacZ/+; en-Gal4/+; UASp35, p53^l/+, p53^l

brk³⁸⁻²⁰-lacZ/+; en-Gal4/+; UASp35/+

dronc^{I29}/dronc^{I24}

hid^{X14}/*hid*⁰⁵⁰¹⁴

hid^{X14}, *p53*¹/*hid*⁰⁵⁰¹⁴, *p53*¹

Analysis of X-irradiation induced changes in apoptosis, cell cycle, and gene expression.

Wandering third instar larvae were mock treated or X-irradiated with 4000 rads using a Faxitron RX650 X-ray cabinet system (Faxitron X-ray Corporation). For 12, 16 and 24 hour time points, larvae were mock treated or X-irradiated with 4000 rads as early third instar within *Drosophila* media. Wandering third instar larvae were collected for immunostaining.

Immunostaining and acridine orange staining was largely performed as described previously (Abrams et al. 1993; Brodsky et al. 2000b; Oikemus et al. 2004). Imaginal wing discs from wandering third instar larvae were dissected in 1xPBS and fixed in 4% formaldehyde for 30 minutes at room temperature. Samples were washed in 250µl of 1xPBS + 0.3% Triton-X100 5 times for 5 minutes each and incubated in blocking solution (1xPBS+0.3% Triton-X100+5% Normal Goat Serum) for 1hr. Samples were incubated in primary antibody diluted in blocking solution overnight at 4°C. The posterior expression domain in *en*-Gal4 experiments was marked by expression of endogenous En protein. The following dilutions were used for each antibody: rabbit anti-cleaved caspase-3 (Cell Signaling Technology) 1:100; mouse anti-phospho-H3 (Cell Signaling Technology) 1:500; mouse anti-beta-Gal (Santa Cruz Biotechnology, Inc.) 1:1000; mouse anti-En (Developmental Studies Hybridoma Bank at the University of Iowa) 1:10; guinea pig anti-Hid (Ryoo et al. 2004) (gift from Hyung Don Ryoo, N.Y.U.

Medical Center) 1:500. Following primary antibody incubation, samples were washed 5 times for 5 minutes each and incubated with secondary antibody in blocking solution for two hours at room temperature. The following secondary antibodies were used: anti-mouse Alexa 488 (Molecular Probes) 1:2000, anti-rabbit Alexa 555 (Molecular Probes) 1:2000, anti-guinea pig Alexa 488 (Molecular Probes) 1:2000, anti-mouse FITC (Jackson labs, for staining with anti-phospho-histone H3 primary) 1:250, and anti-mouse Cy5 (Jackson labs) 1:250. Imaginal wing discs were stained with DAPI (4',6-diamidino-2-phenylindole) and mounted in Vectashield (Vector Laboratories). Wide field images of imaginal wing disc were acquired using a Zeiss Axioplan imaging fluorescence microscope equipped with an ORCA-ER digital camera (Hamamatsu) and Axiovision 4.5 software. Confocal images were acquired with a Leica TCS SP2 confocal microscope. For each genotype and time point at least 10 wing discs were scored.

Confocal microscopy and quantitative analysis of cleaved-caspase staining. To quantify the amount of cleaved caspase-3 in imaginal wing discs, a z-series was taken through the entire disc at intervals of 1.42 μm with a 20X objective. A 3-D reconstruction of the wing disc was obtained using Imaris and Imaris Measurement Pro image analysis software (version 5.0.1, x64, Bitplane). A high intensity threshold was used to create isosurfaces of regions that stained positive for cleaved caspase-3. A low intensity threshold was used to create an isosurface of the background staining observed throughout the entire disc. Examples of raw images, low intensity isosurfaces and high intensity isosurfaces are shown in Figure 1E. Percent positive for cleaved caspase-3 was

calculated as the total volume of high intensity isosurfaces divided by volume of the low intensity isosurface. For each genotype and time point at least 5 wings were scored.

Minute assay. Newly laid eggs were collected for 48 hours. Seven days after beginning egg collections, larvae in *Drosophila* media dispersed on a petri dish were X-irradiated with 1000 rads or mock treated and then transferred to new vials. Wandering third instar larvae were picked out of the vials over the next 48 hours. One week later, adults were scored for the number of bristles each contained. For each genotype and treatment at least 125 flies were scored. Data from 5 different irradiation experiments were pooled.

CHAPTER IV
DISCUSSION

This chapter will discuss the experimental design and caveats to the experiments in chapters II and III. The conclusions of the experiments, as well as the contributions and future directions of the field will also be discussed.

Sumoylation of *Drosophila* p53

Apoptosis is an irreversible process and therefore needs to be tightly controlled. Because p53 is an important mediator of apoptosis, it also is tightly regulated through extensive post-translational modifications. Although the functional significance of these modifications *in vivo* is not well understood, these modifications are thought to regulate the activity of p53. Mammalian p53 is modified by SUMO on the C-terminal domain, however the functional consequence of this modification is unclear. Cell culture studies have shown that sumoylation of mammalian p53 can increase, decrease, or not change the transcriptional activity of p53. In Chapter II, we show that sumoylation of p53 is conserved from *Drosophila* to mammals. In contrast to mammals, *Drosophila* p53 is sumoylated on two sites, one N-terminal and one C-terminal. In *Drosophila* S2 cells, mutation of both sumoylation sites reduces the transcriptional activity of p53. A key finding in this study is that the two sumoylation sites of *Drosophila* p53 are required for the efficient induction of damage-induced apoptosis in the eye imaginal disc. This result is the first *in vivo* evidence that the sumoylation sites of p53 are required for optimal p53 function and its ability to induce apoptosis.

Functional Significance of *Drosophila* p53 at Nuclear Bodies. In mammals, the promyelocytic leukaemia (PML) tumor suppressor protein acts as a scaffold protein to properly assemble PML-nuclear bodies. PML-nuclear bodies contain many proteins including p53, and have been implicated in a variety of cellular functions including apoptosis (Takahashi et al. 2004; Bernardi and Pandolfi 2007). In our study, we found that *Drosophila* p53 localizes to nuclear bodies that are similar to mammalian PML bodies. Possible functions of *Drosophila* p53 at nuclear bodies are discussed below.

Like mammals, *Drosophila* p53 forms nuclear dots that colocalize with human PMLIV, SUMO, and the *Drosophila* homolog of Daxx (DLP). In mammals, PML is considered the organizer of nuclear bodies (Takahashi et al. 2004; Bernardi and Pandolfi 2007). Although flies do not have a PML homolog, the fact that p53, SUMO, DLP, and human PML IV all colocalize to nuclear bodies suggests that some aspects of these nuclear bodies are conserved. Mammalian p53 is recruited to PML bodies, regardless of its sumoylation status. In contrast to mammals, our experiments showed that localization of *Drosophila* p53 to nuclear bodies does appear to depend on sumoylation. It is tempting to speculate that in the absence of a PML homolog, sumoylation is required to localize p53 to these nuclear bodies. However it is difficult to make these conclusions because both SUMO and p53 were highly overexpressed in our experiments.

A key unresolved question in this study is what is the function of p53 in these nuclear bodies. In mammals, studies have shown that many proteins that are sumoylated are found in PML bodies. In general, sumoylation does not appear to be required for recruitment to PML bodies, rather sumoylation may be a consequence of localizing to

nuclear bodies (Takahashi et al. 2004). There is also evidence to suggest that other post-translational modifications of p53 take place in PML bodies. In response to DNA damage, Chk2 is recruited to PML bodies, where it enhances phosphorylation of p53, resulting in increased protein stability (Yang et al. 2002). Other studies have shown that in response to oncogenic stress, p53 and the acetyltransferase CBP are recruited to PML bodies, which is required for the efficient acetylation of p53, leading to an increase in its transcriptional activity (Pearson et al. 2000). These results suggest that PML bodies serve as centers for enzymatic modifications including sumoylation, acetylation, and phosphorylation (Takahashi et al. 2004). Therefore, one potential function of these nuclear bodies is to regulate p53 activity by coordinating post-translational modifications.

PML nuclear bodies are not only associated with enzymatic modification, but also may be sites of transcriptional regulation. Several lines of evidence indicate that PML bodies can positively regulate transcription, including the fact that PML bodies lie near highly acetylated chromatin, nascent RNA has been detected near PML bodies, and many transcription factors colocalize to nuclear bodies (Boisvert et al. 2000; Zhong et al. 2000; Tashiro et al. 2004). There is also evidence that PML bodies can repress transcriptional activity, such as co-repressors and heterochromatic protein-1 (HP-1) localize to nuclear bodies (Seeler et al. 1998; Zhong et al. 2000; Tashiro et al. 2004). Because the content of PML nuclear bodies is always changing, PML bodies may positively or negatively regulate transcription depending on the cellular context (Bernardi and Pandolfi 2007). Therefore, it is possible that transcriptional activity of *Drosophila* p53 could be either positively or negatively regulated in these bodies depending on the cellular context and

the content of these nuclear bodies. We speculate that in the context of DNA damage, p53 may be positively regulated in two different ways. First, *Drosophila* p53 may be sumoylated and phosphorylated in these bodies. Secondly, these bodies may contain other coactivators that synergize with p53 to facilitate the transactivation of pro-apoptotic genes. Additionally, it is possible that in the absence of cellular stress p53 may be repressed in these bodies. Evidence for this hypothesis is the fact that the *Drosophila* homolog of Daxx (DLP), a transcriptional repressor, also colocalizes to these bodies and has also been shown to physical interact with *Drosophila* p53 (Bodai et al. 2007).

Sumoylation sites are required for optimal transcriptional activity of p53 in *Drosophila* S2 cells. We demonstrate that mutation of both sumoylation sites of *Drosophila* p53 reduces its transcriptional activity, but not its ability to bind DNA. Interestingly, mutation of a single site does not significantly reduce the transcriptional activity of p53. These results suggest that both sumoylation sites of p53 are required for optimal transcriptional activity. However, there are a few limitations to these assays. First, the reporter assay and the DNA binding assay were performed on plasmid DNA, rather than genomic DNA. These assays would give more direct insight into the function of sumoylation if they were performed using genomic DNA. Secondly, it is unclear if this altered transcriptional activity is actually due to the inability of p53 to be sumoylated or undergo another post-translational modification at that same site, such as methylation or acetylation. To address this concern, we fused SUMO to p53KRKR to try and rescue transcriptional activity. However, a caveat to this experiment was that fusing SUMO to

the wild-type p53 protein also hindered its transcriptional activation, thus making it difficult to interpret the results of this experiment.

There are a few possible ways sumoylation of *Drosophila* p53 could affect its transcriptional activity. First, sumoylation of p53 could alter the ability of p53 to act on a promoter. For example, binding of *Drosophila* p53 to an irradiation responsive enhancer region upstream of the *rpr* gene is required for *rpr* expression and apoptosis after treatment with IR (Brodsky et al. 2000a). Recently, this irradiation responsive enhancer region was also shown to be required for the induction of the adjacent gene, *hid* (Zhang et al. 2008). Perhaps, sumoylation of p53 could be required for p53 to act either on the *hid* or *rpr* promoter. Examination of the transcriptional differences between wild type p53 and p53KRKR *in vivo* will provide insight into how sumoylation affects transcriptional activity of p53. Secondly, sumoylation of p53 could cause a conformational change and allow it to bind a coactivator, thus altering activity on a promoter. Immunoprecipitation experiments could be performed to identify factors that bind to the sumoylatable form of p53. These factors could be required to enhance p53 transcriptional activity.

Sumoylation sites of *Drosophila* p53 affect *in vivo* function. We also examined the affects of mutating the sumoylation sites of *Drosophila* p53 *in vivo*. p53KRKR showed reduced expression levels compared to wild-type p53, when overexpressed using an eye specific promoter. We examined several independent insertions of p53KRKR transgene and found that they all showed reduced expression levels of nuclear p53 compared to the wild-type transgene, indicating that decreased levels of p53 is not due to

the position of the genomic insertion. These results suggest that the sumoylation sites are required for optimal stability of p53 or nuclear localization of p53. There are a few examples of sumoylation affecting stability of p53 family members. For instance, sumoylation of p63 α and p73 α decreases its stability (Minty et al. 2000; Ghioni et al. 2005). Similarly, neddylation, another ubiquitin-like modification, decreases levels of activated p53 in *C. elegans* (Gao et al. 2008). Future studies should focus on how sumoylation of *Drosophila* p53 affects its stability because this could be an important aspect for p53 to be able to induce apoptosis.

Changes in the induction of apoptosis were observed between wild-type p53 and p53KRKR. Overexpression of p53KRKR showed delayed induction of apoptosis compared to wild-type p53. Based on our studies in *Drosophila* S2 cells, the delay in apoptosis could be occurring due to decreased transcriptional activity. However, the delay could also be due to reduced expression levels of p53KRKR. Additionally, we tried to rescue damage-induced apoptosis in a p53 mutant by mildly overexpressing wild-type p53 and p53KRKR. Wild-type p53 rescued damage-induced apoptosis to normal levels. p53KRKR was able to induce some apoptosis after damage, but it was significantly decreased compared to wild type. Because we are able to detect the presence of transgenic p53KRKR, but not endogenous levels of p53, we speculate that there are sufficient levels of p53 to induce apoptosis after DNA damage. Therefore, we believe that this decrease in damage-induced apoptosis is due to reduced transcriptional activity of p53KRKR; rather than reduced expression levels of p53KRKR.

Finally, we made another observation about *Drosophila* p53 that was not discussed in detail in Chapter II. We observed an increase in p53 levels after IR in the developing eye by immunofluorescence (Figure 2.2). This is inconsistent with a previous result, which showed by western blot analysis that p53 levels were not increased after damage in the *Drosophila* embryo (Brodsky et al. 2004). The increase in *Drosophila* p53 levels in the eye is independent of the ability of p53 to be sumoylated. This result raises the possibility that, like mammals, *Drosophila* p53 protein levels are increased after DNA damage. It will be interesting to determine if p53 is stabilized at the protein level or induced at the mRNA level following IR. Although the *Drosophila* genome contains no Mdm-2 homolog, it is worth examining if *Drosophila* p53 is ubiquitinated and if the proteosomal degradation pathway is required to regulate p53 protein levels.

Previous studies about the sumoylation of p53 were performed in cell culture, where proteins were highly overexpressed. Cell culture studies have many conflicting reports about how sumoylation affects p53 function (Hay 2005). Additionally, mouse models where the C-terminal lysine residues have been mutated show surprisingly mild effects on DNA damage responses, suggesting that post-translational modifications are not critical for p53 function *in vivo* (Feng et al. 2005; Krummel et al. 2005). It is possible that these mild effects are due to other p53 family members compensating for loss of p53 function or other sites are being modified and both sites need to be mutated to observe significant changes. Our study shows the first *in vivo* evidence that the sumoylation sites of p53 are required for optimal levels damage-induced apoptosis. We speculate that we observe such a dramatic effect for two reasons. First, *Drosophila* contains a single p53

homolog. Secondly, we mapped two sumoylation sites and our data suggests that it requires that both of them need to be mutated in order to observe effects on p53 function.

Our study of *Drosophila* p53 and sumoylation has demonstrated that *Drosophila* provides a good model system for studying the regulation and post-translational modifications of p53 *in vivo*. We demonstrated that the sumoylation sites of *Drosophila* p53 are required for efficient transactivation function and optimal ability to induce apoptosis. Future studies should focus on how sumoylation of p53 alters transactivation function and what the function of p53 at nuclear bodies is *in vivo*. Ultimately, how signaling pathways regulate the sumoylation of p53 and under what circumstances p53 is sumoylated *in vivo* should be determined. Development of new reagents such as an antibody that can detect endogenous p53 will be required for understanding this process. These results will be critical for understanding the regulation of p53 and how it induces apoptosis.

p53-independent apoptosis limits DNA damage-induced aneuploidy

While p53 plays a conserved role in metazoans connecting the DNA damage response pathway to the core apoptotic machinery, delayed or reduced levels of apoptosis following chromosome damage are still observed in the absence of p53 function. In Chapter III, we show that p53-independent apoptosis in *Drosophila* requires the pro-apoptotic gene *hid*, the apical caspase, *Dronc* and effector caspases inhibited by the baculovirus protein p35. The JNK pathway is required and limiting for this response and is required for induced expression of HID protein following IR. The cycle checkpoint gene *grp* and the JNK phosphatase *puckered* are both negative regulators of this response and mutations in these genes act additively to sensitize *p53* mutant cells to IR. Induction of p53-independent apoptosis is required to limit accumulation of aneuploid cells following IR. We propose that p53-independent and p53-dependent apoptosis act to eliminate cells with defective genomes. The ATM/Chk2/p53-dependent pathway terminates cells with broken chromosomes. We speculate that p53-independent apoptosis eliminates cells that no longer have broken chromosomes, but have been repaired incorrectly. We propose this mechanism acts to eliminate unfit cells from a tissue. In flies, we demonstrated that it eliminates defective bristle cells. In mammals, it may act to eliminate cells with genomic instability that could lead to transformation (Figure 4.1).

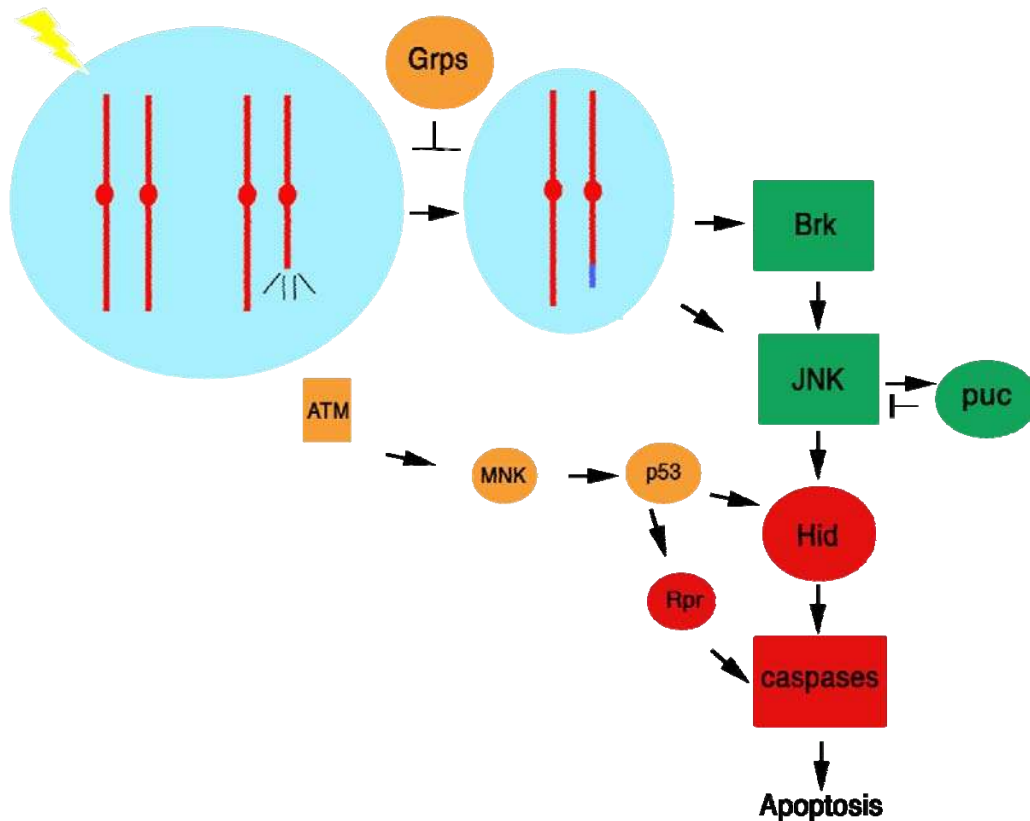


Figure 4.1. Model for p53-dependent and p53-independent apoptosis. In response to DNA damage, cells sense DNA breaks and activate repair, cell cycle delay and apoptosis. DNA damage detection by the ATM and ATR kinases activate p53 to induce apoptosis (orange boxes). p53 regulates the proapoptotic genes *hid* and *rpr*, resulting in caspase activation (red boxes). In parallel, *grp* mediates a cell cycle arrest allowing time for DNA repair. Incorrect repair of chromosome breaks followed by progression through mitosis will result in cells with reduced copy number of large chromosome regions (segmental aneuploidy). Haploinsufficiency of genes in these regions can induce apoptosis by ectopic *brk* expression, JNK pathway activation, and induction of *hid* expression.

Conclusions

This section reviews the conclusions in Chapter III and the limitations to the experimental design. Experiments to better understand the model for *Drosophila* p53-independent apoptosis will also be discussed.

Initial observations and apoptotic assays. Our first observation that p53-independent apoptosis occurred in *Drosophila* was through studies of the DNA damage response protein ATM. Mutation of *atm* causes high levels of telomere fusions and spontaneous apoptosis in imaginal wing discs (Oikemus et al. 2004; Silva et al. 2004; Song et al. 2004). The spontaneous apoptosis can mostly be suppressed by a second mutation in *p53*, however some apoptosis still remains, indicating that there is some p53-independent apoptosis occurring (Oikemus et al. 2004). We examined if we could generate p53-independent apoptosis in response to IR. We observed that p53-dependent apoptosis occurs at 4hrs after treatment with IR, while p53-independent apoptosis occurs between 16 and 24hrs, as assayed by cleaved caspase-3, TUNEL, and acridine orange. From this experiment, we concluded there are two apoptotic responses after treatment with IR, a p53-dependent response that occurred early, and a p53-independent response that occurred later. We were interested in studying the mechanism by which p53-independent apoptosis was occurring. The conclusions of that study and the experimental design and limitations are discussed below.

To test the role of other genes in p53-independent apoptosis we developed a standard for examining the apoptotic response as well as a quantitative apoptotic assay. We primarily measure the induction of apoptosis using the cleaved caspase-3 antibody. Cleaved caspase-3 is an activated form of the caspase-3 protein that can cleave numerous downstream substrates to induce apoptosis and is thought to serve as terminal marker for cells undergoing apoptosis (Bao and Shi 2007). The cleaved caspase-3 antibody recognizes the activated form of caspase-3 and cross reacts with *Drosophila* effector caspases (anti-cleaved caspase-3, Cell Signaling Technology). Although some studies in *Drosophila* embryos have implied that the cleaved caspase-3 antibody can detect other caspases and possibly other apoptotic proteins (Xu et al. 2005; Xu et al. 2006), it is an accurate marker for Drice (functional homolog of mammalian caspase-3) activation in irradiated imaginal wing discs (Muro et al. 2006). Additionally, we examined apoptosis using two additional assays, acridine orange and the TUNEL assay (not shown), to ensure the apoptotic response we are measuring is accurate.

To be able to accurately detect differences in the level of p53-independent apoptosis due to various genetic manipulations, we developed a quantitative apoptotic assay. For this assay, we quantified the levels of apoptosis by calculating the percent volume of the wing disc staining positive for cleaved caspase-3 (Figure 3.1). One caveat to this method is that depending the stage of apoptosis a cell is in, the volume of cleaved caspase-3 staining may be different. For instance, in a cell that has recently activated apoptosis, the cleaved caspase-3 staining will likely be larger because the cell has not started to condense. A cell in late stages of apoptosis will be condensed and have a

smaller volume of cleaved caspase-3 staining. This difference in cleaved caspase-3 volume could make some experiments difficult to interpret, especially when there is only a slight difference in volume of cleaved caspase-3 between genotypes. An ideal assay for quantifying apoptosis would be to compare the number of cells in an imaginal wing disc to the number of cells staining positive for an apoptotic marker, such as TUNEL. We have made several attempts at performing this type of assay. One problem has been being able to accurately count the number of cells in an imaginal wing disc because they are so tightly compact. Future work should focus on optimizing this type of assay.

Requirement of *Drosophila hid*, and initiator and effector caspases during p53-independent apoptosis. p53-independent apoptosis requires the initiator caspase, *dronc* (functional homolog of mammalian caspase-9) and effector caspases, inhibited by the baculovirus protein p35. We observed no apoptosis in *dronc* mutant wing discs at late and early time points, suggesting that *dronc* is required for all damage-induced apoptosis. Similarly, in discs expressing the anti-apoptotic baculovirus protein p35, which inhibits the partially redundant effector caspases, Drice and Dcp-1 (functional homologs of mammalian caspase-3)(Xue and Horvitz 1995; Kondo et al. 2006; Xu et al. 2006; Baum et al. 2007; Lannan et al. 2007), no TUNEL or acridine orange staining was observed, suggesting effector caspases are required for p53-independent apoptosis. Because p35 inhibits effector caspases following proteolytic cleavage, cleaved caspase-3 can be observed in cells expressing p35 (Yu et al. 2002). We observed increased accumulation

of cleaved caspase-3 at late time points in a *p53* mutant overexpressing p35, suggesting that these cells would undergo death in the absence of p35.

p35 could potentially be inhibiting other caspases required for damage-induced apoptosis. To directly determine if the effector caspases, *drice* and *dcp-1*, are required for p53-independent apoptosis, mutants of *drice* and *dcp-1* should be examined. Because *drice* and *dcp-1* double mutants are lethal (Xu et al. 2006), it is not possible to examine the role of the double mutant during p53-independent apoptosis. Although it has been suggested that these caspases are partially redundant in function (Xu et al. 2006), only *drice* is required for damage-induced apoptosis in the imaginal wing (Muro et al. 2006)(Kondo et al. 2006). Therefore, it is possible that *drice* is the only effector caspase required for any damage-induced apoptosis in the wing. To directly test this hypothesis, we should examine levels of p53-independent apoptosis in a *drice* mutant.

The pro-apoptotic gene, *hid* is required and upregulated during p53-independent apoptosis. We were unable to detect HID during p53-independent apoptosis under normal conditions, likely due to HID only being expressed for a short period of time during apoptosis. To be able to detect HID protein expression, we expressed p35 during p53-independent apoptosis. By expressing p35, cells were able to enter the apoptotic pathway, but not die, thus apoptotic proteins can accumulate. Under these conditions, we observed HID accumulating in p53 mutant cells 16 and 24hrs after treatment with IR. We speculate that cells upregulating HID are the cells that are undergoing apoptosis for two reasons. First, *hid* mutants show almost no p53-independent apoptosis at late time points, suggesting that *hid* is required for this response. Additionally, we observe an

accumulation of HID colocalizing with cells accumulating cleaved caspase-3, suggesting that these cells are dying (data not shown). Because *hid* mRNA can be expressed in living cells (Bergmann et al. 1998), future experiments should also examine if *hid* mRNA is also increased during p53-independent apoptosis. This result would provide further insight into the regulation of *hid* during p53-independent apoptosis.

Taken together, the requirement of *hid*, *dronc*, and effector caspases suggests that *Drosophila* p53-independent apoptosis is acting through a core apoptotic pathway. These results are unlike some p53-independent apoptotic pathways that have been described in cell culture models and zebrafish, where caspase-2 is activated and bypasses the core apoptotic response in mammals (Sidi et al. 2008).

The JNK pathway is required and limiting for p53-independent apoptosis. We were interested in testing the role of the JNK pathway during p53-independent apoptosis for two reasons. First, in response to certain cellular stresses JNK has been shown to target *hid* (Luo et al. 2007). Additionally, some studies have suggested that overexpression of the JNK inhibitor, *puckered*, inhibits apoptosis due to loss of a *Minute* gene (Moreno et al. 2002a). From our study, we conclude that the JNK pathway is both required and limiting for p53-independent apoptosis. We also demonstrate that JNK directly targets the upregulation of HID during this process. However, there were some limitations to the reagents used to test the role of the JNK pathway in this response. First, to measure JNK pathway activation we examined levels of *puckered* (*puc-lacZ*), a transcriptional target of the JNK pathway. However, it is possible that other signaling

pathways could also target *puckered*. A more direct test of JNK pathway activation would be to examine levels of phospho-JNK, using a phospho-JNK antibody. Additionally, when the JNK pathway was inhibited by overexpression of *puckered*, some apoptosis still remained. There are two possible reasons why some p53-independent apoptosis was still occurring. First, it is possible that other signaling pathways are upregulating pro-apoptotic genes to induce apoptosis. Secondly, overexpression of *puckered* may not be able to completely inhibit JNK activity. Evidence for this hypothesis is that we still observed some JNK pathway activation, by increased levels of *puc-lacZ*, when *puckered* is overexpressed. Another caveat to this experiment is that overexpression of *puckered* could potentially act to inhibit other MAPK pathways. To address this concern, more direct reagents, such as a transgene expressing a JNK RNAi construct or JNK mutant clones, should be used to inhibit JNK and test the requirement of the JNK pathway during p53-independent apoptosis.

The role of the DNA damage response proteins during p53-independent apoptosis. Previously described mechanisms of p53-independent apoptosis rely on signaling from upstream DNA damage response proteins (Urist et al. 2004; Reinhardt et al. 2007; Sidi et al. 2008). Therefore, we hypothesized that it was upstream DNA damage response proteins that were activating JNK. To test for the requirement of DNA damage response proteins during *Drosophila* p53-independent apoptosis we examined p53-independent apoptosis in various double and triple mutant combinations of the upstream DNA damage response proteins. Surprisingly, we found that p53-independent

apoptosis was unaffected by these mutations, suggesting that the DNA damage response proteins are not required for this response and that this response may not be activated by DNA damage. These results do not absolutely rule out a role for other, untested components of DNA damage response pathway in p53-independent apoptosis, but this is unlikely because the damage signal has subsided long before p53-independent apoptosis begins to occur (unpublished result).

Not only are core components of the DNA damage response pathway not required for apoptosis in response to IR or unprotected telomeres, mutation of some of these genes can increase apoptosis. Many of these genes contribute to repair of IR-induced breaks, which could provide a simple mechanism for increased apoptosis following IR (Hari et al. 1995; Brodsky et al. 2000b; Ciapponi et al. 2004; Ciapponi et al. 2006; Oikemus et al. 2006). However, unlike some of the upstream components of the signaling response, *grp* is required for cell cycle arrest following IR, but cells mutant for *grp* or for *grp* and *mnk* do not show an increased frequency of chromosome breaks following IR (Jaklevic and Su 2004; Oikemus et al. 2006), suggesting that it may be *grp* role in cell cycle arrest that is suppressing p53-independent apoptosis.

Our results demonstrated that mutation of *grp* accelerated JNK pathway activation and apoptosis, suggesting cell cycle progression is correlated with p53-independent apoptosis. This result raises two important questions. First, do these cells exit mitosis before undergoing cell death or do they undergo mitotic catastrophe. Mitotic catastrophe has many definitions, however we define it as cells dying in a mitotic state. Some mechanisms of mammalian p53-independent apoptosis occurs through mitotic

catastrophe, as assayed by colocalization of cleaved caspase-3 and the mitotic marker phospho-histone-3 (Reinhardt et al. 2007). Although we cannot definitively state these cells are not dying in mitosis, we did not observe colocalization of phospho-histone-3 and cleaved caspase-3 in our experiments, suggesting that *Drosophila* p53-independent apoptosis is not occurring through the same pathway as previously described. Additionally, studies in *Drosophila* embryos have shown that mitotic catastrophe is chk2-dependent (Takada et al. 2003), and p53-independent apoptosis is not dependent on chk2. Secondly, to determine if cell cycle progression is required for p53-independent apoptosis, we tried to both genetically and chemically inhibit the cell cycle in imaginal wing discs. Unfortunately, inhibition of the cell cycle resulted in the induction of apoptosis and we were unable to measure the p53-independent apoptosis in those experiments.

p53-independent apoptosis limits aneuploid cells as assayed by the *Minute* phenotype. Because cell cycle progression correlated with p53-independent apoptosis, we hypothesized that cells with segmental aneuploidy were being generated, and these cells were being eliminated by this mechanism. An assay that can measure aneuploid cells is the *Minute* assay. Using this assay, we were able to observe how many segmentally aneuploid cells were being generated and how many were being eliminated by this mechanism. This section will describe how the *Minute* assay provides a marker for cells with segmental aneuploidy and the conclusions from this assay.

DNA damage-induced chromosome rearrangements or large deletions followed by cell cycle progression result in cells that are haploid for a large part of their genome, which is known as segmental aneuploidy or damage-induced aneuploidy. Because *Minute* genes are scattered all throughout the genome, almost any large deletion would likely result in deletion of a *Minute* gene. Therefore, the *Minute* bristle phenotype provides a marker for cells with segmental aneuploidy. Previously, it has been shown that larvae treated with IR produce adults with *Minute* bristle and these *Minute* bristles are due to large deletions (Baker et al. 1978). However, there are other ways that the *Minute* phenotype could potentially be generated in response to DNA damage. For instance, a gain of function mutation in an inhibitor of bristle development or a loss of function mutation in both copies of a gene that positively regulates bristle development could generate the *Minute* phenotype. However, we believe these types of mutations would be less likely to occur. Additionally, if these types of mutations were generating the *Minute* phenotype, then it would be just as likely that gain of function mutations in genes positively regulating bristle development or loss of function mutations in suppressors of bristle development would occur and should result in longer bristles or flies with extra bristles. Neither of these phenotypes were observed in our analysis, suggesting that those types of mutations are not contributing to the *Minute* bristle phenotype. Lastly, the *Minute* phenotype could be generated due to damaged ribosomal proteins. Although this is formally possible, we speculate that protein damage occurs so early in larval development that it would be repaired or replaced by the time bristles were being synthesized. The *Minute* assay does provide a read out for cells with segmental

aneuploid, however we recognize the limitations of this assay. Ultimately, the development of a more direct assay to measure large deletions across the entire genome in the imaginal wing disc is essential for understanding how cells with damage-induced aneuploidy are eliminated.

We examined the contribution of p53-dependent and p53-independent apoptosis for eliminating cells with segmental aneuploidy using the *Minute* assay. We observed an increase in the amount of *Minute* bristles recovered after treatment with IR, when p53-independent apoptosis is inhibited. This result demonstrates that p53-independent apoptosis acts to limit the formation of damage-induced aneuploid cells. Interestingly, we observed no increase in the amount of *Minute* cells in a *p53* mutant compared to wild type after treatment with IR. These results suggest that p53-dependent apoptosis is not essential for eliminating aneuploid cells; rather p53 is activated in response to breaks and cannot distinguish between breaks that would be repaired correctly or incorrectly. Taken together, these results suggest that in flies that p53-independent apoptosis is essential for maintaining genomic stability, whereas p53-dependent apoptosis is not essential for eliminating this type of genomic instability after DNA damage. The contribution of p53 in maintaining genomic stability in mammals will be discussed later in this chapter.

Role of *brinker* during p53-independent apoptosis. Previously, it has been shown that clones of *Minute* cells in a wild-type background ectopically express *brinker* (Moreno et al. 2002a). In addition, wild-type clones in a wing disc overexpressing *myc*, upregulate *brinker* (Moreno and Basler 2004). In these previously published

experiments, ectopic expression of *brinker* results in JNK-dependent apoptosis (Adachi-Yamada and O'Connor 2002; Moreno et al. 2002a; Moreno and Basler 2004). Additionally, clonal populations can survive if ectopic expression of *brinker* is suppressed, suggesting that the cells upregulating *brinker* are dying (Moreno et al. 2002a; Moreno and Basler 2004). We tested the role of *brinker* during p53-independent apoptosis. Consistent with the recovery of *Minute* bristles in the adult fly and the requirement of JNK during p53-independent apoptosis, we observed increased expression of *brinker* in the imaginal wing disc after treatment with IR. However, we could not show colocalization of *brinker* with HID or other markers of p53-independent apoptosis, we speculate it is because *brinker* is transiently expressed during p53-independent apoptosis. Additionally, we tried to suppress p53-independent apoptosis by suppressing ectopic expression of *brinker*. However, we were unable to interpret the results of those experiments because expression of transgenes that suppress ectopic expression of *brinker* induced significant amounts of apoptosis.

Upregulation of *brinker* after treatment with IR was observed in both wild type and *p53* mutant imaginal discs at late time points only. The fact that *brinker* is not induced at early time points in a wild-type wing disc suggests that upregulation of *brinker* is not a non-specific consequence of cells undergoing damage-induced apoptosis. It is tempting to speculate that *brinker* is a marker for the *Minute* cells being generated in response to DNA damage. If we assume that the cells upregulating *brinker* are undergoing p53-independent apoptosis, then these results suggest that p53-independent apoptosis does not depend on the absence of p53 to occur. Essentially, both p53-

dependent and p53-independent apoptosis are occurring after damage in a wild-type wing disc. We speculate that both these pathways act to eliminate different types of damage after treatment with IR in a wild-type wing disc.

How does segmental aneuploidy trigger p53-independent apoptosis?

An interesting question that arises from this study is what is the upstream signal that activates p53-independent apoptosis. Results from Titen and Golic demonstrate that significant aneuploidy is required for p53-independent apoptosis in response to generation of dicentric chromosomes (Titen and Golic 2008). Additionally, our results suggest that p53-independent apoptosis limits the formation of cells with segmental aneuploidy. Therefore, we speculate that there is something about a cell being segmental aneuploid that activates JNK to induce apoptosis. Potential hypotheses for how segmental aneuploidy induces apoptosis and experiments to test those hypotheses will be discussed below.

There are a few possible ways that segmental aneuploidy could trigger apoptosis. One possible way is that it is a cumulative effect of reduced dosage of many genes. For instance, loss of a chromosome arm or several large deletions containing essential genes may not be tolerable to the cell, thus committing it to death. Another possible way segmental aneuploidy triggers apoptosis is through loss of a *Minute* gene. *Minute* genes are located throughout the genome and clones of *Minute* cells undergo apoptosis due to competition with their wild-type neighbors (Moreno et al. 2002a; Marygold et al. 2007).

Therefore, we hypothesized that chromosomal rearrangements followed by mitosis will likely result in a daughter cell that is missing at least one *Minute* gene, which could potentially act as the for trigger p53-independent apoptosis. Experiments to address this model will be discussed below.

Experiments from Titen and Golic suggest that p53-independent apoptosis is due to generation of significant aneuploidy. They observed p53-independent cell death 18-30 hours after dicentric chromosomes were generated. Interestingly, p53-independent cell death was only observed when dicentrics were generated on the X or autosomal chromosomes. The Y chromosome, which is only essential for male fertility, did not show p53-independent apoptosis when dicentrics were generated. However, generation of dicentrics on Y chromosome with a duplication of the 4th chromosome on the tip did induce p53-independent apoptosis (Titen and Golic 2008). Interestingly, the 4th chromosome contains a single *Minute* gene, *rps3A*. Therefore, if p53-independent apoptosis was due to loss of *rps3A*, then apoptosis should be suppressed by a transgene expressing *rps3A*. Although this experiment would give some insight into the signal for p53-independent apoptosis, it would not be definitive in the case of DNA damage or telomere fusions on autosomes, where many different rearrangements are being induced. For instance, when dealing with severe amounts of telomere fusions or DNA damage both loss of a *Minute* gene and cumulative loss of several genes could both be contributing to p53-independent apoptosis. Additional experiments to distinguish between these two models will be discussed below.

Previous experiments showed that clones of *Minute* cells were eliminated in a wild-type wing disc (cell competition)(Morata and Ripoll 1975). However, *Minute* clones could survive in the adult wing if they were induced in a *Minute* background of similar strength (Simpson and Morata 1981). These results suggested that cell competition was due to the growth differential, and once there was no growth differential these cells could survive. Additionally, the developmental delay in flies transheterozygous for two *Minute* genes is the same as the delay as the more severe *Minute*, indicating that *Minute* mutations are not additive (Schultz 1929). Therefore, we originally thought if IR-induced, p53-independent apoptosis was occurring due to loss of a *Minute* gene, then we should be able to suppress it in a *Minute* background. However, there are several caveats to this experiment. First, original experiments demonstrating that competition could be suppressed by growing clones in a *Minute* background only compared *Minutes* of a similar strength (Simpson and Morata 1981). It is unknown how well clones of a severe *Minute* would survive in a less severe *Minute* background. This result is of particular importance for our experiment, because IR could be inducing a number of chromosomal rearrangements, leading to loss of strong or haplolethal *Minutes*. Secondly, it is not known how *Minute* cells respond to IR. For instance, these cells may not contain the translational capacity to initiate the appropriate responses to DNA damage. These cells may inefficiently repair damaged DNA or may not be able to induce the appropriate pro-apoptotic signals, making the results of these experiments difficult to interpret.

Lastly, *Minute* flies show an increase in JNK signaling and spontaneous apoptosis, indicating these cells are normally under stress, even when they are not in a

competitive environment (Coelho et al. 2005)(L.M. McNamee, unpublished result)(Figure 3.11). We have termed this non-competitive apoptosis. This result is still consistent with the idea that loss of a ribosomal protein gene is sufficient to induce apoptosis. It will be important to determine if non-competitive and competitive apoptosis are induced through the same signaling pathway, and then determine if those pathways are required for p53-independent apoptosis. Similar to competitive apoptosis, our data shows that JNK signaling is increased in *Minute* wing discs, indicating the JNK may be playing a role in non-competitive apoptosis. Additionally, non-competitive apoptosis appears to be independent of p53, and so far no one has reported that p53 is required for competitive apoptosis (Figure 3.11). It is possible that competitive and non-competitive cell death occurs through the same signaling pathway and perhaps competitive cell death has increased kinetics due to surrounding wild-type neighbors.

Other genes that are required for cell competition should also be tested for their requirement in non-competitive apoptosis. For instance, engulfment genes, *draper*, *wasp* and *phosphatidyserine receptor* are required to induce competitive cell death. Wild-type cells begin engulfing *Minute* cells to activate the apoptotic program (Li and Baker 2007). Although engulfment has been shown to contribute to cell death (Hoepfner et al. 2001; Reddien et al. 2001), these results indicate that engulfment is required during cell competition and the cell-autonomous cell-death program is insufficient to remove M/+ cells by itself (Li and Baker 2007). Therefore, if engulfment genes were required for both competitive and non-competitive apoptosis, then requirement for engulfment genes in p53-independent apoptosis would provide further evidence that these cells are being

eliminated due to loss of a *Minute* gene.

Finally, identification of new genes in competitive and non-competitive apoptosis could identify signals that are specific to the induction of apoptosis in a *Minute* cell. A genetic screen, identifying new genes affecting cell competition, found a mutation on the 3rd chromosome that is homozygous viable and maps to the cytogenetic location 69C2-70D1 (Tyler et al. 2007). It should be determined if this mutation is also required for non-competitive apoptosis and what gene is affected by this mutation. If this gene were specific to death of a *Minute* cell, then it would be useful in determining if p53-independent apoptosis was occurring due to loss of a ribosomal protein gene.

Aneuploidy and haploinsufficiency of ribosomal protein genes in mammals

This section will highlight what is known about responses to aneuploidy and haploinsufficiency of ribosomal protein genes in mammals. Additionally, how our model for p53-independent apoptosis could apply to mammalian DNA damage responses is discussed.

In principle, there are two different ways a cell can become aneuploid. First, errors in cell division that result in loss or gain of an entire chromosome. Secondly, large deletions and rearrangements followed by mitosis results in daughter cells that are missing a large portion of a chromosome, which is referred to as segmental aneuploidy.

Typically, these types of rearrangements are a result of unprotected telomeres or DNA damage (Geigl et al. 2008).

In mammals, responses to aneuploidy and polyploidy have largely been described in terms of loss or gain of an entire chromosome. Cell culture experiments have demonstrated that p53 and p73 act to limit aneuploid and polyploid cells. Deletion of both p53 and p73 results in deregulated cell cycle activity and a defective G2/M checkpoint, indicating that p53 and p73 suppress aneuploidy and polyploidy by inhibiting mitosis (Talos et al. 2007; Tomasini et al. 2008). These results suggest that p53 and p73 play a role in maintaining genomic stability, at least in terms of loss or gain of an entire chromosome. Due to the lack of technology, there is no assay to accurately analyze segmental aneuploidy throughout the entire genome, thus how cells deal with these types of chromosome rearrangements is largely unanswered (Geigl et al. 2008).

Although it is unclear how cells deal with segmental aneuploidy, it is well established in zebrafish and mice that haploinsufficiency of a ribosomal protein gene activates p53-dependent apoptosis (Panic et al. 2007; Danilova et al. 2008; McGowan et al. 2008). Mice that are haploinsufficient for *rps6* die at embryonic day 5.5 due to increased levels of apoptosis. Deletion of p53 in *rps6*^{-/+} mice can delay apoptosis and death until embryonic day 12.5, but the animal will eventually be terminated, suggesting that there are also p53-independent mechanisms for eliminating these cells (Panic et al. 2006). These results raise the possibility that p53 detects and eliminates cells that have lost a ribosomal protein gene before there is a phenotype, and that p53-independent

mechanisms are activated in response to cellular defects due to loss of a ribosomal protein gene.

As in *Drosophila*, mammalian ribosomal protein genes are widely distributed throughout the genome. Therefore, haploinsufficiency of a ribosomal protein gene may be the trigger for apoptosis in response to damage-induced aneuploidy. If we assume that loss of a ribosomal protein activates apoptosis in response to damage-induced aneuploidy, then p53 would likely be required for the efficient response in mammals. However, in the absence of p53, loss of one or several ribosomal protein genes due to damage-induced aneuploidy may elicit delayed p53-independent apoptosis.

Aneuploidy, haploinsufficiency of ribosomal protein genes, and tumorigenesis

This section will focus on potential consequences of not eliminating aneuploid cells and how segmental aneuploidy and loss of a ribosomal protein gene could potentially lead to cancer. Lastly, I speculate on what types of cancers might arise due to loss of a ribosomal protein gene and propose experiments that may aid in identifying if loss of a ribosomal protein gene can lead to cancer if these cells are not eliminated.

One of the most important questions in this study is what is the consequence of not eliminating cells with aneuploidy or segmental aneuploidy. Aneuploidy is often observed in cancer, however it is controversial whether or not aneuploidy drives the formation of tumors. Some argue it is required for initiation, while others believe it is

necessary for progression, and others hypothesize it is merely a side effect of a tumor. Alternatively, cells that have rampant aneuploidy do not appear to contribute to tumorigenesis because they die after 6 divisions due to the severe loss of chromosomes (Weaver and Cleveland 2006).

Although it is controversial whether aneuploidy leads to cancer, segmental aneuploidy is a well-established cause of tumor development (Weaver and Cleveland 2006). It has previously been shown that telomere dysfunction leading to non-reciprocal translocations results in epithelial cancers in *p53* mutant mice (Artandi et al. 2000). This result suggests that segmental aneuploidy can drive tumorigenesis and *p53* can be critical for suppressing the formation of these tumors. Thus providing some evidence that *p53* may be able to detect and eliminate cells with this type of genomic instability in mammals.

What still remains unclear is what is it about a cell being segmental aneuploid that drives tumorigenesis and could haploinsufficiency of a ribosomal protein gene be contributing to this response. One potential model is that transformation that resulted from segmental aneuploidy would not show loss of a ribosomal protein gene. Cells lacking a ribosomal protein gene would not have the growth potential to lead to cancer and would likely be eliminated by *p53*-dependent or *p53*-independent apoptosis. This model would predict that it would only be rearrangements that did not lose a ribosomal protein gene. These rearrangements would likely result in loss of tumor suppressors genes or gain of function mutations in oncogenes to promote tumorigenesis.

Another model about how segmental aneuploidy could lead to transformation is through loss of a ribosomal protein gene. We speculate that tumors could arise from segmental aneuploidy that did show reduced copy of a ribosomal protein gene if accompanied by other mutations, such as deletion of other pro-apoptotic genes or gain of function in oncogenes. This is an interesting hypothesis because many human diseases that are caused by haploinsufficiency of a ribosomal protein gene show increased risk of developing cancers (Flygare and Karlsson 2007; Ebert et al. 2008). How these cancers arise due to lack of a ribosomal protein gene is not understood. Perhaps, loss of a ribosomal protein gene might further promote tumorigenesis because these cells may be defective in DNA repair and tumor suppression, thus allowing the cell to further destabilize the genome and lead to transformation. Additionally, loss of a ribosomal protein gene accompanied by gain of function mutations in an oncogene could lead to accelerated growth, even in the absence of optimally functioning translation machinery.

One way to determine if loss of ribosomal protein genes drives tumor formation is to examine genome content of tumors. Examination of genome content in tumors could help identify how frequently loss of a ribosomal protein gene occurs. One possible result would be that loss of a ribosomal protein gene would frequently be seen in tumors, suggesting that this event could be driving tumorigenesis. Alternatively, loss of a ribosomal protein gene would not be observed at a high frequency, indicating that maintaining normal copy number of ribosomal protein genes may be essential for driving tumor formation. Lastly, there might be no correlation between loss of a ribosomal

protein gene in tumors, suggesting that there is another aspect about segmental aneuploidy that drives tumor formation.

It is possible that loss of a ribosomal protein gene may only lead to certain types of cancer. For instance, patients with Diamond Black Fan anemia exhibit high levels of leukemias and osteosarcomas (Flygare and Karlsson 2007). Additionally, zebrafish haploinsufficient for several different ribosomal protein genes usually die early due to lymphomas and malignant peripheral nerve sheath tumors (Amsterdam et al. 2004). One possible reason for increases in leukemias and lymphomas could be because these cells proliferate at a high rate and require increased amounts of protein synthesis. Defects in DNA repair and other cellular functions combined with increased need to proliferate could lead to cancer. Leukemias and lymphomas may be good candidates to screen genome content to see if loss of a ribosomal protein gene is frequently observed.

DNA damage-induced aneuploidy in mammals and potential therapeutic targets

This section will focus on experiments to address if segmental aneuploidy and loss of a ribosomal protein gene play a role in mammalian DNA damage responses. Based on our studies in *Drosophila*, potential therapeutic targets for sensitizing cells with segmental aneuploidy will also be discussed.

Going forward it should be determined what pathways contribute to the elimination of cells with damage-induced aneuploidy. An assay that can detect

segmental aneuploidy throughout the genome will be essential for identifying the signal that activates apoptosis due to segmental aneuploidy. This type of assay will also be essential for identifying the circumstances in which segmental aneuploidy leads to cancer. In flies, loss of a *Minute* gene provides an assay to detect segmental aneuploidy. Unfortunately, in mammals there is currently no way to detect segmental aneuploidy in cells. Development of a technology that could detect loss of a ribosomal protein gene may also provide a useful read out for segmental aneuploidy in mammals. Ultimately, the development of a technology that can detect and measure all different types of changes in genome content in a single cell would be the most informative.

Another way to determine if loss of a ribosomal protein gene triggers apoptosis in response to damage-induced aneuploidy is to identify the signaling pathway that induce apoptosis in response to haploinsufficiency of a ribosomal protein gene. If we identify the components of the pathway that are specific to the elimination of these cells, then we can ask if that pathway contributes to damage-induced apoptosis. One potential approach for identifying the genes required for induction of apoptosis in response to haploinsufficiency of a ribosomal protein gene would be to perform an shRNA screen. Once the genes required for the apoptotic pathway have been identified, genetic experiments can be performed to determine the order of the pathway. We hope that we would identify components of the pathway that are specific to the elimination of a cell that is haploinsufficient for a ribosomal protein gene. If these components also contributed to damage-induced apoptosis, then it would provide strong evidence that haploinsufficiency of a ribosomal protein gene triggers some damage-induced apoptosis .

A better understanding of the mechanism that eliminates cells with damage-induced aneuploidy could result in potential therapeutic targets to sensitize tumor cells to DNA damage. In our model, we identified that mutation of genes involved in cell cycle arrest and inhibitors of JNK pathway sensitize cells undergo p53-independent apoptosis in flies. Therefore, targeting JNK pathway activation and cell cycle progression in mammals may provide potential therapeutic targets to sensitize tumors that are normally resistant to damage-induced apoptosis. In mammals, mutation of Chk1 has been shown to sensitize cells to damage-induced apoptosis and many Chk1 inhibitors are in clinical trials (Tse et al. 2007). The JNK pathway has been shown to have opposing roles in promoting tumorigenesis. Some have reported that mutation of an upstream kinase, MK4, in the JNK pathway is frequently observed in certain types of tumors, whereas JNK1 deficiency has been shown to reduce proliferation and vascularization in other tumor types (Johnson and Nakamura 2007). Therefore, hyperactivation of the JNK pathway may only be beneficial in certain tumor types. It is tempting to speculate that tumors that arise from loss of a ribosomal protein gene might be more susceptible to this type of treatment.

How cells respond to segmental aneuploidy is clearly an important area of study. It seems that subtle changes in genome content can drive tumorigenesis. Unfortunately, in mammals there is no technology that can detect segmental aneuploidy throughout the genome. This obstacle has made it difficult to understand how cells react to damage-induced aneuploidy. Our study and results from Titen and Golic demonstrate that cells effectively deal with damage-induced aneuploidy by activating p53-independent

apoptosis. Future studies should focus on how damage-induced aneuploidy triggers apoptosis in mammals because this could be an important mechanism for inhibiting transformation and developing therapies to treat cancer cells.

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