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Drosophila p53 Is a Structural and Functional Homolog of the Tumor Suppressor *p53*

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Summary

The importance of p53 in carcinogenesis stems from its central role in inducing cell cycle arrest or apoptosis in response to cellular stresses. We have identified a *Drosophila* homolog of *p53* ("*Dmp53*"). Like mammalian p53, Dmp53 binds specifically to human p53 binding sites, and overexpression of Dmp53 induces apoptosis. Importantly, inhibition of Dmp53 function renders cells resistant to X ray–induced apoptosis, suggesting that Dmp53 is required for the apoptotic response to DNA damage. Unlike mammalian p53, Dmp53 appears unable to induce a G1 cell cycle block when overexpressed, and inhibition of Dmp53 activity does not affect X ray–induced cell cycle arrest. These data reveal an ancestral proapoptotic function for p53 and identify *Drosophila* as an ideal model system for elucidating the p53 apoptotic pathway(s) induced by DNA damage.

Introduction

p53 is a tightly regulated transcription factor that induces cell cycle arrest or apoptosis in response to cellular stresses such as DNA damage and oncogene activation (Ko and Prives, 1996; Levine, 1997; Giaccia and Kastan, 1998; Kamijo et al., 1998). Loss or inactivation of the *p53* tumor suppressor gene is the single most common mutation in human cancer (Hainaut et al., 1998). Thus, inactivation of p53 function may provide a selective advantage to tumor cells through accrued mutations leading to deregulated cell proliferation and resistance to cell death (Graeber et al., 1996; Kinzler and Vogelstein, 1996).

Numerous studies have established that growth arrest and apoptosis are independent functions of p53 (reviewed in Gottlieb and Oren, 1996). p53-dependent G1 arrest occurs largely through transcriptional induction of $p21^{WAF1}$, which prevents entry into S phase by inhibiting G1 cyclin–dependent kinase activity (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Deng et al., 1995). However, p21 is not required for p53-dependent apoptosis (Wagner et al., 1994). In fact, p21 may protect against p53-induced apoptosis in at least some cell types (Waldman et al., 1996; Wang and Walsh, 1996; Gorospe et al., 1997; Bissonnette and Hunting, 1998; Yu et al., 1998; Asada et al., 1999).

Induction of apoptosis by p53 is critical for the tumor suppressor function of p53. There appear to be multiple mechanisms through which p53 promotes apoptosis. For example, p53 can transcriptionally activate the proapoptotic genes *Bax* (Miyashita and Reed, 1995), *Fas* (Owen-Schaub et al., 1995), and *IGF-BP3* (Buckbinder et al., 1995), as well as a set of genes that may promote apoptosis through the formation of reactive oxygen species (Polyak et al., 1997). Furthermore, there is evidence that p53 can induce apoptosis in the absence of its transcriptional activation function (reviewed in Ko and Prives, 1996).

The recent discovery of two *p53*-related genes, *p63* and *p73*, has revealed an additional level of complexity to studying p53 function in vertebrates (Kaelin, 1999). Both genes encode proteins with transactivation, DNA-binding, and tetramerization domains, and some isoforms of p63 and p73 are capable of transactivating p53 target genes and inducing apoptosis. It was initially thought that only p53 was induced in response to DNA damage and other stress signals. There is now evidence, however, that p73 is also activated by some forms of DNA damage in a manner that is dependent upon the c-Abl tyrosine kinase (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999). These data suggest that p53-independent apoptotic pathways may be mediated by other p53 family members.

A better understanding of p53-regulated pathways could lead to improved strategies for treating p53-deficient tumors. However, the apparent complexity of p53 function in vertebrates has hampered efforts to elucidate these pathways. The fruit fly *Drosophila* has proven to be a powerful tool for the genetic dissection of biochemical pathways. We report the identification of a *Drosophila* homolog of *p53* (the abbreviation "*Dmp53*" will be used here to distinguish *Drosophila p53* from other family members). Dmp53 shares significant amino acid identity with the DNA-binding domain of p53, including conservation of key residues commonly mutated in human cancer. Like *p53*, overexpression of *Dmp53* induces apoptosis, and inhibition of Dmp53 function renders cells resistant to X ray–induced apoptosis. In contrast to mammalian *p53*, *Dmp53* overexpression does not induce a G1 cell cycle block, and inhibition of Dmp53 activity does not affect X ray–induced cell cycle arrest. These data suggest that the ancestral function of p53 may have been restricted to eliminating damaged cells by apoptosis and identify *Drosophila* as an ideal model system for dissecting p53-mediated apoptotic pathways.

Results

Identification of *Dmp53*

We identified a partial *Drosophila* cDNA with sequence similarity to the *p53* family of genes as a result of a

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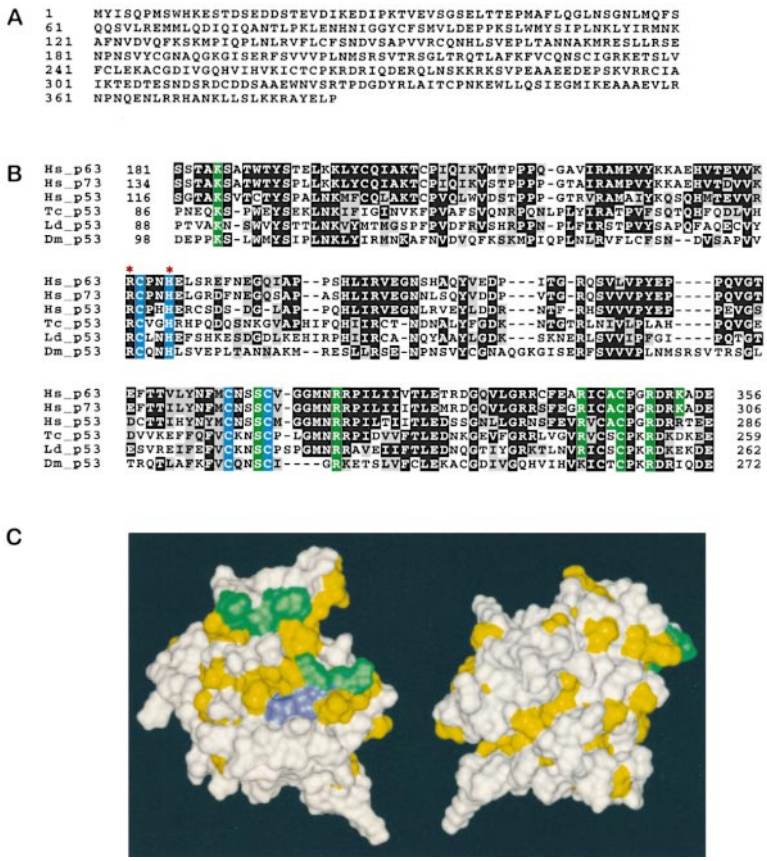

Figure 1. Dmp53 Sequence Conservation and Predicted Structure

(A) Predicted amino acid sequence of Dmp53. (B) Alignment of DNA-binding domains of p53-related proteins from human (Hs), *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc), and *Leptinotarsa decemlineata* (Ld). Positions at which 50% or more of the residues are identical are shaded in black, and conservative substitutions are shaded in gray. Positions are left unshaded if one residue is conserved among the three human proteins and a different residue is conserved among the three insect proteins (unless it represents a conservative substitution). Residues that form the zinc ion coordination site in p53 are indicated in blue. DNA contact residues are indicated in green. Red asterisks mark residues altered in the Dmp53 dominant-negative mutations R155H and H159N. (C) Two views of a surface representation model of the Dmp53 DNA-binding domain (residues 90–275). The model is derived from the crystal structure of human p53 (Cho et al., 1994). The left view shows the predicted DNA-binding face of Dmp53, the right view shows the protein rotated 180°. Colored residues are those conserved between Dmp53 and the vertebrate p53 family (38% similarity, including conservative substitutions). Residues in green correspond to DNA contact residues in human p53. Residues in blue highlight the zinc ion coordination site. All other conserved residues are shown in yellow; most of these are involved in formation of the DNA-binding pocket or in structural packing.

large-scale expressed sequence tag (EST) project. Northern blot analysis using the cDNA probe revealed a single 1.6 kb transcript in embryos, larvae, and adults (data not shown). The complete sequence of the *Dmp53* mRNA was obtained by rapid amplification of cDNA ends (RACE) and sequencing of a 1.6 kb cDNA clone. The cDNA encodes a putative 385 amino acid protein (Figure 1A). Hybridization of a *Dmp53* probe to polytene chromosomes localized the gene to chromosome 3, band 94D. We also identified additional insect *p53*-related genes through EST sequencing projects in the flour beetle (*Tribolium castaneum*) and Colorado potato beetle (*Leptinotarsa decemilineata*).

Human p53 is a 393 amino acid protein composed of three main functional domains: an amino-terminal acidic transactivation domain, a central DNA-binding domain, and a carboxy-terminal tetramerization domain. Significant similarity between Dmp53 and the vertebrate p53 family is limited to the DNA-binding domain and includes residues identified in human p53 as critical for DNA sequence recognition and coordination of a zinc ion (Figures 1B and 1C) (Cho et al., 1994). We generated a three-dimensional model of the Dmp53 DNA-binding domain based on the human p53 crystal structure (Figure 1C) (Cho et al., 1994). Conserved surface residues predominantly cluster in the DNA-binding site, while most of the remaining conserved residues are buried and involved in stabilizing the tertiary fold of the domain. This suggests that, despite limited sequence identity, the Dmp53 DNA-binding domain may adopt a tertiary structure similar to the human p53 DNA-binding domain.

There is no sequence similarity between Dmp53 and other p53 family members in the carboxyl termini, yet this region of Dmp53 contains secondary structures characteristic of p53-related proteins. Mammalian p53 binds DNA as a homotetramer, and self-association is mediated by a β sheet and amphipathic α helix located in the carboxyl terminus of the protein (Clore et al., 1994; Lee et al., 1994; Jeffrey et al., 1995). A similar β sheet (residues 320–322 and 332–337) and amphipathic α helix (residues 341–359) are predicted in the carboxyl terminus of Dmp53. Consistent with this prediction, a yeast two-hybrid assay revealed that Dmp53 interacts with itself but not with human p53 (data not shown). Like the carboxyl terminus, the amino terminus of Dmp53 shows no sequence similarity with other p53 family members. However, this region is highly divergent among p53 family members, except for conserved residues critical for binding of MDM2 to human p53 (Lin et al., 1994; Kussie et al., 1996). Interestingly, the residues critical for MDM2 binding are not conserved in Dmp53.

p53-related sequences have been described previously in invertebrates, including clams (Barker et al., 1997; Van Beneden et al., 1997) and squid (Schmale and Bamberger, 1997). Squid p53 more closely resembles p63 or p73 because of its long C-terminal extension that contains the SAM domain characteristic of the p63/p73 subfamily (Figure 2). This led to the suggestion that p53 may have evolved from an ancestral p73-like molecule (Kaghad et al., 1997). Dmp53 lacks a carboxy-terminal SAM domain, and there is no evidence for a SAM domain–containing alternative exon within the 100 kb of

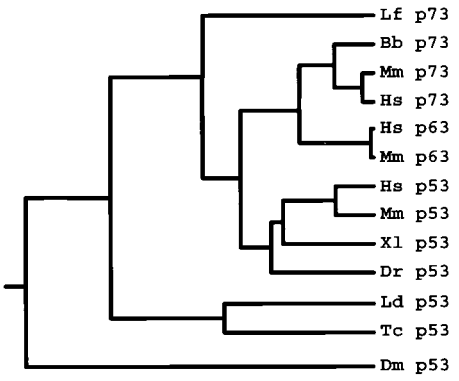

Figure 2. Phylogenetic Analysis of the *p53* Gene Family

Phylogenetic tree generated with Clustal W shows the relationship of p53-related proteins from *Loligo forbesi* (Lf), *Barbus barbus* (Bb), *Mus musculus* (Mm), *Homo sapiens* (Hs), *Xenopus laevis* (Xl), *Danio rerio* (Dr), *Leptinotarsa decemlineata* (Ld), *Tribolium castaneum* (Tc), and *Drosophila melanogaster* (Dm).

genomic sequence downstream of *Dmp53*. The beetle p53 proteins also lack any similarity to the carboxy-terminal domains of p63 and p73. Given that the insect *p53*-related genes represent the most evolutionarily distant members of the *p53* gene family known to date, we suggest that the *p63/p73* subfamily arose from an ancestral *p53*-like gene after the split of the arthropod and vertebrate lineages.

Dmp53 Binds to Human p53 Recognition Sites in *p21* and *GADD45*

To determine whether the sequence similarity of Dmp53 and p53 may reflect a conserved function as a DNA binding transcription factor, we tested whether Dmp53 can bind to a double-stranded DNA molecule containing a p53 recognition site using an electrophoretic mobility shift assay. Figure 3 shows that Dmp53 binds specifically to oligonucleotides containing p53 binding sites from the human *p21* and *GADD45* genes, demonstrating that both DNA binding and target site specificity have been conserved through more than 500 million years of evolution. This interaction is specific, as addition of unlabelled wild-type *GADD45* oligoduplex DNA competes for Dmp53 binding, whereas unlabelled mutant *GADD45* oligoduplex DNA does not (Figure 3A, lanes 5–8). Moreover, an anti-Dmp53 polyclonal antibody prevents DNA binding by Dmp53 (Figure 3A, lane 4), and an anti-Dmp53 monoclonal antibody supershifts the Dmp53/DNA complex (Figure 3B, lane 4). It is interesting that human p53, which was expressed and tested in an identical assay, bound p53 binding sites only in the presence of the activating antibody PAb421 (data not shown). PAb421 is thought to act by associating with a region in the carboxyl terminus of p53 that normally negatively regulates DNA binding (Hupp et al., 1992). The ability of Dmp53 to bind DNA without any activating treatments may indicate that a similar negative regulatory element does not exist in Dmp53.

We generated mutant alleles of *Dmp53* analogous to the R175H (R155H in Dmp53) and H179N (H159N in Dmp53) tumor-derived mutations in human *p53*. These mutations in human *p53* produce proteins with dominant-negative activity, presumably because they cannot bind DNA but retain a functional tetramerization domain (Brachmann et al., 1996; reviewed in Vogelstein and Kinzler, 1992). Thus, DNA binding by any tetramer that incorporates the mutant protein is disrupted. Both Dmp53R155H and H159N proteins inhibit binding of wild-type Dmp53 to a p53 binding site (Figure 3C, lanes 1–5), although they did not bind to DNA themselves. As described below, these mutant forms of Dmp53 are useful tools to test the function of wild-type Dmp53 in vivo.

Dmp53 Overexpression Induces Apoptosis but Not G1 Arrest

To determine whether Dmp53 can serve the same functions in vivo as human p53, we ectopically expressed Dmp53 in *Drosophila* larval eye discs using *glass*-responsive enhancer elements (Hay et al., 1994). The *glass-Dmp53* (*gl-Dmp53*) transgene expresses Dmp53 in all cells posterior to the morphogenetic furrow. The morphogenetic furrow marks the front of a wave of cellular differentiation that sweeps from the posterior to the anterior of the eye disc during larval development (Wolff and Ready, 1991). Thus, *gl-Dmp53* larvae express Dmp53 in all eye disc cells as they differentiate as well as in a subset of cells behind the furrow that undergo a final round of cell division before terminal differentiation.

Expression of Dmp53 from the *gl-Dmp53* transgene produces viable adults that have small, rough eyes with fused ommatidia (Figure 4B). TUNEL staining of *gl-Dmp53* eye discs shows that this phenotype is due, at least in part, to widespread apoptosis in cells expressing Dmp53 (Figure 4D). Similar results are seen when apoptotic cells are detected by acridine orange or Nile Blue (data not shown). TUNEL-positive cells appear within 15–30 cell diameters of the furrow. Given that the furrow is estimated to move approximately five cell diameters per hour (Wolff and Ready, 1991), this indicates that cells initiate apoptosis within 3–6 hr after Dmp53 is expressed.

The ability of p53 to induce apoptosis in some vertebrate cell types can be inhibited by overexpression of p21. The precise mechanism(s) through which p21 inhibits apoptosis is unknown, but direct inhibitory interactions with procaspase 3 (Suzuki et al., 1999a, 1999b) and apoptosis signal-regulating kinase 1 (Asada et al., 1999) have been reported. To determine if expression of human p21 can similarly suppress Dmp53-induced apoptosis, we coexpressed Dmp53 and p21 in the developing eye disc. p21 expression dramatically suppresses Dmp53-induced apoptosis in the disc (Figures 4I and 4J) as well as the adult rough-eye phenotype (data not shown). This suppression does not appear to involve reduction of p53 protein levels, since matched disc samples from larvae expressing *gl-Dmp53* (Figure 4I) or *gl-Dmp53* plus *gl-p21* (Figure 4J) show similar levels of anti-Dmp53 antibody staining. These data suggest that p53-related proteins in flies and vertebrates trigger apoptosis through similar p21-suppressible pathways. Surprisingly, we were unable to achieve similar inhibition of apoptosis by coexpression of the baculovirus p35 protein, a universal substrate inhibitor of caspases (data

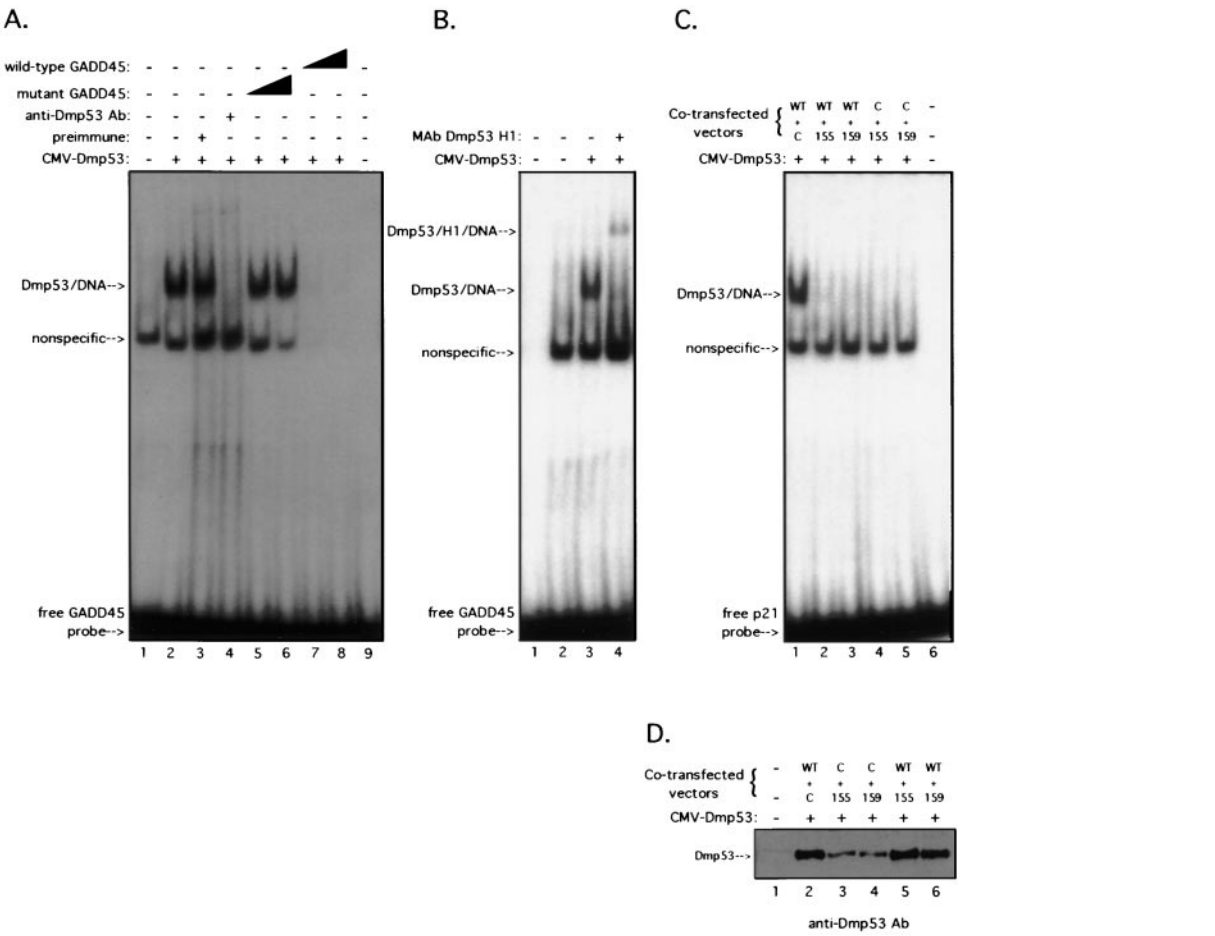

Figure 3. Wild-Type Dmp53 Binds Specifically to Human p53 Binding Sites, and This Binding Is Inhibited by Dmp53R155H and H159N Mutants

H1299 cells were transiently transfected with plasmids expressing either wild-type Dmp53, Dmp53R155H (155), or Dmp53H159N (159). A mammalian whole-cell extract was prepared (see Experimental Procedures), and 20 μg was used for electrophoretic mobility shift assays (EMSA) and Western blot analysis.

(A) EMSA showing the ability of wild-type Dmp53 to bind ^{32}P-labeled oligonucleotides containing the p53-binding element from *GADD45*. Antibodies were added as indicated: lane 3, preimmune serum, and lane 4, polyclonal anti-Dmp53 serum. Wild-type or mutant *GADD45* oligonucleotides were added in 10-fold (lanes 5 and 7) or 30-fold (lanes 6 and 8) molar excess over probe. Lane 1 contains an extract from cells expressing an empty CMV vector. Lanes 2–8 contain extracts expressing wild-type CMV-Dmp53. Lane 9 contains a reaction mixture lacking extract.

(B) EMSA showing the ability of wild-type Dmp53/*GADD45* complexes to be supershifted by the monoclonal anti-Dmp53 H1 antiserum (lane 4). Lane 1 contains a reaction mixture lacking extract. Lane 2 contains an extract from cells expressing an empty CMV vector. Lanes 3 and 4 contain an extract from cells expressing wild-type CMV-Dmp53.

(C) Dmp53 mutant proteins inhibit binding by wild-type Dmp53 to *p21*-binding site oligonucleotides. For each cotransfection (lanes 1–6), a constant amount of DNA (1 μg) was used for each expression vector ("WT," "155," and "159" refer to wild-type Dmp53, Dmp53R155H, and Dmp53H159N, respectively) or control ("C" refers to empty CMV vector) for a total of 2 μg. Lane 6 reaction mixture lacks extract.

(D) Western blot loaded with 20 μg of extract utilized in (C) and probed with anti-Dmp53 polyclonal antibody to detect Dmp53 in cells expressing either wild-type Dmp53 alone (WT + C), Dmp53R155H alone (C + 155), Dmp53H159N alone (C + 159), wild-type Dmp53 plus Dmp53R155H (WT + 155), or wild-type Dmp53 plus Dmp53H159N (WT + 159). Lane 1 contains 20 μg extract expressing an empty CMV vector.

For (A), (B), and (C), "nonspecific" refers to a nonspecific protein/DNA complex present in the H1299 whole-cell extracts.

not shown). Given that p35 has previously been shown to inhibit human p53-induced apoptosis in lepidopteran (Prikhod'ko et al., 1999) and *Drosophila* cells (Yamaguchi et al., 1999), the lack of p35 suppression of apoptosis may reflect different rates and/or levels of Dmp53 and p35 protein accumulation.

In addition to its ability to affect cell death pathways, mammalian p53 can induce cell cycle arrest at the G1 and G2/M checkpoints (Levine, 1997). In the *Drosophila* eye disc, the second mitotic wave is a synchronous, final wave of cell division posterior to the morphogenetic furrow (Tomlinson and Ready, 1987; Wolff and Ready, 1991). This unique aspect of development provides a means to assay for similar effects of Dmp53 on the cell cycle. Transition of these cells from G1 to S phase in wild-type discs can be detected by bromodeoxyuridine (BrdU) incorporation into DNA (Wolff and Ready, 1991) (Figures 4E and 4F). This transition from G1 to S phase is not blocked or delayed by Dmp53 overexpression from the *gl-Dmp53* transgene (Figure 4F). In contrast,

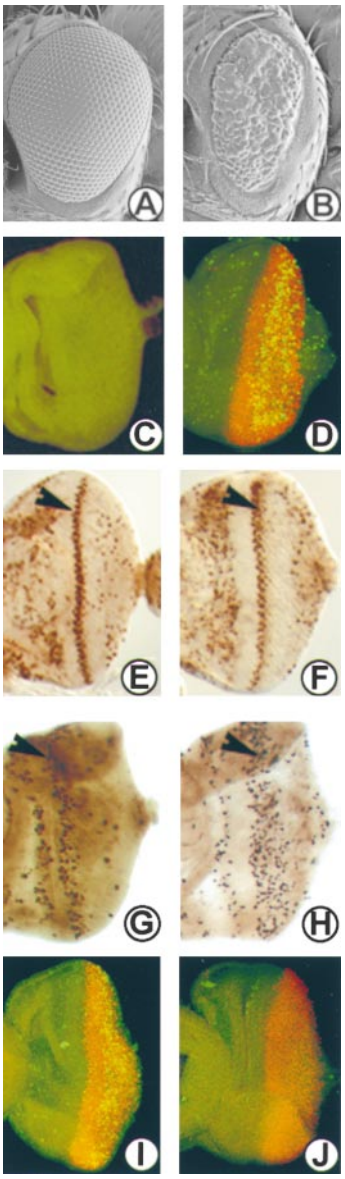

Figure 4. Effects of Dmp53 Overexpression in Eye Discs

(A and B) Scanning electron micrographs of eyes from a wild-type fly (A) or a fly carrying one copy of the *gl-Dmp53* transgene (B).

(C and D) Dmp53 overexpression induces apoptosis. Eye discs were dissected from a wild-type third instar larva (C) and a larva containing two copies of the *gl-Dmp53* transgene (D). Discs are stained by TUNEL (green) to mark apoptotic cells and with anti-Dmp53 antibody to show expression of Dmp53 (red).

(E and F) Dmp53 overexpression does not cause a G1 arrest in the eye disc. A wild-type disc (E) and a disc containing one copy of the *gl-Dmp53* transgene (F) were incubated with BrdU to label cells in S phase (brown). Arrows indicate S phase cells of the second mitotic wave.

(G and H) Dmp53 overexpression alters the pattern of mitoses in cells of the second mitotic wave. A wild-type disc (G) and a disc containing one copy of the *gl-Dmp53* transgene (H) were stained with an anti-phospho-histone antibody that specifically labels mitotic cells (brown). Arrows indicate the normal position of the second mitotic wave.

(I and J) Dmp53-induced apoptosis is suppressed by coexpression of human p21. Discs containing one copy of the *gl-Dmp53* transgene (I) or one copy each of the *gl-Dmp53* and *gl-p21* transgenes (J) were stained by TUNEL (green) and anti-Dmp53 antibody staining (red).

expression of human p21 or a *Drosophila* p21 homolog, dacapo, under control of the same *glass*-responsive enhancer element completely blocks DNA replication in the second mitotic wave (de Nooij and Hariharan, 1995; de Nooij et al., 1996; and data not shown). However, overexpression of Dmp53 does affect M phase in the eye disc. In wild-type discs, an M phase–specific anti-phospho-histone antibody typically stains a distinct band of cells within the second mitotic wave (Figure 4G). In *gl-Dmp53* larval eye discs, this band of cells is present but is significantly broader and more diffuse (Figure 4H), suggesting that Dmp53 alters the entry into and/or duration of M phase.

Reduced Dmp53 Function Blocks X Ray–Induced Apoptosis

We next sought to determine whether loss of Dmp53 function affected apoptosis or cell cycle arrest in response to DNA damage. In order to examine the phenotype of tissues deficient in Dmp53 function, we expressed the dominant-negative *Dmp53* alleles described above as transgenes under the control of tissue-specific promoters. Coexpression of Dmp53R155H with wild-type Dmp53 suppresses the rough eye phenotype that normally results from wild-type Dmp53 overexpression (Figure 5C), confirming that this mutant protein has dominant-negative activity in vivo. The same result was obtained by expressing the Dmp53H159N protein (data not shown). Unlike wild-type *Dmp53*, overexpression of the dominant-negative alleles using the *glass* enhancer (Figure 5A) or a constitutive enhancer (*arm*-GAL4; data not shown) has no visible effect on normal development.

In mammalian systems, p53-induced apoptosis plays a crucial role in preventing the propagation of damaged DNA (reviewed in Lowe, 1995). DNA damage also leads to apoptosis in *Drosophila*. To determine if this response requires the action of Dmp53, we expressed dominant-negative *Dmp53* transgenes in the posterior compartment of the wing disc. Wild-type wing discs show widespread apoptosis detectable by TUNEL staining 4 hr after X irradiation (Figure 5E). When either dominant-negative allele of *Dmp53* is expressed in the posterior compartment of the wing disc, apoptosis is blocked in the cells expressing Dmp53, whereas the anterior compartment displays a normal amount of X ray–induced cell death (Figures 5F and 5G). Thus, induction of apoptosis following X irradiation requires the function of Dmp53. This proapoptotic role for Dmp53 appears to be limited to a specific response to cellular damage, because developmentally programmed cell death in the eye and other tissues (Abrams et al., 1993) is unaffected by expression of either dominant-negative *Dmp53* allele (data not shown).

Although our data strongly suggest that Dmp53 function is required for X ray–induced apoptosis, it does not appear to be necessary for the cell cycle arrest induced by the same dose of irradiation. In the absence of irradiation, a random pattern of mitosis is observed in third instar wing discs of *Drosophila* (Figure 5H). Upon irradiation, a cell cycle block in wild-type discs leads to a significant decrease in anti-phospho-histone staining (Figure 5I; Brodsky et al., 2000a). This cell cycle block is unaffected by expression of dominant-negative Dmp53

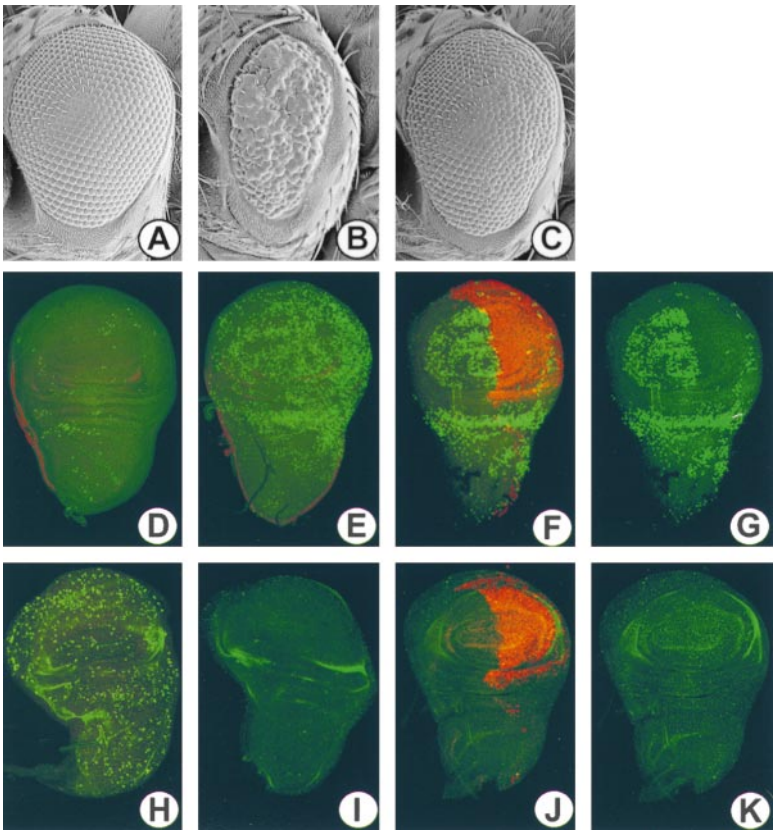

Figure 5. Dominant-Negative Dmp53 Blocks X Ray–Induced Apoptosis but Not X Ray–Induced Cell Cycle Arrest

(A–C) Dmp53R155H suppresses Dmp53 activity in vivo. Scanning electron micrographs of eyes from flies carrying one copy of the *gl-Dmp53R155H* transgene (A), one copy of the wild-type *gl-Dmp53* transgene (B), and one copy each of the *gl-Dmp53* and *gl-Dmp53R155H* transgenes (C).

(D–G) Dmp53H159N blocks X ray–induced apoptosis. Wing discs were dissected from third instar larvae containing an *engrailed-GAL4* transgene. Apoptotic cells are stained by TUNEL (green). Panels show an unirradiated disc (D), a disc dissected 4 hr after X irradiation (E), and a disc dissected 4 hr after X irradiation carrying a *UAS-Dmp53H159N* transgene shown with (F) and without (G) anti-Dmp53 antibody staining (red). The *UAS-Dmp53H159N* transgene is expressed specifically in the posterior compartment of the wing disc under the control of the *engrailed-GAL4* driver.

(H–K) Dmp53H159N does not block X ray–induced cell cycle arrest. Wing discs were dissected from third instar larvae containing an *engrailed-GAL4* transgene. Mitotic cells are stained with anti-phopho-histone antibody (green). Panels show an unirradiated disc (H), a disc dissected 4 hr after X irradiation (I), and a disc dissected 4 hr after X irradiation carrying a *UAS-Dmp53H159N* transgene shown with (J) and without (K) anti-Dmp53 antibody staining (red).

in the posterior of the wing disc (Figures 5J and 5K). Several time points after X irradiation were examined, and all gave similar results, suggesting that both the onset and maintenance of the X ray–induced cell cycle arrest is independent of Dmp53.

Expression of Dmp53 during Development

We characterized the expression of *Dmp53* transcripts during embryogenesis to assess potential roles for Dmp53 during *Drosophila* development (Figure 6). *Dmp53* RNA is maternally loaded into oocytes and is abundant until cellularization of the blastoderm. Zygotic expression of *Dmp53* begins at cellularization and is initially ubiquitous. At midembryogenesis, *Dmp53* RNA levels are highest in the mesoderm and gut, with only low levels of RNA detectable in the epidermal and neural cell layers. As development proceeds, the expression of *Dmp53* becomes progressively more restricted and falls dramatically in all tissues except for the primordial germ cells and a small patch of hindgut cells. Although

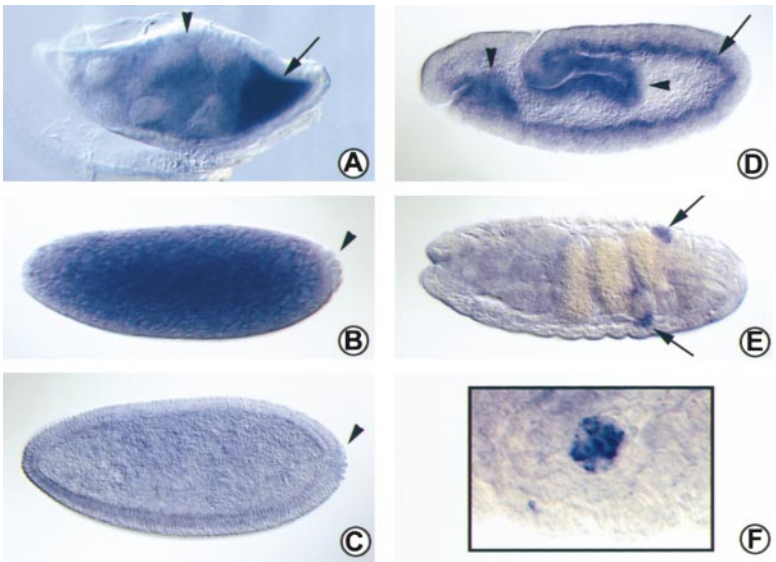

Figure 6. *Dmp53* RNA Expression during Development

(A) Stage 10 egg chamber showing high levels of *Dmp53* transcripts in the nurse cells (arrowhead) and oocyte (arrow).

(B) Stage 3 embryo, showing high levels of maternally loaded *Dmp53* RNA filling the embryo. Note that the posterior pole cells (primordial germ cells) are not stained (arrowheads in [B] and [C]). These cells are transcriptionally quiescent until midembryogenesis (Su et al., 1998).

(C) *Dmp53* maternal RNA levels are reduced by stage 5 (cellular blastoderm).

(D) At stage 10, zygotic expression of *Dmp53* RNA is highest in the mesoderm (arrow) and developing gut (arrowhead).

(E) By stage 16, *Dmp53* expression is dramatically reduced in all tissues with the exception of the primordial germ cells within the two gonads (arrows) and a small portion of the hindgut (not shown).

(F) Close up of *Dmp53* expression within the gonad of a stage 16 embryo.

one must use caution when inferring function from expression data, the high levels of *Dmp53* RNA in germ cells is likely to be significant because germline *p53* expression is a common feature in species ranging from clam (Van Beneden et al., 1997) to human (Bukovsky et al., 1995; Quenby et al., 1999). This conservation of expression suggests an important function for p53 in germline development.

Discussion

We have identified and characterized a *Drosophila* homolog of *p53*. Our data show that, like mammalian p53, Dmp53 is a sequence-specific DNA-binding protein whose function is required to induce apoptosis in cells damaged by X irradiation. Similar results are reported in the accompanying paper by Brodsky et al. (2000b [this issue of *Cell*]). Thus, the well-established proapoptotic activity of vertebrate p53 is an ancient, conserved function for this protein. However, there are also many intriguing differences between the mammalian and fly versions of p53. Perhaps the most noteworthy difference is the likelihood that the ability to serve as a G1 checkpoint regulator is a more recent evolutionary development. It is also probable that the relationship between p53 and MDM2 is a later event in the course of evolution.

Overexpression of Dmp53 in larval eye discs has no effect on transition of cells from G1 to S phase. While we do not know whether high levels of Dmp53 would be capable of affecting G1 in other cell types, this result may reflect differences in the downstream targets of vertebrate and *Drosophila* p53. In vertebrates, p53-mediated G1 arrest is due primarily to transcriptional activation of the cyclin-dependent kinase (cdk) inhibitor p21, which can inhibit the CDKs that phosphorylate and inactivate the retinoblastoma gene product (Rb). Phosphorylated Rb family members release and activate E2F/DP transcription factor complexes that are required for cell cycle progression (reviewed in Nevins et al., 1997). The *cdk-Rb-E2F/DP* pathway is highly conserved, and homologs of these genes regulate the cell cycle in *Drosophila* (de Nooij et al., 1996; Du et al., 1996; Edgar and Lehner, 1996; Lane et al., 1996). Overexpression of *p21* or its *Drosophila* homolog, *dacapo*, in eye discs clearly blocks the cell cycle in G1 (de Nooij and Hariharan, 1995; de Nooij et al., 1996). Therefore, the lack of a G1 arrest in *gl-Dmp53* discs suggests that expression of *dacapo* is not induced by Dmp53. This hypothesis is consistent with reports indicating that *dacapo* is not induced by X irradiation (de Nooij et al., 1996). We speculate that, although all the necessary components of the *p53* and *Rb* pathways appear to be present in *Drosophila*, later events in evolution made *p21* a target of p53 and thus established a dual role for p53 in apoptosis and G1 checkpoint control.

Although overexpression of Dmp53 in the eye disc had no detectable effect on entry into S phase, it did appear to delay entry into M phase. Given that overexpression of human p53 can similarly delay entry into M phase (Agarwal et al., 1995; Stewart et al., 1995), it is possible that this G2 checkpoint function is conserved in *Drosophila*. However, it is also possible that the G2/M phase delay we observed is simply an indirect consequence of the proapoptotic activity of Dmp53. The dominant-negative Dmp53 proteins had no detectable effect on cell cycle arrest in response to X irradiation. However, this negative result does not rule out a function for Dmp53 in G2 arrest because the *ATM/Chk1* pathway is conserved in *Drosophila* (Hari et al., 1995; Fogarty et al., 1997) and may be sufficient to induce G2 arrest in response to radiation. We also cannot rule out the possibility that our dominant-negative mutants might somehow block the apoptotic function of Dmp53 without eliminating a G2 checkpoint function. Thus, it remains an open question whether Dmp53 might play a role in radiation-induced G2 arrest.

As the most evolutionarily distant member of the *p53* gene family to be identified, *Dmp53* may shed light on the selective pressures that have maintained p53-like molecules throughout evolution. The absence of p53 function in mice increases the prevalence of defective embryos and fetuses, particularly after exposure to drugs or radiation (Nicol et al., 1995; Norimura et al., 1996). This embryo-protective role for p53 may have been a selective force for p53 evolution, since it would have a direct impact on reproductive success (Hall and Lane, 1997). Similarly, the expression of *p53* in the germ cells of flies, clams, and mammals strongly suggests selection for germline p53 function during evolution. The absence of p53 in mouse testes results in reduced spontaneous germ cell apoptosis and an increased frequency of abnormal sperm (Beumer et al., 1998; Yin et al., 1998). Thus, p53 may also have an important and conserved role in maintaining the fidelity of the germline through the elimination of defective cells.

X irradiation of imaginal wing discs induces apoptosis that requires functional Dmp53. This poses the question of how Dmp53 activity is regulated in response to radiation. It is well established that mammalian p53 receives signals from a variety of cellular stresses such as various forms of DNA damage, nucleotide deprivation, incomplete DNA synthesis, and hypoxia. These signals are likely to work through a set of signaling pathways that activate and stabilize the p53 protein (reviewed in Giaccia and Kastan, 1998; Prives and Hall, 1999). Although understanding of the different gene products responsible for these various signaling pathways is still in its infancy, there is strong evidence that one pathway to p53, that induced by irradiation, requires functional ATM (reviewed in Morgan and Kastan, 1997). The ATM protein kinase shares homology with other members of the PI3 kinase family, including the *S. pombe* DNA damage mediator kinase Rad3 (Elledge, 1996). Recently, two kinases that are downstream of ATM, CHK1, and CDS1/CHK2 have been shown to phosphorylate and regulate human p53 (Chehab et al., 2000; Shieh et al., 2000). Given that many aspects of the DNA damage checkpoint response are conserved between yeast and mammals, it is possible that Dmp53 might be similarly regulated. Indeed, *Drosophila* homologs of *ATM* (*mei41*) (Hari et al., 1995) and *Chk1* (*grapes*) (Fogarty et al., 1997) have been identified, and it will be interesting to determine their relationship to Dmp53. It should be mentioned here that while ATM has been clearly shown to regulate p53-mediated cell cycle arrest, there is evidence that apoptosis induced by p53 is independent of the function of

ATM (Barlow et al., 1997; Liao et al., 1999). Thus, the study of Dmp53-induced apoptosis in *Drosophila* may uncover new upstream regulators of p53 activity.

The ability of mammalian p53 to induce apoptosis has been studied in considerable detail. As mentioned in the introduction, a number of genes (e.g., *Bax*, *IGF-BP3*, *PIG3*, etc.) have been identified that are induced in response to p53 and can cause apoptosis. Genes such as *hid*, *grim*, and *reaper*, known to be involved in apoptosis in *Drosophila* (McCall and Steller, 1997; Abrams, 1999), are being tested for their relationship to Dmp53. Brodsky et al. (2000b) report that *reaper* is, in fact, a downstream target of Dmp53 transcriptional activation. There are reports as well that transcriptional repression may be a necessary component of some aspects of the p53 apoptotic program (Shen and Shenk, 1994; Sabbatini et al., 1995). DNA site selection (SELEX) or DNA arrays will be useful in identifying additional *Drosophila* genes that are targets of Dmp53.

Our discovery of Dmp53 and its conserved role in radiation-induced apoptosis provides a new model system to study the function of a key tumor suppressor gene. Many important cellular pathways and functions have been elucidated through the elegant genetics that *Drosophila* affords. The study of Dmp53 is likely to provide new insights into pathways that regulate mammalian p53-induced apoptosis and may suggest new therapeutic approaches to cancer.

Experimental Procedures

Cloning of Dmp53

A partial *Dmp53* cDNA was found by a BLAST version 2.0 (Altschul et al., 1990) search of an EST database representing embryo, disc, and adult head mRNA (C. K. et al., unpublished data). The full sequence of *Dmp53* was determined from three sources: sequencing a commercially available cDNA clone (Research Genetics, ID #31553); sequencing RACE products from embryo and adult Marathon-ready *Drosophila* cDNA libraries (Clontech); and sequencing genomic DNA from P1 clones *DS01201*, *DS02942*, *DS05102*, and *DS06254* (Berkeley *Drosophila* Genome Project). Dominant-negative versions of *Dmp53* containing the mutations R155H and H159N were generated by PCR-mediated mutagenesis. The *p53*-related genes from *Tribolium castaneum* and *Leptinotarsa decemilineata* were identified by BLAST searches of internally generated EST databases.

Sequence Analysis and Molecular Modeling

The multiple sequence alignment and phylogenetic tree were created using the program ClustalW 1.7 (Thompson et al., 1994; freely available at ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalW). The Genbank identifier numbers for the sequences used in the alignment and/or tree are as follows: *Loligo forbesi p53/73*, gi|1244762; *Barbus barbus p73*, gi|4689086; *Mus musculus p73*, gi|4887145, *p63*, gi|3695094, and *p53*, gi|53571; *Homo sapiens p73*, gi|35214, *p63(KET)*, gi|3970717, and *p53*, gi|35214; *Xenopus laevis p53*, gi|545102; *Danio rerio p53*, 1778019.

Secondary structure predictions were made using the software Simpa (Levin, 1997; http://www.embl-heidelberg.de/argos/simpa/). A low-resolution three-dimensional model for Dmp53 residues 90–275 was built using comparative structural modeling with the program Modeler/InsightII 98.0 (Molecular Simulations, Inc.) (Sali and Blundell, 1993; Sanchez and Sali, 1997). The coordinates for residues 108–298 in the crystal structure of the human p53 DNA-binding domain (PDB code 1TUP) (Cho et al., 1994) were used as the template. Figure 1B shows the alignment used in the calculations.

Electrophoretic Mobility Shift Assays

H1299 cells (American Type Culture Collection) were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. Cells were transfected by a lipopolyamine-based (Transfectam) protocol as described previously (Di Como et al., 1999). Briefly, cells were grown in RPMI1640/10% FBS and transfected with various amounts of DNA. The precipitate was left on the cells for 12 hr, after which fresh media was added for the periods indicated. *CMV-Dmp53*, *CMV-Dmp53R155H*, and *CMV-Dmp53H159N* express full-length *Dmp53*, *Dmp53R155H*, and *Dmp53H159N* cDNAs from the cytomegalovirus (CMV) promoter in *pRc/CMV* (Invitrogen).

H1299 cells were transfected in six-well, 9.6 cm^2 plates with 2 μg of DNA and harvested at 48 hr after transfection. Cells were lysed in 200 μl of lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% NP40, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.5 mM PMSF, and a mixture of protease inhibitors), and the extracts were centrifuged at 16,000 $\times$ g for 20 min to remove cell debris. Protein concentrations were determined using the Bio-Rad assay using bovine globulin as a standard (Bio-Rad Laboratories, CA). When necessary, total cell extracts were frozen in a dry ice/alcohol bath and stored at -80°C. For Western blot analysis, samples were electrophoresed through an SDS-10% polyacrylamide gel. Protein gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). For Dmp53 detection, an anti-Dmp53 polyclonal antibody was used at a 1/3000 dilution. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham).

Probes were made as follows: synthesized oligonucleotides (Operon Technologies, Inc.) were self-annealed and end-labeled with T4 polynucleotide kinase (PNK) in the presence of [γ-^{32}P]ATP. The oligonucleotide probe containing the *p21 cis*-acting p53-responsive element (el-Deiry et al., 1993) is as follows: 5′-TAGAGC*GAACATGT CCCAACATGTTG*GCGTCG-3′. The oligonucleotide probe containing the wild-type *GADD45 cis*-acting p53-responsive element (Kastan et al., 1992) is as follows: 5′-TAGAGC*GAACATGTCTAAG CATGCTG*GCGTCG-3′. The oligonucleotide probe containing the mutant *GADD45 cis*-acting element is as follows: 5′-TAGAGC *GAAaATtTCTAAGaATtCTG*GCGTCG-3′. The p53 binding site is italicized, and the mutated nucleotides are in lowercase lettering. The oligonucleotides were purified over a denaturing 16% acrylamide gel, and both complementary strands were annealed and purified over a native 10% acrylamide gel.

Electrophoretic mobility shift assays were performed as described (Di Como and Prives, 1998), except for minor changes. Binding reaction mixtures contained 20 μg mammalian whole-cell extract in gel shift buffer (20 mM HEPES [pH 7.9], 25 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 0.5 mM DTT, 0.025% Nonidet P-40, 2 mM spermidine [Sigma], 10% glycerol, 0.1 μg/μl acetylated bovine serum albumin [NEB]) and 120 ng double-stranded poly(d[I-C]) (Boehringher Mannheim) in a final volume of 30 μl. In all cases, volumes were equalized with lysis buffer. Reactions were incubated for 10 min at 24°C, after which 5 ng of ^{32}P-labeled oligonucleotide was added, and the incubation was continued at 24°C for 15 min. Samples were run on 4.5% native polyacrylamide gels at 180–200V (not exceeding 40 mA current) in 0.5$\times$ TBE buffer at 24°C for 1.5 hr. Gels were dried under vacuum at 80°C and exposed to Kodak XAR film at 24°C for 30–60 min.

Drosophila Genetics

Fly culture and crosses were performed according to standard procedures at 22°C–25°C. *Dmp53*, *Dmp53R155H*, and *Dmp53H159N* transgenes were made by cloning a BclI/HincII fragment spanning the *Dmp53* open reading frame into *pExpress-glass* and *pExpress-UAS* transformation vectors. The *pExpress* vector is an adapted version of the *pGMR* vector (Hay et al., 1994), which contains an α-*tubulin* 3′ UTR for increased mRNA stabilization and a modified multiple cloning site. The *pExpress-UAS* vector is similar to *pExpress-glass* except that it contains multiple *UAS-GAL4* binding sites in place of the *glass* binding sites. Standard P element–mediated germline transformation was used to generate transgenic lines containing these constructs. For X irradiation experiments, third instar larvae in vials were exposed to 4000 Rads of X rays using a Faxitron X-ray cabinet system.

Staining of Embryos and Discs

For immunocytochemistry, third instar larval eye and wing discs were dissected in PBS, fixed in formaldehyde at room temperature

(4% for 5 min for anti-phospho-histone or 2% for 30 min for anti-Dmp53 plus TUNEL), permeabilized in PBS + 0.5% Triton (15 min at room temperature), blocked in PBS + 5% goat serum, and incubated with primary antibody (2 hr at room temperature or overnight at 4°C). Anti-Dmp53 monoclonal antibody staining was performed using a 1:2 dilution of hybridoma supernatant. Hybridoma clone H2 was used for anti-Dmp53 staining, and a mixture of supernatant from clones H2, H4, and H5 were used to detect Dmp53R155H and Dmp53H159N. Anti-phospho-histone staining was performed using an Anti-phospho-histone H3 Mitosis Marker (Upstate Biotechnology) at a 1:500 dilution. Goat anti-mouse or anti-rabbit secondary antibodies conjugated to FITC or Texas Red (Jackson Immunoresearch) were used at a 1:200 dilution. Antibodies were diluted in PBS + 5% goat serum. TUNEL was performed using the Apoptag Direct kit (Oncor) per manufacturer's protocol with a 0.5% Triton/PBS permeabilization step. Discs were mounted in anti-fade reagent (Molecular Probes), and images were obtained on a Leica confocal microscope. BrdU staining was performed as described (de Nooij and Hariharan, 1995). In situ hybridization was performed as described (Van Vactor and Kopczynski, 1999) using digoxygenin-labeled antisense RNA probes.

Generation of Anti-Dmp53 Antibodies

Anti-Dmp53 rabbit polyclonal and mouse monoclonal antibodies were generated by standard methods using as antigen a full-length Dmp53 protein fused to glutathione-S-transferase (GST-Dmp53). Inclusion bodies of GST-Dmp53 were purified by centrifugation following extraction in B-PER buffer (Pierce) and injected subcutaneously into rabbits (Josman Labs) and mice (Antibody Solutions, Inc.) for immunization. The final boost for mouse monoclonal antibody production used intravenous injection of soluble GST-Dmp53 produced by solubilization of GST-Dmp53 in 6 M Guanidine-Hydrochloride and dialysis into phosphate buffer containing 1 M NaCl. Hybridoma supernatants were screened by ELISA using soluble 6× HIS-tagged Dmp53 protein bound to Ni-NTA-coated plates (Qiagen) and an anti-mouse IgG Fc fragment–specific secondary antibody.

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