Dual roles of Drosophila p53 in cell death and cell differentiation

Yun Fan, Tom V. Lee, Dongbin Xu, Zhihong Chen, Anne-Francoise Lamblin, Hermann Steller, and Andreas Bergmann.

1 Department of Biochemistry & Molecular Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard – Unit 1000, Houston, TX 77030
2 University of Minnesota Interdisciplinary Informatics, OVPR, 1500 Gortner Ave, Saint Paul, MN 55108
3 Howard Hughes Medical Institute, The Rockefeller University, 1230 York Ave., New York, NY 10021

Summary

The mammalian p53-family consists of p53, p63 and p73. While p53 accounts for tumor suppression through cell cycle arrest and apoptosis, the functions of p63 and p73 are more diverse and also include control of cell differentiation. The Drosophila genome contains only one p53 homolog, Dp53. Previous work has established that Dp53 induces apoptosis, but not cell cycle arrest. Here, by using the developing eye as a model, we show that Dp53-induced apoptosis is primarily dependent on the pro-apoptotic gene hid, but not reaper, and occurs through the canonical apoptosis pathway. Importantly, similar to p63 and p73, expression of Dp53 also inhibits cellular differentiation of photoreceptor neurons and cone cells in the eye independently of its apoptotic function. Intriguingly, expression of the human cell cycle inhibitor p21 or its Drosophila homolog dacapo can suppress both Dp53-induced cell death and differentiation defects in Drosophila eyes. These findings provide new insights into the pathways activated by Dp53 and reveal that Dp53 incorporates functions of multiple p53-family members.

Introduction

p53 is a well-known tumor suppressor. Depending on cell type and cellular context, activation of p53 can trigger multiple cellular responses including cell cycle arrest and apoptosis (reviewed in 1). Although p53 functions through several mechanisms, it has been best characterized as a transcription factor that activates target genes including the cyclin-dependent kinase (CDK) inhibitor p21 and the pro-apoptotic genes Puma and Noxa.1 In addition, p63 and p73, two p53 paralogs, have been identified in vertebrates (reviewed in 2,3). They can induce apoptosis, but also have additional functions because p63−/− and p73−/− knockout mice show clear developmental defects, in contrast to p53−/− null mice which are viable and develop normally.2,3 The analysis of the p63−/− phenotype revealed that p63 is required for epithelial stem cell maintenance. In the absence of p63, these stem
cells undergo terminal differentiation and do not remain to sustain the epidermis.\textsuperscript{4} \(p63^{-/-}\) mice die shortly after birth. The \(p73^{-/-}\) phenotype is more complex, and also includes differentiation defects of certain populations of neurons in the brain. Further complicating is the observation that there are antagonistic p53 family members which are produced from additional intronic promoters generating N-terminally truncated (ΔN) isoforms (reviewed by \textsuperscript{3,4}). The ΔN isoforms can bind to the full length transactivating (TA) isoforms of p53, p63 and p73, and antagonize their function.\textsuperscript{3,4} Thus, this complexity makes it very difficult to dissect the functional mechanisms of the p53-family members in vertebrates. It is therefore attractive to examine the ancestral function of p53 orthologs in invertebrates such as \textit{Drosophila}.

The \textit{Drosophila} genome contains a single p53 family member, referred to as Dp53.\textsuperscript{5–7} Similar to mammalian p53, Dp53 null mutant flies are viable, fertile and with the exception of an apoptotic defect of primordial germ cells, they have no obvious developmental defects.\textsuperscript{8,9} In contrast to mammalian p53, Dp53 appears unable to induce radiation-induced cell cycle arrest.\textsuperscript{5,6,8} Similarly, mammalian cells lacking p63 and p73 are also unable to induce DNA damage-induced cell cycle arrest.\textsuperscript{10} Consequently, Dp53 and various forms of irradiation do not induce the expression of the \textit{Drosophila} p21 homolog, \textit{dacapo} (\textit{dap}).\textsuperscript{8,11}

Importantly, the pro-apoptotic function of p53 is well-conserved in \textit{Drosophila}. In response to apoptotic stimuli, the pro-apoptotic genes \textit{reaper} (\textit{rpr}), \textit{head involution defective} (\textit{hid}), and \textit{grim} are both necessary and sufficient to induce apoptosis through inhibition of the caspase inhibitor Diap1, which subsequently leads to activation of the initiator caspase Drone and two major effector caspases, DrICE and Dcp-1 (reviewed by \textsuperscript{12}). In response to radiation-induced DNA damage, Dp53 activates the transcription of \textit{rpr} to initiate apoptosis.\textsuperscript{6} In this process, \textit{hid} is also induced, but the details are less clear.\textsuperscript{8,11,13}

Expression of Dp53 in developing \textit{Drosophila} eyes induces massive cell death.\textsuperscript{5,7} However, the Dp53-induced eye phenotype cannot be completely blocked by expression of \textit{p35}, a potent inhibitor of DrICE and Dcp-1, suggesting that an effector caspase-independent mechanism of Dp53-induced apoptosis may exist in \textit{Drosophila}. There is precedence for a potential caspase-independent function of p53. Overexpression of the \textit{C.elegans} p53 homolog, \textit{cep-1}, caused wide-spread cell death independently of caspase activation.\textsuperscript{14}

Here, we further examined the phenotypes obtained by expression of Dp53 in \textit{Drosophila} eyes. We show by mutant analysis that only \textit{hid}, but not \textit{rpr}, is required for Dp53-induced apoptosis in this system. In addition, expression of Dp53 can activate the canonical caspase-dependent apoptosis pathway in \textit{Drosophila}. Consistently, and in contrast to previous reports, we found that \textit{p35} can block cell death induced by expression of Dp53. However, inhibition of apoptosis does not rescue the Dp53-induced rough and small adult eye phenotype. We show that expression of Dp53 causes differentiation defects of various cell types including photoreceptor neurons and cone cells independently of its pro-apoptotic function. These differentiation defects imply that Dp53 may also have genetic features of mammalian p63 and p73 proteins. Intriguingly, expression of the \textit{p53} target gene, human p21, or its \textit{Drosophila} homolog \textit{dap} can suppress Dp53-induced cell death as well as cell differentiation defects. These findings reveal that Dp53 incorporates functions of multiple
mammalian p53-family members and provide new insights into the pathways activated by Dp53.

Results

Expression of \textit{Dp53} induces cell death through the canonical apoptosis pathway in \textit{Drosophila} eyes

Expression of \textit{Dp53} in the fly eye either directly under control of the eye-specific \textit{GMR} promoter (\textit{GMR-Dp53}) or using a modified UAS-Gal4 system (\textit{GMR-Gal4 GUS-Dp53}; referred to as \textit{GMR\textgreater{}GUS-Dp53})8 induces small and rough eyes with glossy appearance (Figure 1a–c).5,7,8 This eye ablation phenotype is induced specifically by \textit{Dp53} as it can be fully rescued by co-expression of a dominant negative form of \textit{Dp53}, \textit{Dp53\textgreater{}H159N} (ref. 5), or by \textit{Dp53} RNAi (data not shown).

It has been reported that the eye ablation phenotype of \textit{GMR-Dp53} cannot be rescued by co-expression of the caspase inhibitor p35, an inhibitor of the effector caspases DrICE and Dcp-1 (ref.5, see Figure 2h). This observation may suggest that \textit{GMR-Dp53} causes the eye ablation phenotype independently of caspase activation. Therefore, we examined the pro-apoptotic function of \textit{Dp53} in more detail. First, we labeled \textit{GMR-Dp53} and \textit{GMR-GAL4\textgreater{}GUS-Dp53} eye imaginal discs from late third instar larvae with an antibody detecting activated caspases (Cas3*). The obtained labeling pattern (Figure 1d–f) resembles the TUNEL pattern in these discs (Figure 1g–i) and corresponds to the expression domain of \textit{GMR}.15 Therefore, \textit{Dp53} can induce caspase activation.

Next, we asked whether the three major pro-apoptotic genes, \textit{rpr}, \textit{hid}, and \textit{grim}, are required for \textit{Dp53}-induced cell death. A deficiency, \textit{Df(3L)H99} (referred to as \textit{H99}) which deletes these three genes,16 was used for mosaic analysis. In late 3\textsuperscript{rd} instar \textit{GMR\textgreater{}GUS-Dp53} eye discs, \textit{Dp53}-induced cell death as detected by TUNEL is completely blocked in \textit{H99} mutant clones (arrows, Figure 2a,a'). Furthermore, \textit{Dp53}-induced cell death is also absent in mutant clones of the initiator caspase \textit{dronc} (Figure 2b,b') or its adaptor \textit{ark} (Figure 2c,c') which encode the apoptosome components of the canonical apoptotic pathway.17–21 These results indicate that the canonical \textit{Drosophila} apoptotic pathway indeed mediates \textit{GMR-Dp53}-induced cell death.

We next asked why expression of the caspase inhibitor p35 does not suppress the \textit{GMR-Dp53}-induced eye phenotype5 (Figure 2h) and examined whether expression of p35 can block \textit{Dp53}-induced apoptosis at the cellular level. Strikingly, simultaneous expression of p35 and \textit{Dp53} under control of the same \textit{GMR-GAL4} driver strongly inhibits \textit{Dp53}-induced apoptosis in the developing eye disc (Figure 2d,d'). The suppression of \textit{Dp53} by p35 is not restricted to eye imaginal discs and can also be observed in wing imaginal discs (Supplemental Figure S1).

Taken together, these data suggest that \textit{Dp53} activity triggers apoptosis through the canonical apoptotic pathway including pro-apoptotic genes and activated caspases. Intriguingly, we also noticed that, although \textit{GMR-Dp53}-induced cell death is completely blocked in \textit{H99}, \textit{dronc} or \textit{ark} mutant clones, and is strongly suppressed by expression of
p35, the resulting adult eyes are not or only partially rescued indicated by their rough and glossy appearance (compare Figure 2e–h with Figure 1b,c). This is in striking contrast to the strong suppression of the GMR-hid- and GMR-reaper-induced eye ablation phenotypes by loss of drone and ark, or expression of p35.22–26 Therefore, this analysis raises two questions. First, which of the H99 genes rpr, hid or grim is required for caspase activation and apoptosis in GMR-Dp53 eye discs? Second, why is the eye ablation phenotype of GMR-Dp53 not rescued when apoptosis is blocked?

Hid is the major effector of Dp53-induced apoptosis in the Drosophila eye

To examine which pro-apoptotic genes mediate GMR-Dp53-induced apoptosis, we first examined the expression of hid in GMR-Dp53 eye discs. Compared to wild type eye discs (Figure 3a–a″), the protein level of Hid is strongly increased in the area where GMR drives expression of Dp53 and apoptosis (Figure 3b′,b″; see also Figure 2d). Because Dp53 encodes a transcription factor we tested whether this increase of Hid protein is due to increased hid transcription using a hid-lacZ reporter transgene (see Material and Methods). In GMR-Dp53 eye discs, hid is indeed transcriptionally induced (Figure 3b,b″). hid expression in response to Dp53 is not only present in developing eye discs but was also found in wing discs (Supplemental Figure S2b,b′) compared to controls (Supplemental Figure S2a).

Because rpr has been shown to be a direct target of Dp53 in response to X-ray induced DNA damage,6 we also analyzed expression of rpr in GMR-Dp53. However, high background expression levels of the rprXRE-lacZ (XRE – X-ray response element) reporter transgene prevented us from assessing rpr expression in eye discs (data not shown). Nevertheless, we were able to detect increased reporter expression of the rprXRE-lacZ transgene upon expression of Dp53 in wing discs (Supplemental Figure S2d,d′) compared to controls (Supplemental Figure S2c). Therefore, both hid and rpr are transcriptionally induced by Dp53 in imaginal eye and wing discs.

From the H99 mutant analysis (Figure 2a), we know that one or more of the H99 genes are important for GMR-Dp53-induced apoptosis. To identify which gene is required for Dp53-induced apoptosis, we tested individual mutants. Surprisingly, loss of rpr by using a combination of deletions (H99/Df(3L)XR38),27 or a null mutant of rpr, rpr87, (ref.13) did not significantly affect the level of GMR-Dp53-induced apoptosis (Figure 3c,c′,d,d′). In contrast, null mutants of hid suppress most of GMR-Dp53-induced cell death (Figure 3e,e′) indicating that Hid is the primary mediator of apoptosis induced by GMR-Dp53.

GMR-Dp53 causes cell differentiation defects independently of its apoptotic function

Although GMR-Dp53 induces apoptosis mainly through hid and its downstream canonical apoptotic pathway, the adult GMR-Dp53 eye phenotype cannot be rescued by blocking the apoptotic pathway (compare Figure 2e–h to Figure 1b,c). It is therefore reasonable to examine whether eye-specific expression of Dp53 can cause developmental defects other than apoptosis. It has been suggested that Dp53 expression may also cause differentiation defects.28 To investigate this possibility, we examined differentiation of various cell types in Dp53-expressing eye discs by using the cellular differentiation markers ELAV (labels all
photoreceptor neurons R1-R8), Rough (Ro; R2-R5) and Seven-up (Svp; R2, R5, R1 and R6). The expression of these differentiation markers in wild-type and GMR-Dp53 eye discs is shown in Supplemental Figure S3. Although differentiated photoreceptor neurons are slightly disorganized at the late larval stage, differentiation of all types of photoreceptor neurons as visualized by these differentiation markers appears largely normal in GMR-Dp53 (Figure S3b–b‴). In contrast, the numbers of R7 photoreceptor neurons and cones cells labeled by the markers Prospero (Pros) and Cut, respectively, are strongly reduced compared to wild type (Figures 4a–d′). Therefore, expression of Dp53 under control of the GMR promoter affects differentiation of R7 and cone cells. To further examine the differentiation defect, GMR-Dp53 pupal eye discs were analyzed. In pupal GMR-Dp53 eye discs, ommatidia are severely mis-organized, as indicated by enlarged interommatidial space, ommatidial fusions (arrows, Figure 4f) and reduced number of cone cells in each ommatidium. Altogether, these data indicate that GMR-Dp53 causes differentiation defects in developing Drosophila eyes.

Because Dp53 induces apoptosis, we wondered whether these differentiation defects are caused by the pro-apoptotic function of Dp53, and analyzed cell differentiation in Dp53-expressing, but apoptosis-deficient background. In control experiments, inhibition of apoptosis in otherwise wild-type background does not affect cell differentiation (data not shown). Although cell death is blocked in H99 or dronc mutant tissues (see Figures 2a,b and 5b), R7 photoreceptor differentiation is not rescued in GMR-Dp53 eye discs (Figure 5a′,b′). Similar results were obtained during pupal development (Figures 5c–e′). This analysis suggests that expression of Dp53 causes differentiation defects independently of its pro-apoptotic role.

Next, we investigated why differentiation of R7 and cone cells was affected by GMR-Dp53, but not that of other photoreceptor neurons. R7 and cone cells are the last cell types to be specified in the larval eye disc. In contrast, photoreceptors R8, R2, R3, R4 and R5 are specified earlier at around the time when GMR induces expression of Dp53. Thus, it is possible that Dp53 is expressed too late to block differentiation of photoreceptor neurons R8 and R2-R5. We examined this possibility by inducing Dp53-expressing clones at earlier stages. To block Dp53-induced apoptosis and thus to obtain Dp53-expressing clones, the caspase inhibitor p35 was expressed simultaneously with Dp53. Under these conditions, all types of photoreceptor neurons as indicated by ELAV-labeling are missing in Dp53/p35 expressing tissues (arrows, Figures 5f′-f‴). Expression of p35 alone does not affect photoreceptor and cone cell differentiation (data not shown). Thus, these observations suggest that Dp53 can only block differentiation if it is expressed before the onset of differentiation. In summary, these data show that expression of Dp53 can interfere with differentiation of all cell types in developing Drosophila eyes independently of its pro-apoptotic function.

**Human p21 and Drosophila Dap suppress both GMR-Dp53-induced apoptosis and cell differentiation defects**

It has been reported that expression of human p21 can suppress Dp53-induced apoptosis in Drosophila.5 Because we showed above that Dp53-induced cell differentiation defects and
apoptosis are independent of each other (see above), we further examined whether and how human p21 can rescue GMR-Dp53-induced phenotypes in more detail. Expression of p21 under GMR control (GMR-p21) causes a rough eye phenotype due to decreased cell proliferation (Figure 6a).29 GMR-p21 does not or only mildly induce cell death and does not affect photoreceptor differentiation (Figure 6b,c). Consistently with previous observations, GMR-p21 suppresses GMR-Dp53-induced apoptosis in larval eye discs (Figure 6e). More importantly, in contrast to p35, GMR-p21 rescues both the R7 differentiation defect in larval eye discs and the eye ablation phenotype of GMR-Dp53 adults (Figure 6d,f). Similarly, although to a lesser extent, expression of the Drosophila homolog of p21, dacapo (dap),30,31 also suppresses both Dp53-induced cell death and cell differentiation defects (Figures 6g–i). As described above, because expression of Dp53 induces expression of the apoptotic gene hid, we examined whether GMR-p21 can modulate GMR-Dp53-induced expression of hid. Indeed, the protein level of Hid is reduced when human p21 is co-expressed in Dp53-expressing eyes (compare Figure 6j to Figure 3b‴). This observation suggests that p21 interferes with Dp53 upstream of hid to suppress Dp53-induced apoptosis in Drosophila (Figure 6k).

**Discussion**

In this study, we used the developing Drosophila eye as an in vivo model to analyze the function of Dp53 at the cellular level. Our study makes three important points. First, Hid is the major effector of GMR-Dp53-induced apoptosis, triggering the canonical caspase-dependent apoptotic pathway. Second, Dp53 induces differentiation defects of all cell types in the eye. This activity is independent of the pro-apoptotic role of Dp53, and reminiscent of mammalian p63 and p73. Third, these dual roles of Dp53 can be inhibited by expression of human p21 or its Drosophila homolog dap. In the following, we discuss these observations in detail.

**Hid is the major effector of GMR-Dp53-induced apoptosis**

Previous studies of radiation-induced cell death have shown that the pro-apoptotic genes rpr and hid are transcriptionally induced by Dp53.6,8,11,32,33 The fast induction (within 30 min) of hid and rpr suggested that they may be direct targets of Dp53.8 A radiation-responsive enhancer containing a typical p53-binding consensus site was identified in the upstream regulatory region of rpr.6 GMR-Dp53 induces reporter expression from the same radiation-responsive enhancer suggesting that induction of rpr by Dp53 is context-independent. However, despite expression of rpr, loss of rpr does not significantly influence GMR-Dp53-induced apoptosis. In contrast, complete loss of hid significantly abrogated GMR-Dp53-induced apoptosis suggesting that Hid is the major mediator of GMR-Dp53. This is consistent with previous findings that heterozygosity of hid partially suppresses radiation-induced apoptosis.8 Therefore, it appears that Dp53—whether its expression is induced by irradiation or by the heterologous GMR promoter—induces apoptosis by similar molecular mechanisms.

It is unclear why hid plays a more important role for GMR-Dp53-induced apoptosis than rpr in this system. Simple expression of rpr may not be sufficient for apoptosis induction and
additional activation may be required. However, this possibility appears unlikely because expression of rpr from the GMR-promoter is sufficient to induce apoptosis. Alternatively, the developing eye may be more prone to hid-induced apoptosis because those cells that die by developmental apoptosis in the eye, die primarily by hid-induced apoptosis. Such a tissue-specific requirement has also been reported for rpr, which is required for apoptosis of abdominal neuroblasts in the central nervous system. Thus, it will be interesting to investigate the pro-apoptotic requirements of Dp53 in other tissues including neuroblasts.

**Dp53 has a conserved function in regulating cell differentiation**

Our analysis indicates that Dp53 blocks cell differentiation of photoreceptor neurons and cone cells independently of its pro-apoptotic role. Interestingly, Dp53 can only block differentiation if it is expressed before the onset of differentiation. Notably, p53, p63 and p73, have also been implicated in the control of cell differentiation (reviewed in 1,3). However, in the cases reported expression of the p53 family members in undifferentiated cells actually induces differentiation instead of inhibiting it as shown here for Dp53. For example, the TA isoform of mouse p53 induces differentiation of mouse embryonic stem cells. Nevertheless, the antagonizing ΔN isoforms of p63 and p73 have been found to promote stem cell proliferation. For example, ΔNp63 is highly expressed in epidermal stem cells, and loss of p63 triggers these cells to terminally differentiate suggesting that ΔNp63 inhibits differentiation (reviewed in 3,4). Likewise, expression of ΔNp73 inhibits myogenic differentiation. ΔNp63 and ΔNp73 can also interfere with p53-induced differentiation programs. Importantly, the Dp53 gene also has an internal promoter and the originally identified Dp53 gene including the one used in this study actually corresponds to the ΔN isoform of Dp53. Therefore, our finding that expression of Dp53 suppresses cell differentiation is consistent with the inhibitory role of ΔN isoforms in cell differentiation. Therefore, Dp53 has similar genetic properties to mammalian ΔNp63 and ΔNp73 isoforms. This statement is also supported by the observation that mammalian cells lacking p63 and p73 are unable to induce DNA damage-induced cell cycle arrest, similar to Dp53. However, both mammalian p53 and Drosophila Dp53 lack the SAM domain at the C-terminus, which is characteristic for p63 and p73. The SAM domain supports oligomerization of p63 and p73. The absence of the SAM domain may indicate that Dp53 is more related to mammalian p53 rather than to p63 and p73. Nevertheless, BLAST searches with Dp53 revealed higher similarity to mammalian p63 and p73 than to p53. Furthermore, it was recently shown that the SAM domain of Dp53 was replaced during evolution by a helix domain which also supports oligomerization. Thus, both genetically and functionally, Dp53 resembles p63 and p73 more than p53.

**p21 and Dacapo antagonize an early step of Dp53 activation**

Depending on the cell type, expression of human p21 can suppress p53-induced apoptosis (reviewed by 39). Because after DNA damage p53 induces cell cycle arrest through induction of p21, it is thought that p21-mediated suppression of p53-induced apoptosis would give cells the opportunity to repair damaged DNA first, before induction of apoptosis, depending on the extent of DNA damage. We extend these observations further and show
here that human p21 not only suppresses Dp53-induced apoptosis, but also suppresses the Dp53-induced block of cell differentiation. Remarkably, the suppression of Dp53-induced phenotypes is more efficient by human p21 than by Drosophila Dap. It is surprising that this control of Dp53 activity is conserved in flies, given that Dp53 is not required for radiation-induced cell cycle arrest and also does not induce dap expression.\textsuperscript{5,6,8,11} However, the fact that Dap exerts at least some anti-Dp53 activity suggests that human p21 did not acquire this activity recently in evolution. It rather appears that Dap may have partially lost it because it is not induced by Dp53 and thus there was no selective pressure for Dap to maintain its anti-Dp53 activity during evolution, hence the weaker suppression.

The molecular mechanisms by which p21 suppresses p53-induced apoptosis are unclear and somewhat contradictory (reviewed in 39). According to several studies, the anti-apoptotic function of p21 appears to be mediated through binding and inhibition of caspase-3, ASK1, JNK, p38 and CDKs.\textsuperscript{39} However, our study provides three lines of evidence that p21-mediated suppression of Dp53 occurs upstream of hid expression. First, the Dp53-induced block of cell differentiation which is independent of hid, is suppressed by p21. Second, Dp53-induced expression of hid is strongly reduced by p21. Finally, the apoptotic phenotype of GMR-hid is not affected by coexpression of p21 (data not shown). Thus, these data support the notion that p21 and Dap suppress GMR-Dp53 upstream of hid. p21 does not affect the protein levels of Dp53 in GMR-Dp53 (data not shown) suggesting that p21 does not interfere with Dp53 expression, translation or stability.

Because p21 suppresses Dp53 at a very early step and because Dp53 is thought to directly bind to the hid promoter,\textsuperscript{8} we would suggest that p21 directly interferes with the ability of Dp53 to induce gene expression. Alternatively, it is also possible that the suppression of Dp53 by p21 is indirect, and that Dp53 requires a cell cycle-competent environment for hid expression. Further studies are needed to clarify these questions.

In summary, these findings reveal that Dp53 incorporates functions of multiple mammalian p53-family members and provide new insights into the pathways activated by Dp53. It will now be interesting to identify the mechanisms by which Dp53 inhibits cell differentiation and how p21 overcomes it.

**Materials and Methods**

**Fly Strains and Crosses**

All stocks were reared at room temperature. The GMR promoter is described in 15. dronc\textsuperscript{229,22} ark\textsuperscript{G8,24} Df(3L)H99,16 Df(3L)XR38,27 hidWR+XL,26 GMR-p21,29 and UAS-dap,30 are as described. engailed-GAL4 (en-GAL4), eyeless-Flipase (ey-FLP), heat shock-Flipase (hs-FLP), FRT80 P[ubiGFP], UAS-p35, GMR-Dp53, GUS-dp53 and UAS-p53\textsuperscript{H159N} were obtained from the Bloomington Stock Center. The GMR-p21 and UAS-dap lines were kindly provided by I. Hariharan.

**Generation of the hid-lacZ and rpr\textsuperscript{XRE}-lacZ reporter lines**

hid-lacZ—The 30kb genomic DNA upstream of the hid start site was cloned in three parts with approximately 10kb each and a small region of overlap around 100–200bp. These three
DNA fragments were then cloned and inserted into a *Drosophila* reporter plasmid, pCaSpeR-hs43-*lacZ*, respectively. Each reporter construct was named based on the distance upstream of the *hid* start site e.g. *hid*^{20-10}*-lacZ* contains regulatory DNA 20-10 kb upstream of the start site. Transgenic flies were then generated using standard procedures. The *hid*^{20-10}*-lacZ* reporter was found to respond in GMR-*Dp53* eye discs (Figure 3b) and was utilized in this study as the *hid* reporter.

**rpr^{XRE-lacZ}**—Previous work led to the identification of a 2.2 Kb NdeI-BglII genomic interval necessary for the activation of *rpr* gene expression in response to X-ray and UV radiation exposure (AFL and HS, unpublished). Using sequence specific primers with BamHI restriction sites, the NdeI-BglII 2.2 kb X-ray interval was amplified by PCR and cloned directly into the BamHI site of 1.3 *rpr-LacZ* reporter vector 40 for P element–mediated germline transformation. Transgenic embryos submitted to X-ray treatment showed a strong up-regulation of *lacZ* expression and activity in response of X-ray and UV radiation exposure (AFL and HS, unpublished).

**Mosaic Analysis**

To examine *H99*, *dronc* or *ark* clones in *Dp53*-expressing eye discs, late 3rd instar larvae of the following genotype were analyzed: (1) *w* eyFLP; GMR>GUS-*Dp53*/+; *H99* FRT80/ P[ubiGFP] FRT80. (2) *w*; GMR>GUS-*Dp53*/+; *dronc* P[ubiGFP] FRT80. (3) *w*; *ark* G8 FRT42/P[ubiGFP] FRT42; GMR-*Dp53*/+. For mosaic analysis with clones expressing *Dp53* and p35 simultaneously, larvae of the following genotype were heat shocked for 30min at 37°C, raised at room temperature and analyzed 48h later: *w hsFLP*/+; *tub>*GFP>*GAL4/UAS-p35; UAS-*Dp53*/+. In each of these experiments, more than 20 representative clones were analyzed.

**Immunohistochemistry**

Eye-antennal imaginal discs from late 3rd instar larvae or mid-pupa (45–50 hour after puparium formation) were dissected and labeled with the following antibodies: rabbit anti-Dlg and mouse anti-Rough (kindly provided by K. Choi), rabbit anti-Seven-up (a gift from R. Schulz), rabbit anti-cleaved Caspase-3 (Cell Signaling Technology), mouse anti-*Dp53*, rat anti-ELAV, mouse anti-Pros, mouse anti-Cut and mouse anti-βGAL (all obtained from the DSHB, U. of Iowa). Secondary antibodies were donkey Fab fragments from Jackson ImmunoResearch. The TUNEL assay kit is from Roche. Images were taken with either a Zeiss Axiosmager equipped with ApoTome technology or a confocal microscope.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We apologize to our colleagues whose work could not be cited due to space restrictions. We would like to thank John Abrams, Michael Brodsky, Kwang Choi, Bruce Hay, Iswar Hariharan, Hyung Don Ryoo, Robert Schulz, Kristin White, the Bloomington Stock Center in Indiana, and the Developmental Studies Hybridoma Bank (DSHB) in Iowa, for fly stocks and reagents. This work was supported by the NIH (R01GM068018, R01GM081543, R01GM074977 to AB, and R01GM06124 to HS). The content is solely the responsibility of the authors and does
not represent the official views of the NIH. We gratefully acknowledge support by the Robert A. Welch Foundation (G-1496).

### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ASK1         | Apoptosis signal-regulating kinase 1 |
| Cas3*        | cleaved caspase-3 |
| CDK          | Cyclin-dependent kinase |
| cep-1        | c. elegans p53-like protein |
| dap          | dacapo |
| Dcp-1        | Death caspase-1 |
| ΔN           | Delta N |
| Df(3L)H99    | Deficiency (3L)H99 |
| Dp53         | Drosophila p53 |
| DrICE        | Drosophila interleukin-1 converting enzyme |
| Dronec       | Drosophila Nedd2-like Caspase |
| ELAV         | embryonic lethal abnormal vision |
| en           | engrailed |
| ey           | eyeless |
| FLP          | Flippase |
| FRT          | Flippase recombination target |
| GFP          | Green fluorescent protein |
| GMR          | Glass multimer reporter |
| GUS          | GMR UAS |
| hid          | head involution defective |
| hs           | heat shock |
| JNK          | Jun kinase |
| pros         | prospero |
| ro           | rough |
| rpr          | reaper |
| SAM          | sterile alpha motif |
| Svp          | seven-up |
| TA           | Transactivating |
| tub          | tubulin |
| TUNEL        | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
UAS  upstream activating sequence
XRE  X-ray response element
R1–R8  Photoreceptor neurons 1–8

References

1. Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell. 2009; 137:413–431. [PubMed: 19410540]
2. Pietsch EC, Sykes SM, McMahon SB, Murphy ME. The p53 family and programmed cell death. Oncogene. 2008; 27:6507–6521. [PubMed: 18955976]
3. Stiewe T. The p53 family in differentiation and tumorigenesis. Nature reviews. 2007; 7:165–168.
4. Murray-Zmijewski F, Lane DP, Bourdon JC. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. Cell death and differentiation. 2006; 13:962–972. [PubMed: 16601753]
5. Ollmann M, Young LM, Di Como CJ, Karim F, Belvin M, Robertson S, et al. Drosophila p53 is a structural and functional homolog of the tumor suppressor p53. Cell. 2000; 101:91–101. [PubMed: 10778859]
6. Brodsky MH, Nordstrom W, Tsang G, Kwan E, Rubin GM, Abrams JM. Drosophila p53 binds a damage response element at the reaper locus. Cell. 2000; 101:103–113. [PubMed: 10778860]
7. Jin S, Martinek S, Joo WS, Wortman JR, Mirkovic N, Sali A, et al. Identification and characterization of a p53 homologue in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:7301–7306. [PubMed: 10860994]
8. Brodsky MH, Weinert BT, Tsang G, Rong YS, McGinnis NM, Golic KG, et al. Drosophila melanogaster MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. Molecular and cellular biology. 2004; 24:1219–1231. [PubMed: 14729967]
9. Yamada Y, Davis KD, Coffman CR. Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monocarboxylate transporter. Development. 2008; 135:207–216. [PubMed: 18057102]
10. Flores ER, Tsai KY, Crowley D, Sengupta S, Yang A, McKeon F, et al. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature. 2002; 416:560–564. [PubMed: 11932750]
11. Akdemir F, Christich A, Sogame N, Chapo J, Abrams JM. p53 directs focused genomic responses in Drosophila. Oncogene. 2007; 26:5184–5193. [PubMed: 17310982]
12. Xu D, Woodfield SE, Lee TV, Fan Y, Antonio C, Bergmann A. Genetic control of programmed cell death (apoptosis) in Drosophila. Fly. 2009; 3. [PubMed: 19164943]
13. Moon NS, Di Stefano L, Morris EJ, Patel R, White K, Dyson NJ. E2F and p53 induce apoptosis independently during Drosophila development but intersect in the context of DNA damage. PLoS Genet. 2008; 4:e1000153. [PubMed: 18688282]
14. Derry WB, Putzke AP, Rothman JH. Caenorhabditis elegans p53: role in apoptosis, meiosis, and stress resistance. Science (New York, NY). 2001; 294:591–595.
15. Ellis MC, O’Neill EM, Rubin GM. Expression of Drosophila p53 protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. Development. 1993; 119:855–865. [PubMed: 8187644]
16. White K, Grether ME, Abrams JM, Young L, Farrell K, Steller H. Genetic control of programmed cell death in Drosophila. Science (New York, NY). 1994; 264:677–683.
17. Dorsty L, Colussi PA, Quinn LM, Richardson H, Kumar S. DRONC, an ecdysone-inducible Drosophila caspase. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96:4307–4312. [PubMed: 10200258]
18. Zhou L, Song Z, Tittel J, Steller H. HAC-1, a Drosophila homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. Molecular cell. 1999; 4:745–755. [PubMed: 10619022]
19. Kanuka H, Sawamoto K, Inohara N, Matsuno K, Okano H, Miura M. Control of the cell death pathway by Dapaf-1, a Drosophila Apaf-1/CED-4-related caspase activator. Molecular cell. 1999; 4:757–769. [PubMed: 10619023]

20. Rodriguez A, Oliver H, Zou H, Chen P, Wang X, Abrams JM. Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. Nature cell biology. 1999; 1:272–279. [PubMed: 10559939]

21. Mills K, Daish T, Harvey KF, Pfleger CM, Hariharan IK, Kumar S. The Drosophila melanogaster Apaf-1 homologue ARK is required for most, but not all, programmed cell death. The Journal of cell biology. 2006; 172:809–815. [PubMed: 16533943]

22. Xu D, Li Y, Arcaro M, Lackey M, Bergmann A. The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in Drosophila. Development. 2005; 132:2125–2134. [PubMed: 15800014]

23. Daish TJ, Mills K, Kumar S. Drosophila caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. Dev Cell. 2004; 7:909–915. [PubMed: 15572132]

24. Srivastava M, Scherr H, Lackey M, Xu D, Chen Z, Lu J, et al. ARK, the Apaf-1 related killer in Drosophila, requires diverse domains for its apoptotic activity. Cell death and differentiation. 2007; 14:92–102. [PubMed: 16645639]

25. Xu D, Wang Y, Willecke R, Chen Z, Ding T, Bergmann A. The effector caspases drICE and dcp-1 have partially overlapping functions in the apoptotic pathway in Drosophila. Cell death and differentiation. 2006; 13:1697–1706. [PubMed: 16645642]

26. Grether ME, Abrams JM, Agapite J, White K, Steller H. The head involution defective gene of Drosophila melanogaster functions in programmed cell death. Genes Dev. 1995; 9:1694–1708. [PubMed: 7622034]

27. Peterson C, Carney GE, Taylor BJ, White K. Reaper is required for neuroblast apoptosis during Drosophila development. Development. 2002; 129:1467–1476. [PubMed: 11880355]

28. Kondo S, Senoo-Matsuda N, Hiromi Y, Miura M. DRONC coordinates cell death and compensatory proliferation. Molecular and cellular biology. 2006; 26:7258–7268. [PubMed: 16980627]

29. de Nooij JC, Hariharan IK. Uncoupling cell fate determination from patterned cell division in the Drosophila eye. Science (New York, NY). 1995; 270:983–985.

30. de Nooij JC, Hariharan IK. A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during Drosophila embryogenesis. Cell. 1996; 87:1237–1247. [PubMed: 8980230]

31. Lane ME, Sauer K, Wallace K, Jan YN, Lehner CF, Vaessen H. Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. Cell. 1996; 87:1225–1235. [PubMed: 8980229]

32. Lee CY, Clough EA, Yellon P, Teslovich TM, Stephan DA, Baehrecke EH. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. Curr Biol. 2003; 13:350–357. [PubMed: 12593803]

33. Zhou L, Steller H. Distinct pathways mediate UV-induced apoptosis in Drosophila embryos. Dev Cell. 2003; 4:599–605. [PubMed: 12689597]

34. White K, Tahaoglu E, Steller H. Cell killing by the Drosophila gene reaper. Science (New York, NY). 1996; 271:805–807.

35. Yu SY, Yoo SJ, Yang L, Zapata C, Srinivasan A, Hay BA, et al. A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye. Development. 2002; 129:3269–3278. [PubMed: 12070100]

36. Bourdon JC, Fernandes K, Murray-Zmijewski F, Liu G, Diot A, Xirodimas DP, et al. p53 isoforms can regulate p53 transcriptional activity. Genes Dev. 2005; 19:2122–2137. [PubMed: 16131611]

37. Yang A, Kaghad M, Caput D, McKeon F. On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet. 2002; 18:90–95. [PubMed: 11818141]

38. Ou HD, Lohr F, Vogel V, Mantele W, Dotsch V. Structural evolution of C-terminal domains in the p53 family. EMBO J. 2007; 26:3463–3473. [PubMed: 17581633]
39. Janicke RU, Sohn D, Essmann F, Schulze-Osthoff K. The multiple battles fought by anti-apoptotic p21. Cell Cycle. 2007; 6:407–413. [PubMed: 17312393]

40. Jiang C, Lamblin AF, Steller H, Thummel CS. A steroid-triggered transcriptional hierarchy controls salivary gland cell death during Drosophila metamorphosis. Molecular cell. 2000; 5:445–455. [PubMed: 10882130]
Figure 1. Expression of Dp53 induces massive cell death in Drosophila eyes
Shown are adult eyes (a–c) and late 3rd instar larval eye imaginal discs (d–i). Here, and in the following figures, posterior is to the right.

(a–c) Compared to wild type (wt) (a), expression of Dp53 under control of the GMR promoter (GMR-Dp53) (b) or the GMR-Gal4 driver (GMR>GUS-Dp53) (c) causes small adult eyes with rough and glossy appearance.

(d) Wild type eye disc labeled with anti-cleaved Caspase-3 antibodies (Cas3*). A few cells are Cas3*-positive.

(e,f) GMR-Dp53 (e) and GMR>GUS-Dp53 (f) eye discs labeled with Cas3* antibodies. Massive cell death is induced in the posterior half of the eye disc where GMR drives expression of Dp53.

(g) Wild-type eye disc labeled with TUNEL. Only a few dying cells label with TUNEL.

(h,i) GMR-Dp53 (h) and GMR>GUS-Dp53 (i) eye discs labeled with the TUNEL assay. Massive dying cells are induced by expression of Dp53.
Figure 2. Dp53 induces cell death through the canonical apoptosis pathway in Drosophila
Shown are late 3rd instar larval eye imaginal discs (a–d′) and adult eyes (e–h).
(a–c) Mosaic eye discs labeled with GFP (green) and TUNEL (red). Clones are marked by absence of GFP. H99 clones (a,a′) and dronc mutant clones (b,b′) are generated in GMR>GUS-dp53 background and ark mutant clones are generated in GMR-dp53 background (c,c′). Cell death induced by Dp53 is blocked in H99, dronc or ark mutant clones (arrows).
(d,d′) GMR>GUS-Dp53/UAS-p35 eye discs labeled with anti-Dp53 antibodies (green) and TUNEL (red). Dp53 is expressed in the posterior eye disc (d) and cell death induced by Dp53 is strongly suppressed by expression of P35 (d′).
(e) H99 mutant mosaic eye in GMR>GUS-Dp53 background.
(f) dronc mutant mosaic eye in GMR>GUS-Dp53 background.
(g) ark mutant mosaic eye in GMR-Dp53 background.
(h) GMR>GUS-p53/UAS-p35 adult eye.
Figure 3. **hid is the major effector of GMR-Dp53-induced apoptosis**

Shown are late 3rd instar larval eye imaginal discs labeled with Cas3* (green), anti-Hid antibodies (red) and βGAL (blue) (a,b), or Cas3* (green) and TUNEL (red) (c,d).

(a–a″) Wild type disc containing the hid-lacZ reporter. Cell death and hid reporter expression are at low levels.

(b–b″′) GMR-Dp53 disc containing the hid-lacZ reporter. Increased levels of Hid protein (b″) and hid reporter (b″′) as well as massive cell death (b′) is detectable in GMR-Dp53.

(c,c′ and d,d′) GMR>GUS-Dp53 in rpr homozygous mutant background (XR38/H99 in c,c′ and rpr87/H99 in d,d′). Dp53-induced cell death is not significantly altered in rpr mutants.

(e,e′) GMR>GUS-Dp53 in hid homozygous mutant background (hidWR+X1/H99). Dp53-induced cell death is strongly reduced as indicated by Cas3* and TUNEL labeling.
Figure 4. Differentiation of R7 and cone cells is disrupted in GMR-Dp53 eye discs

Shown are late 3\textsuperscript{rd} instar eye imaginal discs (a–d') and mid-pupal eye discs (e,f).

(a–b') Eye discs labeled with the R7 photoreceptor marker Pros (red) and Cas3\textsuperscript{*} (green). Compared to wild-type (a), the number of R7 cells as indicated by Pros labeling is strongly reduced in GMR-Dp53 discs (b,b').

(c–d') Eye discs labeled with the cone cell marker Cut (red) and Cas3\textsuperscript{*} (green). Compared to wild-type (c), the number of cone cells as indicated by Cut staining is strongly reduced in GMR-dp53 discs (d,d').
(e,f) Pupal discs labeled with Cut (red) and the cellular membrane marker Dlg (green). In wild-type (e), ommatidia are well-organized and contain four cone cells each. In contrast, in GMR-Dp53 discs (f), the global organization of ommatidia is severely disrupted. Loss of cone cells in some ommatidia and ommatidial fusion (arrows) as indicated by aggregated cone cells are observed.
Figure 5. **GMR-Dp53 induces cell differentiation defects independently of its pro-apoptotic role**

Shown are late 3rd instar eye imaginal discs (a–b′ and f–f″) and mosaic mid-pupal eye discs (c–e′).

(a,a′) A **GMR>GUS-Dp53** disc with **H99** mutant clones labeled with GFP (green) and Pros (red). **H99** clones are marked by absence of GFP. The number of R7 cells does not increase in **H99** clones where apoptosis is blocked.

(b,b′) A homozygous **dronc** mutant **GMR>GUS-Dp53** disc labeled with TUNEL (green) and Pros (red). Dp53-induced cell death is strongly suppressed with only a few dying cells left (b). However, despite inhibition of apoptosis, R7 differentiation is not restored (b and b′).
Mosaic wild type pupal disc labeled with GFP (green), Dlg (red) and Pros (gray). H99 clones are marked by absence of GFP. Although the number of interommatidial cells is increased in H99 clones (arrows), the ommatidial organization is normal and there is a single R7 cell (gray) in each ommatidium (c).

GMR>GUS-Dp53 pupal discs with H99 mutant clones labeled with GFP (green) and Dlg (red) (d), or GFP (green) and Pros (red) (e). H99 clones are marked by absence of GFP. A strongly increased number of interommatidial cells (arrows) is observed in H99 clones (d,d′; arrows). Importantly, the disrupted organization of ommatidia (d′), and the disorganization and reduced number of R7 cells (e′) in GMR>GUS-Dp53 discs is not rescued in H99 clones.

Mosaic discs labeled with GFP (green), the neuronal marker ELAV (red) and anti-Dp53 antibodies (blue). Clones simultaneously expressing Dp53 and p35 are marked by absence of GFP and by anti-Dp53 labeling (blue). In these clones, although cell death is blocked by p35, differentiation of photoreceptor neurons is blocked as indicated by lack of ELAV staining (arrows).
Figure 6. Expression of human p21 or *Drosophila* Dap suppresses both GMR-Dp53-induced apoptosis and cell differentiation defects

(a) GMR-p21/+ adult eye. A little roughening is visible.

(b,c) Control larval eye discs of GMR-p21/+ at the late 3rd instar stage labeled with Cas3* (b) and the neuronal marker ELAV (c). GMR-p21 induces no or little apoptosis (b, arrow), and differentiation of photoreceptor neurons is largely normal (c).

(d) Adult eye of GMR-Dp53/GMR-p21. Expression of human p21 rescues Dp53-induced eye phenotype (compare to Figure 1b).
Late 3rd instar eye discs of GMR-Dp53/GMR-p21 labeled with Cas3* (e) or Pros (f). Dp53-induced cell death is largely suppressed (e, arrowhead) and the number of R7 cells is restored (f).

Adult eye of GMR>GUS-Dp53/UAS-dap. Expression of dacapo (dap) partially rescues the Dp53-induced eye ablation phenotype (compare to Figure 1c).

Late 3rd instar eye discs of GMR>GUS-Dp53/UAS-dap labeled with Cas3* (h) or Pros (i). Dp53-induced cell death is partially suppressed (h, arrowhead) and the number of R7 cells is partially restored (i).

Late 3rd instar eye disc of GMR-Dp53/GMR-p21 labeled with anti-Hid antibody. The level of Dp53-induced Hid is reduced in response to expression of human p21 (compare to Figure 3b').

Expression of Dp53 suppresses cell differentiation independently of its roles in apoptosis. Dp53 activates apoptosis mainly through the pro-apoptotic gene Hid and its downstream canonical apoptosis pathway in Drosophila. Expression of human p21 or its Drosophila homolog Dacapo (Dap) suppresses both p53-induced cell differentiation defects and Hid-induced cell death.