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Dual roles of *Drosophila* p53 in cell death and cell differentiation

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The mammalian p53 family consists of p53, p63 and p73. Whereas 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 *Drosophila* p53 (Dp53) induces apoptosis, but not cell-cycle arrest. In this study, using the developing eye as a model, we show that *Dp53*-induced apoptosis is primarily dependent on the pro-apoptotic gene, *head involution defective* (*hid*), but not *reaper* (*rpr*), 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* (*dap*) 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.
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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 Vousden and Prives[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 Pietsch *et al.*[2] and Stiewe[3]). They can not only induce apoptosis, but also have additional functions because $p63^{-/-}$ and $p73^{-/-}$ knockout mice show clear developmental defects, in contrast to $p53^{-/-}$-null mice that 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.[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 that are produced from additional intronic promoters generating N-terminally truncated (ΔN) isoforms (reviewed by Stiewe[3] and Murray-Zmijewski *et al.*[4]). The ΔN isoforms can bind with the full-length transactivating (TA) isoforms of p53, p63 and p73, and antagonize their functions.[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 *Drosophila*.

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

Importantly, the pro-apoptotic function of p53 is well conserved in *Drosophila*. In response to apoptotic stimuli, the pro-apoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*) and *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, *Drosophila* Nedd2-like caspase (Dronc), and two major effector caspases, *Drosophila* interleukin-1 converting

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Abbreviations: 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; Dronc, *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
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enzyme (DrICE) and death caspase-1 (Dcp-1) (reviewed by Xu *et al.*[12]). In response to radiation-induced DNA damage, Dp53 activates the transcription of *rpr* to initiate apoptosis.[6] In this process, *hid* is also induced, but the details are less clear.[8,11,13]

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

In this study we further examined the phenotypes obtained by expression of *Dp53* in *Drosophila* eyes. We show by mutant analysis that only *hid,* but not *rpr,* is required for Dp53-induced apoptosis in this system. In addition, expression of *Dp53* can activate the canonical caspase-dependent apoptosis pathway in *Drosophila*. Consistently, and in contrast to previous reports, we found that 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 *p53* target gene, human p21, or its *Drosophila* homolog *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.

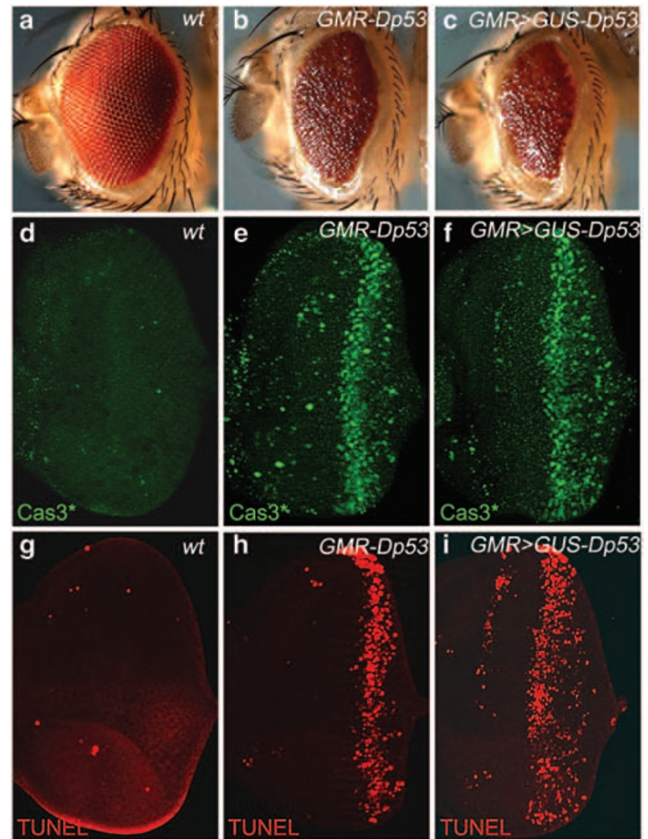

Figure 1 Expression of *Dp53* induces massive cell death in *Drosophila* eyes. Shown are adult eyes (**a**–**c**) and late third instar larval eye imaginal discs (**d**–**i**). In this figure, and in the following figures, posterior is to the right. (**a**–**c**) Compared with wild type (*wt*) (**a**), 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 in which *GMR* drives expression of *Dp53*. (**g**) Wild-type eye disc labeled with TUNEL. Only a few dying cells are labeled 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*

Results

Expression of *Dp53* induces cell death through the canonical apoptosis pathway in *Drosophila* eyes. Expression of *Dp53* in the fly eye either directly under control of the eye-specific *glass multimer reporter* (*GMR*) promoter (*GMR–Dp53*) or using a modified upstream activating sequence (UAS)-Gal4 system (*GMR-Gal4 GMR UAS* (*GUS*)–*Dp53*; referred to as *GMR*> *GUS–Dp53*)[8] induces small and rough eyes with glossy appearance (Figures 1a–c).[5,7,8] This eye ablation phenotype is induced specifically by *Dp53* as it can be fully rescued by co-expression of a dominant-negative form of *Dp53*, $Dp53^{H159N}$ (see Ollmann *et al.*[5]), or by *Dp53* RNAi (data not shown).

It has been reported that the eye ablation phenotype of *GMR–Dp53* cannot be rescued by co-expression of the caspase inhibitor p35, an inhibitor of the effector caspases DrICE and Dcp-1[5] (see Figure 2h). This observation may suggest that *GMR–Dp53* causes the eye ablation phenotype independently of caspase activation. Therefore, we examined the pro-apoptotic function of Dp53 in more detail. First, we labeled *GMR–Dp53* and *GMR*> *GUS–Dp53* eye imaginal discs from late third instar larvae with an antibody detecting activated caspases (cleaved caspase-3 (Cas3*)). The obtained labeling pattern (Figures 1d–f) resembles the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) pattern in these discs (Figures 1g–i) and corresponds to the expression domain of *GMR*.[15] Therefore, Dp53 can induce caspase activation.

Next, we asked whether the three major pro-apoptotic genes, *rpr*, *hid* and *grim*, are required for Dp53-induced cell death. Deficiency (3L)H99 (*Df(3L)H99;* referred to as *H99*), which deletes these three genes,[16] was used for mosaic analysis. In late third instar *GMR*> *GUS–Dp53* eye discs, Dp53-induced cell death, as detected by TUNEL, is completely blocked in *H99* mutant clones (arrows, Figure 2a, a′). Furthermore, Dp53-induced cell death is also absent in mutant clones of the initiator caspase *dronc* (Figure 2b, b′) or its adaptor *ark* (Figure 2c, c′), which encode the apoptosome components of the canonical apoptotic pathway.[17–21]

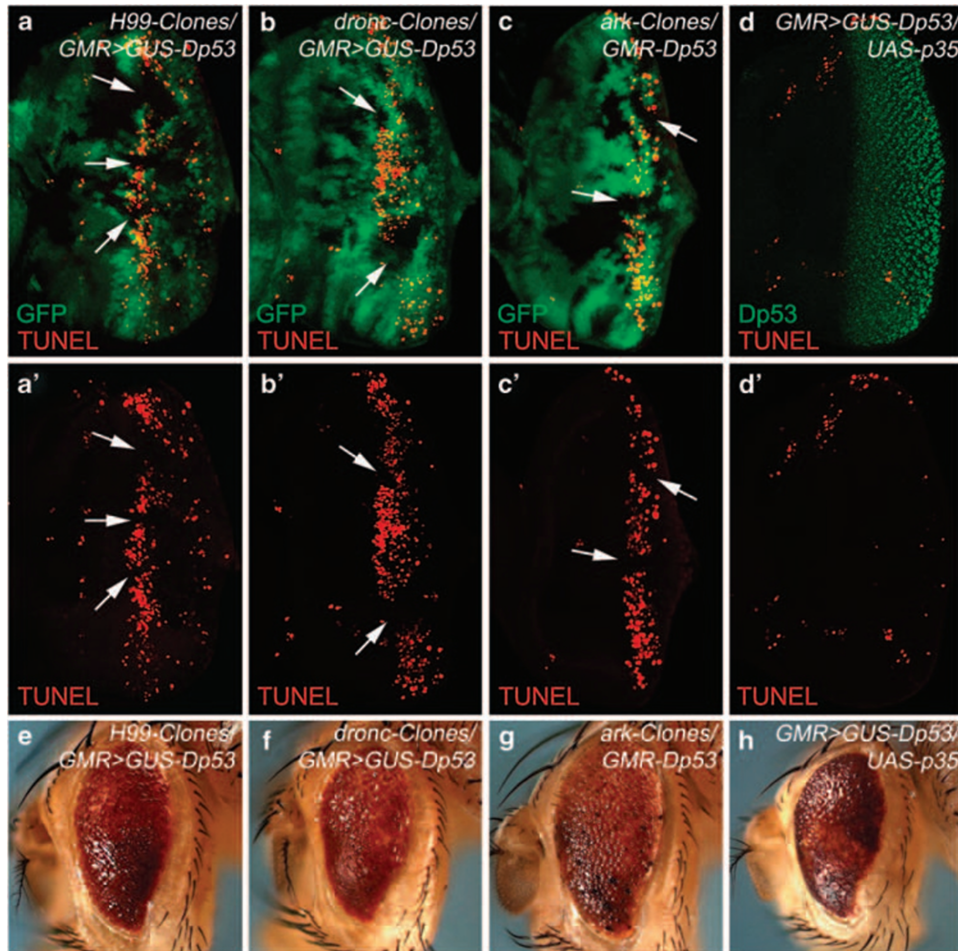

Figure 2 Dp53 induces cell death through the canonical apoptosis pathway in *Drosophila*. Shown are late third instar larval eye imaginal discs (**a**–**d**′) and adult eyes (**e**–**h**). (**a**–**c**) Mosaic eye discs labeled with GFP (green) and TUNEL (red). Clones are marked by absence of GFP. *H99* 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

These results indicate that the canonical *Drosophila* apoptotic pathway indeed mediates *GMR–Dp53*-induced cell death.

We next asked why expression of the caspase inhibitor p35 does not suppress the *GMR–Dp53*-induced eye phenotype[5] (Figure 2h) and examined whether expression of p35 can block Dp53-induced apoptosis at the cellular level. Strikingly, simultaneous expression of p35 and Dp53 under control of the same *GMR–GAL4* driver strongly inhibits Dp53-induced apoptosis in the developing eye disc (Figure 2d, d′). The suppression of Dp53 by p35 is not restricted to eye imaginal discs and can also be observed in wing imaginal discs (Supplementary Figure S1).

Taken together, these data suggest that Dp53 activity triggers apoptosis through the canonical apoptotic pathway, including pro-apoptotic genes and activated caspases. Intriguingly, we also noticed that although *GMR–Dp53*-induced cell death is completely blocked in *H99*, *dronc* or *ark* mutant clones, and is strongly suppressed by expression of p35, the resulting adult eyes are not or only partially rescued, as indicated by their rough and glossy appearance (compare Figures 2e–h with Figures 1b and c). This is in striking contrast to the strong suppression of the *GMR-hid*- and *GMR-reaper*-induced eye ablation phenotypes by loss of *dronc* and *ark,* or expression of p35.[22–26] Therefore, this analysis raises two questions. First, which of the *H99* genes, *rpr*, *hid* or *grim,* are 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 with wild-type eye discs (Figure 3a–a‴), the protein level of Hid is strongly increased in the area in which *GMR* drives expression of *Dp53* and apoptosis (Figure 3b, b″; see also Figure 2d). Because *Dp53* encodes a transcription factor, we

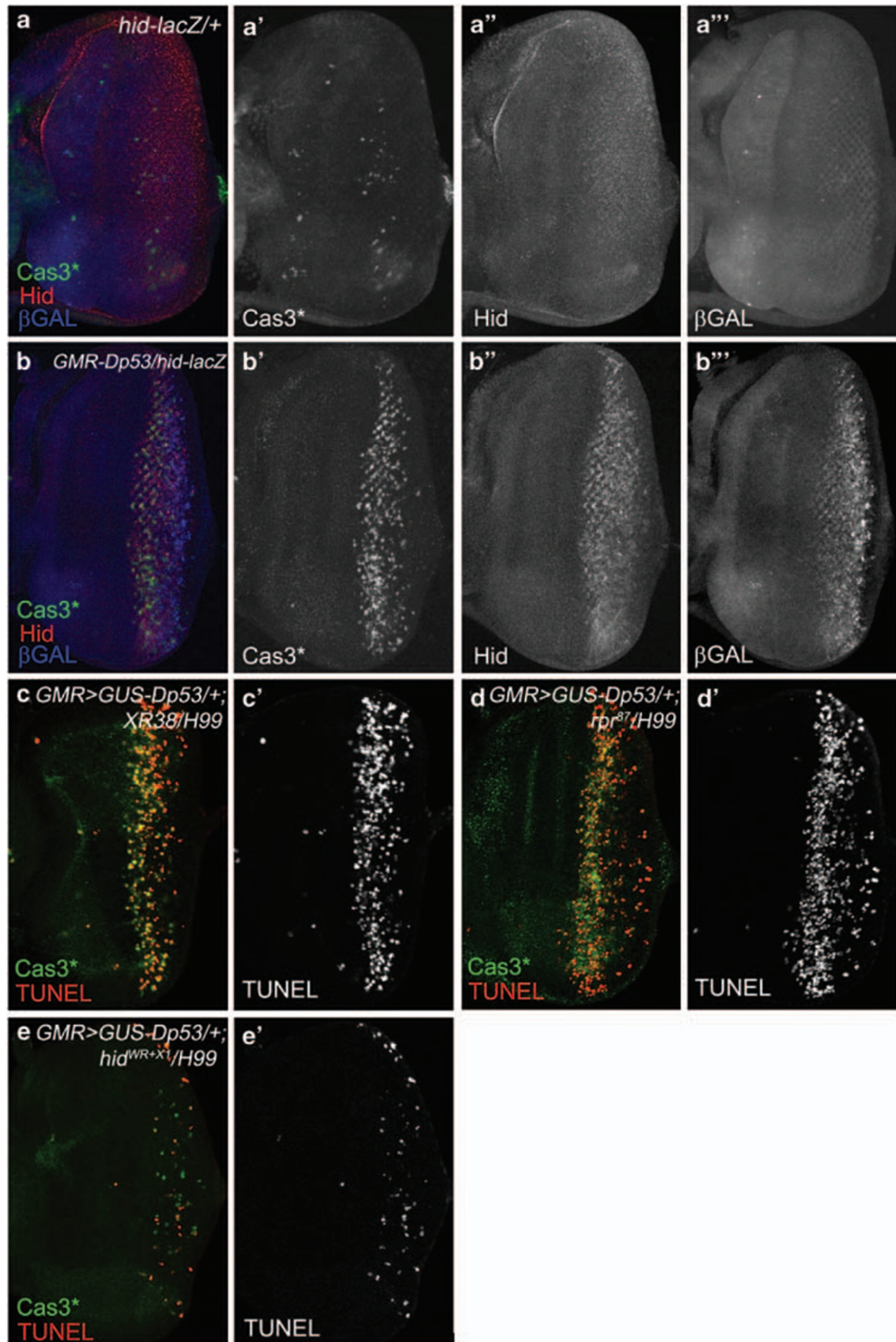

Figure 3 *hid* is the major effector of *GMR–Dp53*-induced apoptosis. Shown are late third instar larval eye imaginal discs labeled with Cas3* (green), anti-Hid antibodies (red) and β-GAL (blue) (**a, b**), or Cas3* (green) and TUNEL (red) (**c, d**). (**a–a‴**) Wild-type disc containing the *hid-lacZ* 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 *rpr*^{87}*/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 (*hid*$^{WR+X1}$*/H99*). Dp53-induced cell death is strongly reduced as indicated by Cas3* and TUNEL labeling

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 was not only present in developing eye discs but was also found in wing discs

(Supplementary Figure S2b, b′) when compared with controls (Supplementary 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 rpr^{XRE}-*lacZ* (X-ray response element (XRE)) 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 rpr^{XRE}-*lacZ* transgene upon expression of *Dp53* in wing discs (Supplementary Figure S2d, d′) compared with controls (Supplementary 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*, rpr^{87} (see Moon *et al.*[13]), did not significantly affect the level of *GMR–Dp53*-induced apoptosis (Figure 3c, c′ and 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 Figures 2e–h with Figures 1b and 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 analyze this possibility, we examined differentiation of various cell types in Dp53-expressing eye discs by using the cellular differentiation markers: Embryonic Lethal Abnormal Vision (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 Supplementary 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* (Supplementary 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 with 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 misorganized, 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

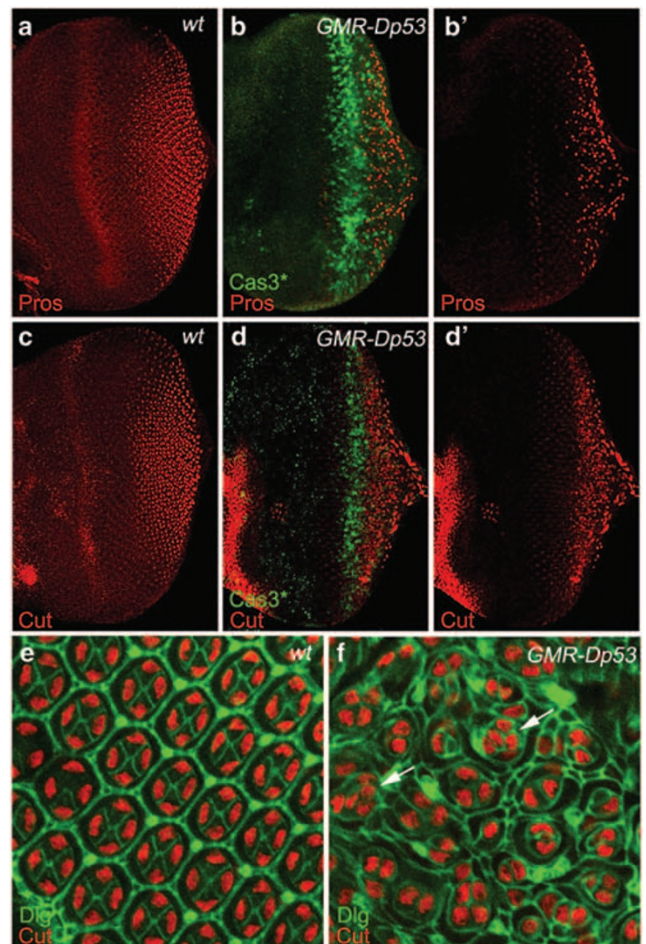

Figure 4 Differentiation of R7 and cone cells is disrupted in *GMR–Dp53* eye discs. Shown are late third instar eye imaginal discs (**a–d′**) and mid-pupal eye discs (**e, f**). (**a–b′**) Eye discs labeled with the R7 photoreceptor marker Pros (red) and Cas3* (green). Compared with wild type (**a**), 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* (green). Compared with 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

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, and 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

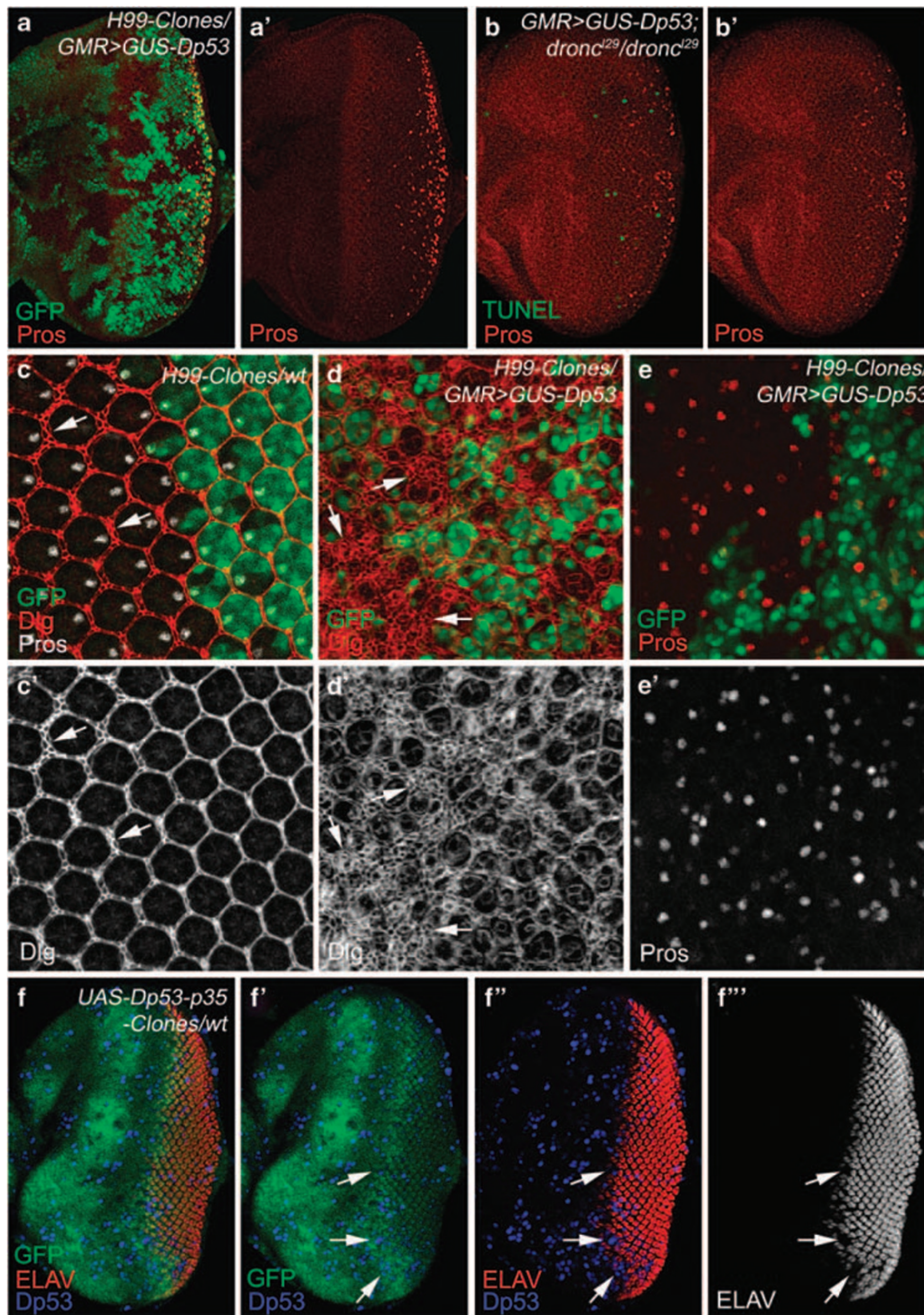

Figure 5 *GMR–Dp53* induces cell differentiation defects independently of its pro-apoptotic role. Shown are late third instar eye imaginal discs (**a–b′** and **f–f‴**) and mosaic mid-pupal eye discs (**c–e′**). (**a, a′**) A *GMR> GUS–Dp53* disc with *H99* mutant clones labeled with GFP (green) and Pros (red). *H99* clones are marked by absence of GFP. The number of R7 cells does not increase in *H99* clones in which 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′**). (**c, c′**) 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**). (**d–e′**) *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. (**f–f‴**) 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)

expression of *Dp53* causes differentiation defects independently of its pro-apoptotic role.

Next, we analyzed 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,-Figure 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 (Figures 6b and 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 (Figures 6d and 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 with Figure 3b‴).

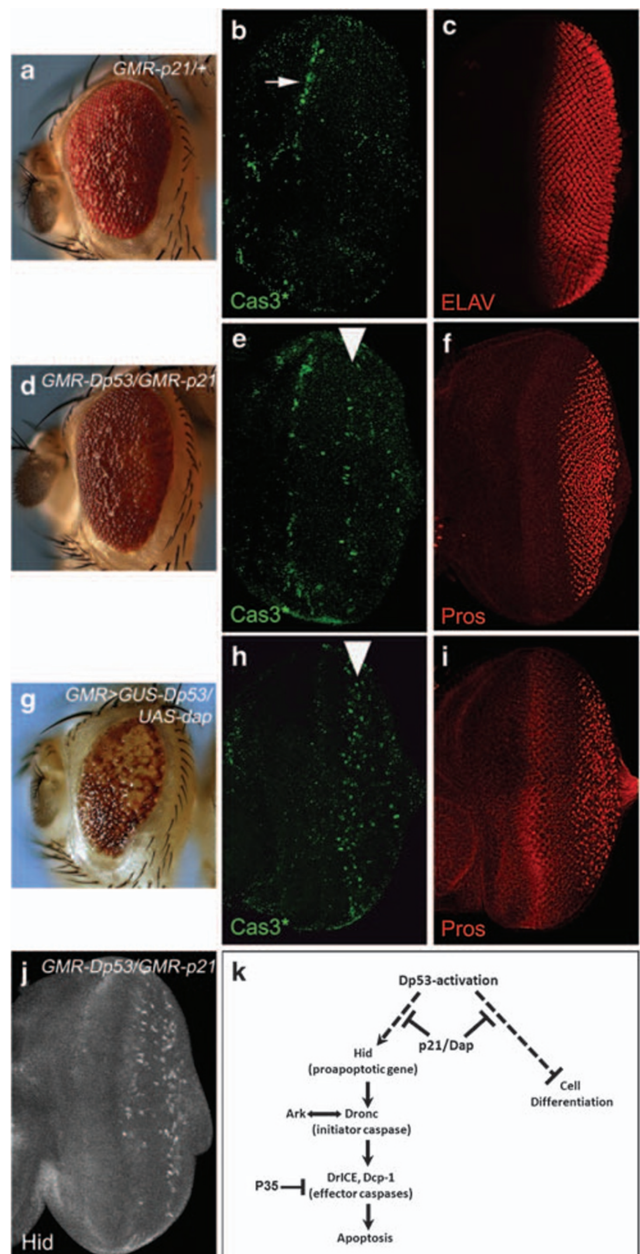

Figure 6 Expression of human p21 or *Drosophila* Dap suppresses both *GMR–Dp53*-induced apoptosis and cell differentiation defects. (**a**) *GMR–p21/+* adult eye. A little roughening is visible. (**b, c**) Control larval eye discs of *GMR–p21/+* at the late third instar stage labeled with Cas3* (**b**) 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 (compared with Figure 1b). (**e, f**) Late third 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**). (**g**) Adult eye of *GMR> GUS–Dp53/UAS-dap*. Expression of *dacapo* (*dap*) partially rescues the *Dp53*-induced eye ablation phenotype (compared with Figure 1c). (**h, i**) Late third 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**). (**j**) Late third 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 (compared with Figure 3b″). (**k**) 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

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 independent of context. 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 seems 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* has a more important role in *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 seems unlikely because expression of *rpr* from the *GMR* promoter is sufficient to induce apoptosis.[34] 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.[35] Such a tissue-specific requirement has also been reported for *rpr*, which is required for apoptosis of abdominal neuroblasts in the central nervous system.[27] Thus, it will be interesting to analyze 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 Vousden and Prives[1] and Stiewe[3]). However, in the cases reported, the expression of the p53 family members in undifferentiated cells actually induces differentiation instead of inhibiting it, as shown in this study for Dp53. For example, the TA isoform of mouse p53 induces differentiation of mouse embryonic stem cells.[3,4] 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 Stiewe[3] and Murray-Zmijewski *et al.*[4]). Similarly, expression of ΔNp73 inhibits myogenic differentiation.[3,4] ΔNp63 and ΔNp73 can also interfere with p53-induced differentiation programs.[3,4]

Importantly, the *Dp53* gene also has an internal promoter[36] and the originally identified *Dp53* gene, including the one used in this study, actually corresponds to the ΔN isoform of Dp53.[36] 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,[10] similar to Dp53. However, both mammalian p53 and *Drosophila* Dp53 lack the sterile alpha motif (SAM) domain at the C-terminus, which is characteristic for p63 and p73.[37] The SAM domain supports oligomerization of p63 and p73. The absence of the SAM domain may indicate that Dp53 is more related with mammalian p53 rather than with p63 and p73. Nevertheless, BLAST searches with Dp53 revealed higher similarity with mammalian p63 and p73 than with p53.[37] Furthermore, it was recently shown that the SAM domain of Dp53 was replaced during evolution by a helix domain, which also supports oligomerization.[38] 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 Janicke *et al.*[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.[39] We extend these observations further and show in this study 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.[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 seems 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, and hence the weaker suppression.

The molecular mechanisms by which p21 suppresses p53-induced apoptosis are unclear and somewhat contradictory (reviewed in Janicke *et al.*[39]). According to several studies, the anti-apoptotic function of p21 seems to be mediated through binding and inhibition of caspase-3, apoptosis signal-regulating kinase 1 (ASK1), Jun kinase (JNK), p38 and CDKs.[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 co-expression 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,[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 Ellis *et al.*[15] *dronc*^{l29},[22] *ark*^{G8},[24] *Df(3L)H99*,[16] *Df(3L)XR38*,[27] *hid*$^{WR+X1}$,[26] *GMR–p21*,[29] and *UAS-dap*[30] are as described previously. The *engailed-GAL4 (en-GAL4)*, *eyeless-flippase* (*ey-FLP*), *heat shock-flippase* (*hs-FLP*), flippase recombination target (*FRT)80 P[ubi-GFP]*, *UAS–p35*, *GMR–Dp53*, *GMR > GUS-Dp53* and *UAS-Dp53*^{H159N} were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA). The *GMR–p21* and *UAS-dap* lines were kindly provided by I Hariharan.

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

hid-lacZ. The 30 kb genomic DNA upstream of the *hid* start site was cloned in three parts with approximately 10 kb each and a small region of overlap around 100–200 bp. 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, for example, *hid*$^{20-10}$*-lacZ* contains regulatory DNA 10–20 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 used in this study as the *hid* reporter.

rpr^{XRE}*-lacZ.* Previous work led to the identification of a 2.2-kb *Nde*I–*Bgl*II genomic interval necessary for the activation of *rpr* gene expression in response to X-ray and UV radiation exposure (Lamblin and Steller, unpublished). Using sequence-specific primers with *Bam*HI restriction sites, the *Nde*I-*Bgl*II 2.2 kb X-ray interval was amplified by PCR and cloned directly into the *Bam*HI 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 upregulation of *lacZ* expression and activity in response to X-ray and UV radiation exposure (Lamblin and Steller, unpublished).

Mosaic analysis. To examine H99, dronc or ark clones in Dp53-expressing eye discs, late third instar larvae of the following genotype were analyzed: (1) *w eyFLP; GMR > GUS–Dp53/+ ; H99 FRT80/P[ubi-GFP] FRT80;* (2) *w; GMR > GUS–Dp53/+ ; dronc*^{l29} *FRT80/P[ubi-GFP] FRT80;* and (3) *w; ark*^{G8} *FRT42/P[ubi-GFP] FRT42; GMR–Dp53/+*. For mosaic analysis with clones expressing Dp53 and p35 simultaneously, larvae of the following genotype were heat shocked for 30 min at 37°C, raised at room temperature and analyzed 48 h later: *w hsFLP/+ ; tubulin (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 third instar larvae or mid-pupa (45–50 h 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, Danvas, MA, USA), mouse anti-Dp53, rat anti-ELAV, mouse anti-Pros, mouse anti-Cut and mouse anti-βGAL (all obtained from the DSHB, University of Iowa, Iowa City, IA, USA). Secondary antibodies were donkey Fab fragments from Jackson ImmunoResearch (West Grove, PA, USA). The TUNEL assay kit is from Roche (Indianapolis, IN, USA). Images were taken with either a Zeiss AxioImager equipped with ApoTome technology (Carl Zeiss MicroImaging, Thornwood, NY, USA) or a confocal microscope.

Conflict of interest

The authors declare no conflict of interest.

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