In vivo p53 function is indispensable for DNA damage-induced apoptotic signaling in *Drosophila*

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Abstract **p53 is a representative tumor suppressor whose dysfunction is a major cause of human cancer syndrome. Here we isolated flies lacking *Dmp53*, which encodes the single *Drosophila* orthologue of mammalian p53 family. *Dmp53* null mutants well developed into adults, only displaying mild defects in longevity and fertility. However, genomic stability and viability of *Dmp53* mutants dramatically decreased upon ionizing irradiation. Moreover, mutating *Dmp53* abolished irradiation-induced apoptosis and *reaper* induction. These results indicate that Dmp53 is a central component of DNA damage-dependent apoptotic signaling.**
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Key words: Apoptosis; p53; Genomic stability; Cell cycle checkpoint; Reaper; Caspase

1. Introduction

The *p53* gene is a representative tumor suppressor gene, which is mutated in more than half of all human cancers [1], and the Li–Fraumeni syndrome patients with germline mutations of the *p53* gene are predisposed to develop diverse types of cancers [2]. In many cases, mutant forms of p53 accumulate in cancerous cells, acting as a dominant negative subunit of a p53 multimeric complex [3]. The diversity of cancer types arisen by p53 dysfunctions suggests that p53 is not involved in a tissue-specific event, but in some general control of cell survival and proliferation.

The primary function of p53 is to act as a transcription factor, which is inactive in normal growing cells. Various stresses including DNA damage have been implicated to activate p53 by post-transcriptional modifications [4]. Of those molecules involved in this signaling, DNA damage-induced kinases such as ATM [5], Chk1 [6], and Chk2 [7] have been suggested to mediate p53 activation by direct or indirect phosphorylation. Once the p53 protein is activated, it moves into the nucleus, binds to specific enhancers, and regulates the transcription of adjacent genes [4]. These p53-regulated genes influence various cellular outputs, such as cell cycle arrest and apoptosis. For cell cycle arrest, p21$^{waf1/cip1}$ [8] has been con-

sidered to be one of the most important p53 downstream targets. For apoptosis, several mitochondrial proteins including Bax [9] have been suggested. Consequently, p53 prevents the proliferation of DNA-damaged cells and ultimately leads them to apoptosis. Although these researches achieved many progresses in cellular and biochemical characterization of p53, the absence of a genetic system limited the integrated explanation about p53 function in vivo.

Drosophila system has been historically provided as a highly convenient and adequate tool for genetic and histological studies. Notably, DNA damage signaling is also extensively studied in this system; mutations in DNA damage signaling components, such as *mei-41* (ATM), *grapes/Dmchk1* (Chk1) and *Dmchk2* (Chk2) showed a typical mutagen-sensitive phenotype with defects in DNA damage-induced cell cycle checkpoint and apoptosis [10,11]. These signaling molecules are thought to be cellular gatekeepers, which prevent the damaged cells from proliferation, consequently warranting the survival of an organism. Although recent studies have suggested that p53 protein is involved in this signaling pathway [12], convincing evidences including mutant studies have not been thoroughly provided.

In this paper, we generated and characterized the null mutants of *Dmp53*, a recently identified *Drosophila* homolog of mammalian *p53* gene [13–15]. Although Dmp53 is not essential for normal development, null mutants of *Dmp53* show a mutagen-sensitive phenotype with reduced viability and chromosome stability, which was sensitively affected by irradiation-induced DNA damage. We also demonstrated that both the transcriptional activation of *reaper*, a well-known gene to induce cell death, and the subsequent caspase activation were the downstream events of DNA damage-induced Dmp53 activation. Collectively, our results strongly support that Dmp53 is critical for ensuring the genomic integrity and organism survival.

2. Materials and methods

2.1. Generation of Dmp53 *mutant flies*
To find the P-element inserted mutants in the *Dmp53* locus within the collection of 60 000 GenExel EP lines, we constructed 600 different sets of genomic DNA pools each with 100 independent fly lines (GenExel Inc., Korea). Using polymerase chain reaction (PCR) methods (5′ and 3′ primers were designed to complement the P-element inverted terminal repeat sequence and the 5′ flanking region of *Dmp53*, respectively), we found one set of genomic DNA pools containing a P-element insertion line in the *Dmp53* locus. By subsequent

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genomic DNA PCR, a single fly line, $Dmp53^{EP-1}$, with a P-element insertion at the $Dmp53$ locus was isolated. The P-element from $Dmp53^{EP-1}$ was excised by crossing with flies containing Δ2–3 transposase. More than 100 excision lines (scored by the loss of eye color) were established and analyzed by PCR experiments using the primers flanking $Dmp53$ gene.

2.2. Molecular characterization of Dmp53 mutants

$Dmp53$ cDNA probes were obtained from GH11591 clone (IMAGE consortium), and $Dmp53$ gDNA probes were generated by genomic PCR experiments. The EP-specific probe corresponding to the 4733rd ∼ 7180th nucleotides of pP{EP} plasmid (Flybase) was obtained by PCR. An anti-Dmp53 goat polyclonal antibody (Santa Cruz) was used for Western blot analysis. Hybridization and blotting conditions were performed as described previously [16]. Inverse PCR experiments were performed as described in Berkeley Drosophila Genome Project (BDGP) homepage.

2.3. Phenotypic analyses of Dmp53 mutants

For the longevity test, flies of the parental strain (w^{1118}) and the $Dmp53$ homozygous mutant strain ($Dmp53^{E4}$) were maintained in a constant temperature and humidity, and 12/12 h dark/light cycle environment [17]. These flies were transferred to fresh food vials and scored for survival every 3 days. Survival rates at the 10th day were regarded as 100%. For the fertility test, fully mated 3-day-old females of either w^{1118} or $Dmp53^{E4}$ were allowed to lay eggs for the same period (∼ 1 day) on the standard medium. After 15 days, the number of adult progenies was counted for fertility. For radiation sensitivity assays, wandering third instar larvae of w^{1118} and $Dmp53^{E4}$ mutants were treated with the indicated doses of radiation from γ-Co^{60} source. For genomic instability assays, the scoring method for macrochaete defects was adopted from the previous report [10]. All these flies were grown on standard medium at 25°C except for the flies shown in Fig. 1C, lower panel.

2.4. Analysis of imaginal discs and embryos

Imaginal discs dissected from late third instar larvae were fixed with 4% paraformaldehyde, and stained overnight at 4°C with either an anti-active Drice antibody [18] or an anti-phosphospecific histone H3 antibody [13,14]. For acridine orange (AO) staining, imaginal discs were dissected and directly incubated in 1.6 μg/ml AO–phosphate-buffered saline (PBS) solution for 5 min. Fluorescent images were obtained by LSM510 confocal microscope.

3. Results and discussion

To investigate the organism-wide role of p53, we designed a scheme to obtain *Drosophila melanogaster p53* mutants. By PCR-based approach, we found one EP line, with a P-element insertion at the 5′-untranslated region of the *Dmp53* gene (inserted at 173 298th nucleotide of AE003741, Fig. 1A), from the fly library of 60 000 independent EP lines. We confirmed the singularity and the insertion site of the P-element by Southern blot (Fig. 1B) and inverse PCR analyses (data not shown), and named this allele of *Dmp53* 'EP-1'. Because this inserted P-element contains UAS-binding sites in a suitable orientation for the overexpression of *Dmp53* transcript, we first examined the effect of the endogenous *Dmp53* gene overexpression, using eye-specific *gmr*-Gal4 driver and wing-specific *MS1096*-Gal4 driver. The *gmr* driver contains a multimer of Glass-binding sites, which drives gene expression in the posterior region of an eye disc. The *MS1096* driver is the enhancer-trapped GAL4 in *Beadex* locus, which drives strong gene expression in the central pouch region of a wing disc.

When the endogenous *Dmp53* gene was overexpressed in the developing eyes (Fig. 1C) and wings (data not shown), the structures of each organ were disrupted with a significant reduction in its size, and these phenotypes were suppressed by

co-expression of the dominant negative protein, which is the C-terminal fragment of Dmp53 with defective DNA-binding activity [13]. Extensive apoptotic cells (Fig. 1D, upper panel; green spots) were observed in Dmp53-overexpressing region, but the number of mitotic cells (Fig. 1D, lower panel; green spots) was not reduced by Dmp53 overexpression. Therefore, we conclude that the degenerative phenotypes of Dmp53 overexpression are caused by extensive apoptosis, but not by cell cycle checkpoint alteration, which is also supported by previous reports [12–14].

Although there have been considerable numbers of investigation about Dmp53 [12–15], all of them were based on an overexpression system, which has limitations to investigate the endogenous function of Dmp53. Therefore, we decided to generate *Dmp53* loss-of-function mutants by mobilizing the P-element in the $Dmp53^{EP-1}$ line. By the standard genetic scheme for P-element excision and the PCR-based screening, we isolated two deletion lines whose *Dmp53* gene was disrupted, and named them 'E4' and 'E8' after their isolation numbers. Sequencing of the PCR products from each genomic DNA revealed that most of the promoter region of the *Dmp53* gene was missing in the $Dmp53^{E8}$ line (173 312nd ∼ 174 190th nucleotides of AE003741, Fig. 1A), and that the first four exons containing ATG and coding sequences were additionally deleted in the $Dmp53^{E4}$ line (172 225th ∼ 173 740th nucleotides of AE003741, Fig. 1A).

To confirm whether the *Dmp53* mutant alleles are the null alleles for *Dmp53* gene, we assessed the presence of Dmp53 in these mutants. Southern analysis of the homozygous mutants showed that the sequences were completely deleted in each homozygous mutant genome (Fig. 1E). Northern (Fig. 1F), reverse transcription (RT)-PCR (Fig. 1G), and Western (Fig. 1H) analyses further showed that both *Dmp53* transcript and protein were missing in the homozygotic $Dmp53^{E4}$ and $Dmp53^{E8}$, confirming that both alleles are null for *Dmp53*.

Next, we examined the phenotypes of the flies deficient for Dmp53. Surprisingly, homozygotic condition of both alleles gave rise to viable flies with no dramatic defects (data not shown). To catch a clue for endogenous Dmp53 function, we carefully examined the life span and fertility of the *Dmp53* null mutants. Compared to the parental strain, the flies without Dmp53 survived well up to 50 days, but mortal rates were gradually increased as time passed (Fig. 2A). The fertility of *Dmp53* null mutants was also significantly afflicted (Fig. 2B), and this might be due to the presence of some degenerative ovarioles observed in histological analyses of Dmp53-deficient mutants (data not shown). Although Dmp53 has been previously shown to be not essential for the meiotic DNA repair during oogenesis [19], our data suggest that Dmp53 could be involved in other stages of ovary development processes.

Because *Dmp53* mutants did not show dramatic defects under mild condition, we were curious to find out whether a harsh stimulus such as ionizing radiation-induced DNA damage could result in a more distinguishable mutant phenotype. Although the *Dmp53* mutants were almost fully viable under normal conditions, the viability of *Dmp53* mutants dramatically declined as irradiation energy increased (Fig. 2C). Furthermore, macrochaete examination of irradiated flies revealed that chromosome loss took place more frequently in *Dmp53* null mutants than in wild-type (Fig. 2D). Therefore, we conclude that *Dmp53* null mutants are irradiation-sensi-
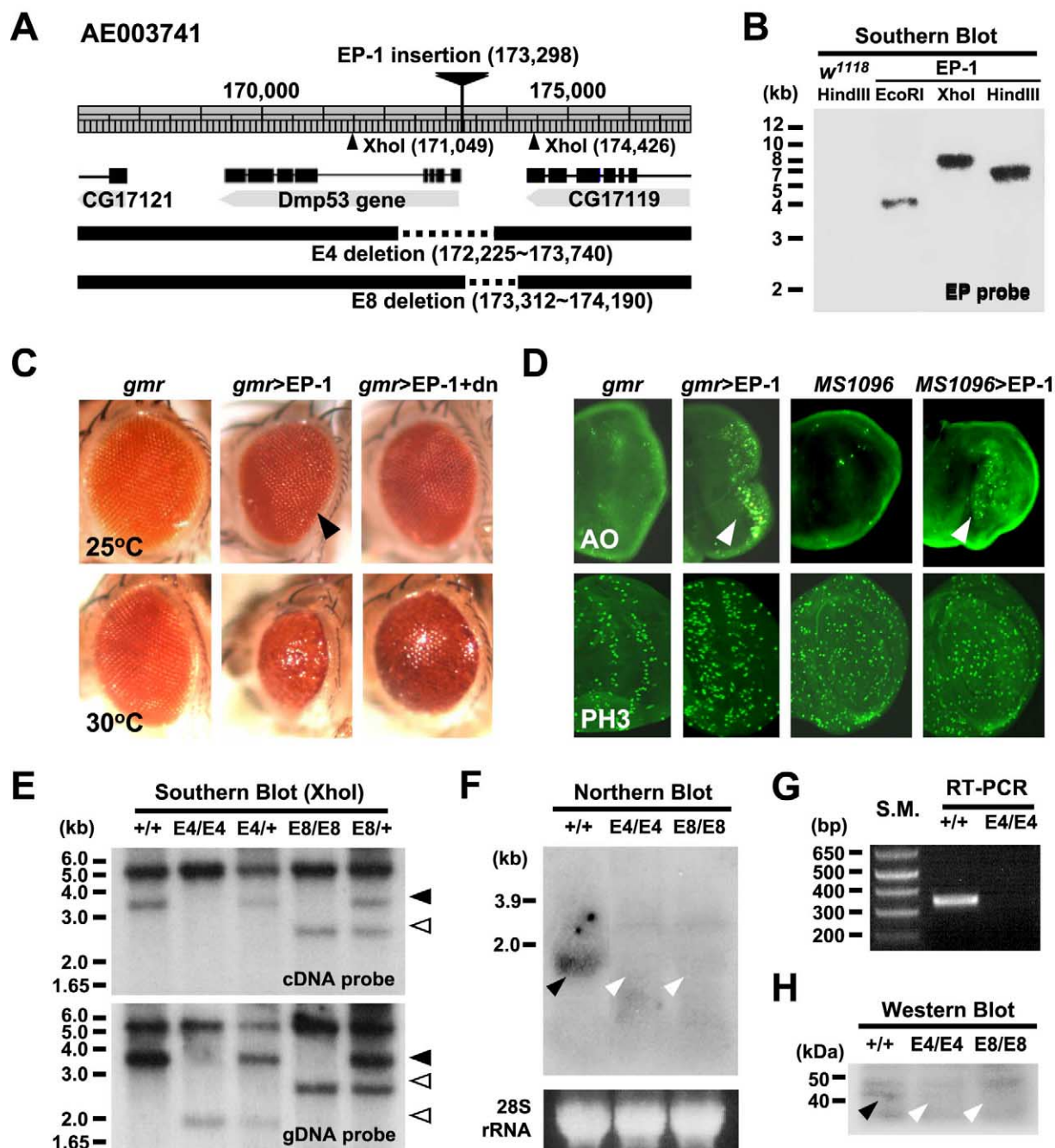
Fig. 1. Isolation and characterization of *Dmp53* mutants. A: Schematic genomic organization of the *Dmp53* locus and transcripts, also showing the molecular status of the *Dmp53* mutants. EP element insertion in *Dmp53^EP−1* mutant (triangle) and genomic deletions in *Dmp53^E4* and *Dmp53^E8* are indicated. Nucleotide numbering has been done according to the AE003741 sequence (*Drosophila* genome release v3.1). B: Southern blot analyses confirming the singularity of P-element in *Dmp53^EP−1*. Genomic DNA from *w^1118* and *Dmp53^EP−1*, which was cut with indicated enzymes, and was blotted and hybridized with a specific EP probe as described in Section 2. The expected band sizes of EP-1 lanes: 4.4 kb for *Eco*RI, 8.4 kb for *Xho*I and 7.2 kb for *Hind*III. C: Eye-specific phenotypes of Dmp53 overexpression driven by *gmr*-Gal4. The genotypes of the flies are *gmr*-Gal4/+ (left), *gmr*-Gal4/+; *Dmp53^EP−1*/+ (middle), and *gmr*-Gal4/+; *Dmp53^EP−1*/UAS-*Dmp53^dn* (right). Each fly has been grown at 25°C (upper panels) or 30°C (lower panels). Cultures in a higher temperature enhanced the degenerative phenotypes of Dmp53 overexpression. The arrowhead indicates the mild degeneration of the eye structure. D: *gmr*-Gal4/+, *gmr*-Gal4/+; *Dmp53^EP−1*/+, *MS1096*-Gal4/+, and *MS1096*-Gal4/+; *Dmp53^EP−1*/+ larvae were analyzed by histological staining. AO staining (upper panels) was used to visualize apoptotic cells, and anti-phosphospecific histone H3 antibody staining (lower panels) was used for visualizing mitotic cells in M phase. Arrowheads indicate the ectopic apoptosis observed in Gal4-expressing regions. E: Southern blot analyses confirmed the deficiencies of *Dmp53* in *Dmp53^E4* and *Dmp53^E8* fly lines. The band responsible for E4/E8 deletion region (3.4 kb, black arrowheads) detected in the wild-type lane (+/+) is absent or reduced to smaller size (1.9 kb for E4 and 2.5 kb for E8, white arrowheads) in *Dmp53* mutant lanes. F: Northern blot analyses revealed the absence of *Dmp53* transcript (1.6 kb, black arrowhead in the wild-type lane) in *Dmp53^E4* and *Dmp53^E8* homozygous adults (white arrowheads in the mutant lanes). G: RT-PCR analyses confirmed that *Dmp53* transcript is absent in *Dmp53^E4* mutant. Primers were designed for the amplification between exons 6 and 7 of the *Dmp53* gene (344 bp signal in wild-type lane). Even through the extensive (∼40 cycles) PCR cycling, we could not detect any *Dmp53* message in the mutant lane. S.M., size marker. H: Dmp53 proteins absent in *Dmp53* null mutants. Western blot analysis was performed using the cell lysates from wild-type, *Dmp53^E4*, and *Dmp53^E8* homozygous larvae. The 42-kDa Dmp53 signal (black arrowhead) is completely missing in both deletion mutants (white arrowheads).
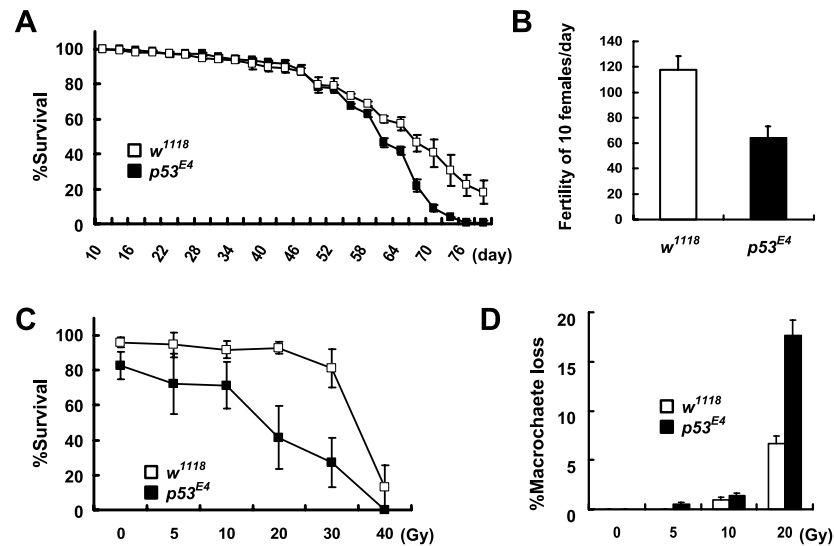
Fig. 2. *Dmp53^{E4}* null mutants show mutagen-sensitive phenotypes. A: Life span of *Dmp53^{E4}* flies (closed square) was mildly reduced, when compared to parental *w^{1118}* strain (open square). Culturing and scoring conditions are described in Section 2. Mean survival % of each genotype from the three independent cultures with 100 males was shown as a graph. Error bars indicate the standard deviation between cultures. B: The fertility of *Dmp53^{E4}* mutant flies was reduced to half compared to its parental *w^{1118}* flies. Mated 3-day-old females were subjected to fertility test. We performed six independent experiments, and the average fertility scores were presented. Error bars indicate standard deviation between experiments. C: The viability of *Dmp53^{E4}* mutant flies (closed square) was sensitively affected by ionizing radiation compared to *w^{1118}* parental strain (open square). The third instar larvae, irradiated with indicated dosages, were incubated for 8 days and scored for adult survivors. Error bars indicate standard deviation between five independent cultures with more than 30 individuals. D: *Dmp53^{E4}* mutants showed increased genomic instability upon irradiation. The macrochaete defect represents genomic instability as previously described [10]. Hatched adults from C were assayed for macrochaete defects. 30 macrochaetes from each of seven adult females were counted. Error bars indicate the standard deviation of the score between the individuals.

tive, and Dmp53 is required for the genomic integrity and survival upon DNA damage.

Interestingly, viable but semi-sterile and mutagen-sensitive phenotypes are also observed in null mutations of *mei-41*, *grapes*, and *Dmchk2* genes in *Drosophila* [10,11]. The products of these genes are homologous to mammalian DNA damage-induced kinases, ATM, Chk1, and Chk2, respectively, which are known to activate mammalian p53 protein [5–7]. As fly mutants of these genes show various defects in DNA damage responses, we attempted to test whether Dmp53 is also required for those processes.

First, eye and wing imaginal discs of wild-type and *Dmp53* null mutants were dissected from third instar larvae 4 h after γ-irradiation, and the apoptotic cells were visualized with AO staining. Unirradiated imaginal discs of wild-type and *Dmp53* null mutants showed few apoptotic cells. However, when irradiated, numerous apoptotic cells were observed throughout the wild-type imaginal discs (Fig. 3A), whereas Dmp53-deficient discs were completely resistant for irradiation-induced apoptosis (Fig. 3B,C). Therefore, we conclude that the endogenous Dmp53 protein is required for irradiation-induced apoptosis.

Although *Dmp53* mutants were defective in irradiation-induced apoptosis, irradiation-induced cell cycle checkpoint was not altered in *Dmp53* mutants. In the absence of irradiation, there were a considerable number of mitotic cells in wild-type (Fig. 3D) and *Dmp53* null mutants (Fig. 3E,F). However, upon irradiation, cells undergoing mitosis in both wild-type and Dmp53-deficient imaginal discs were significantly decreased at a similar level. Therefore, we conclude that irradiation-induced cell cycle arrest does not require Dmp53 activity.

The case of Dmp53 is in stark contrast to the case of DmChk1, which is involved only in irradiation-induced cell cycle arrest but not in apoptosis [11]. Therefore, two different DNA damage response pathways – apoptosis and cell cycle control – could be discriminated; Dmp53 is specific for apoptosis, and DmChk1 is specific for cell cycle control. Mei-41 and DmChk2, which are involved in both DNA damage responses [10,11], are thought to be upstream controllers of Dmp53 and DmChk1. Along with the previous data which imply that DmChk2 directly activates Dmp53 [12], it is very likely that Dmp53 is a central effector of DmChk2, which relays DNA damage-induced apoptotic signal to the cell death machinery.

As the next step, we attempted to reveal the downstream mechanisms in p53-dependent apoptosis upon irradiation. Because caspase activity is required for general apoptosis and involved in irradiation responses [20], we analyzed whether caspases are activated in irradiated wild-type and *Dmp53* mutants as well. As expected, extensive caspase activation was detected in wild-type irradiated imaginal discs (Fig. 3G), with a pattern similar to irradiation-dependent apoptosis (Fig. 3A). However, this caspase activation was completely missing in the *Dmp53* null imaginal discs (Fig. 3H,I), showing that Dmp53 mediates caspase activation in response to the irradiation-induced DNA damage.

Next, we questioned what molecular components relay the signal from the active Dmp53 to the caspase system. Recently, *Drosophila* apoptosis inducers, *grim*, *reaper* and *hid*, have been shown to mediate caspase activation by negative regulation of *Drosophila* inhibitor of apoptosis (DIAP) [18]. And there also was a report that Dmp53 binds a damage response element at the *reaper* locus [13]. Therefore, we analyzed the amount of
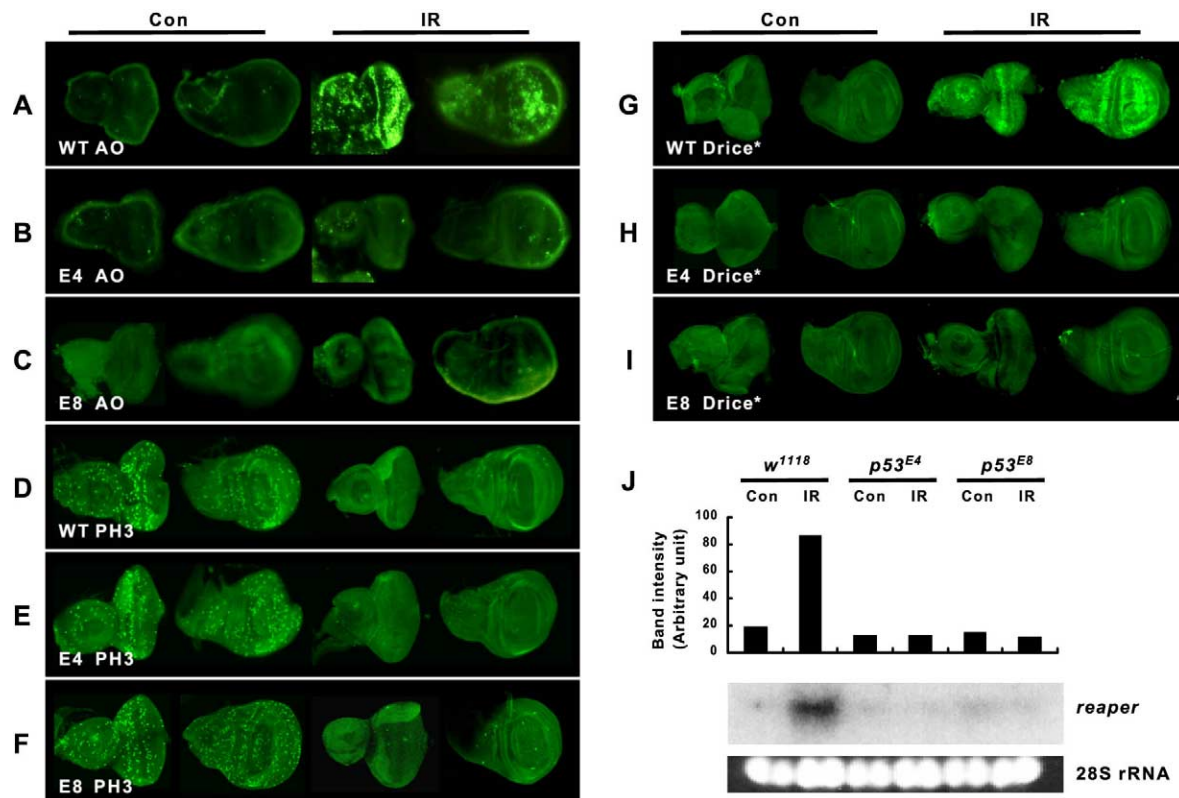
Fig. 3. *Dmp53* null mutants are defective in DNA damage-dependent apoptosis but not the cell cycle checkpoint. Unirradiated (Con) eye (first column) and wing (second column) discs and irradiated (IR) eye (third column) and wing (fourth column) discs were subjected to histological analyses (A–I). The genotypes used for each experiment are w^{1118} (A, D, G), $Dmp53^{E4}$ (B, E, H), and $Dmp53^{E8}$ (C, F, I). A–C: *Dmp53* mutants are defective in irradiation-induced apoptosis. AO staining was used to visualize cells that are undergoing apoptosis. Irradiation induces ectopic apoptosis only in wild-type imaginal discs (A). D–F: *Dmp53* mutants displayed a normal cell cycle arrest by irradiation. Mitotic cells were visualized by staining with anti-phosphospecific histone H3 antibody. Irradiation induces cell cycle arrest in both wild-type (D) and mutant (E and F) imaginal discs. G–I: *Dmp53* mutants are defective in irradiation-dependent caspase activation. An active caspase was detected by anti-active Drice antibody, which specifically detects actively processed Drice (a *Drosophila* caspase homolog) [18]. Caspase activation is observed only in the irradiated wild-type imaginal discs (G). J: Transcriptional activation of *reaper* followed by irradiation is abolished in *Dmp53* null mutants. Total RNAs were isolated from embryos with the genotype indicated. Prior to RNA isolation, the embryos of 3 h after egg laying were untreated (Con) or challenged by irradiation at 4000 Rad (IR), and aged three more hours. The isolated RNA was analyzed by Northern blot analyses (middle panel). 28S ribosomal RNA band was used as a loading control (bottom panel). The amount of *reaper* transcript was quantified and shown as a bar graph (top panel).

reaper transcript in irradiated wild-type and *Dmp53* null mutants. While the *reaper* transcription was highly induced in wild-type irradiated embryos, loss of Dmp53 activity completely blocked this induction (Fig. 3J). Considering the Reaper protein itself is indispensable for irradiation-dependent apoptosis [21], we deduced that the apoptotic activity of Dmp53 upon irradiation is largely dependent on *reaper* transcriptional activation. However, the possibility that other Dmp53 targets could mediate the apoptotic response should not be discounted because there exist conflicting data, which showed that Dmp53 overexpression-induced apoptosis is not completely suppressed by *reaper* null mutation [21]. Nevertheless, we conclude that Dmp53 is an upstream component of Reaper in the DNA damage signaling cascade and controls the cell's apoptotic machinery by regulating the transcription of *reaper*.

Collectively, by characterizing the loss-of-function mutants of Dmp53, we clearly demonstrated that Dmp53 is an important mediator of DNA damage-induced apoptosis. Upon DNA damage, activated Dmp53 transcriptionally induces the *reaper* gene, and Reaper induces caspase activation consequently leading the cells to apoptosis. The presence of the

DNA damage-induced apoptosis ensures the survival and genomic integrity possibly by the elimination of damaged cells. Considering the convenience and adequateness of *Drosophila* system in genetic studies, we believe our p53 null fly model will enable deeper researches in understanding DNA damage responses in metazoan system.

References

[1] Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C. (1994) Cancer Res. 54, 4855–4878.
[2] Malkin, D. et al. (1990) Science 250, 1233–1238.
[3] Sigal, A. and Rotter, V. (2000) Cancer Res. 60, 6788–6793.
[4] Oren, M. (2003) Cell Death Differ. 10, 431–442.
[5] Canman, C.E. et al. (1998) Science 281, 1677–1679.
[6] Shieh, S.Y., Ahn, J., Tamai, K., Taya, Y. and Prives, C. (2000) Genes Dev. 14, 289–300.
[7] Hirao, A. et al. (2000) Science 287, 1824–1827.

[8] el-Deiry, W.S. et al. (1993) Cell 75, 817–825.

[9] Miyashita, T. and Reed, J.C. (1995) Cell 80, 293–299.

[10] Brodsky, M.H., Sekelsky, J.J., Tsang, G., Hawley, R.S. and Rubin, G.M. (2000) Genes Dev. 14, 666–678.

[11] Xu, J., Xin, S. and Du, W. (2001) FEBS Lett. 508, 394–398.

[12] Peters, M. et al. (2002) Proc. Natl. Acad. Sci. USA 99, 11305–11310.

[13] Brodsky, M.H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G.M. and Abrams, J.M. (2000) Cell 101, 103–113.

[14] Ollmann, M. et al. (2000) Cell 101, 91–101.

[15] Jin, S. et al. (2000) Proc. Natl. Acad. Sci. USA 97, 7301–7306.

[16] Lee, J.H., Cho, K.S., Lee, J., Yoo, J. and Chung, J. (2001) Gene 271, 233–238.

[17] Lin, Y.J., Seroude, L. and Benzer, S. (1998) Science 282, 943–946.

[18] Yoo, S.J. et al. (2002) Nat. Cell Biol. 4, 416–424.

[19] Abdu, U., Brodsky, M. and Schupbach, T. (2002) Curr. Biol. 12, 1645–1651.

[20] Hay, B.A., Wolff, T. and Rubin, G.M. (1994) Development 120, 2121–2129.

[21] Peterson, C., Carney, G.E., Taylor, B.J. and White, K. (2002) Development 129, 1467–1476.