Daxx-like Protein of *Drosophila* Interacts with Dmp53 and Affects Longevity and Ark mRNA Level*§†‡

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Daxx-like protein (DLP), the *Drosophila* homolog of Daxx, binds *Drosophila melanogaster* p53 (Dmp53) through its C-terminal region. We generated DLP mutants and found that although DLP expression is developmentally regulated, it is not essential for the execution of the developmental program. The effects DLP mutations show in the loss of heterozygosity assay and on phenotypes resulting from Dmp53 overexpression indicate a genetic interaction between DLP and Dmp53. In contrast to Dmp53 mutants, however, loss of DLP does not result in radiosensitivity indicating that it does not play an essential role in the activation of Dmp53-dependent response after ionizing radiation, and DLP is also not required for the irradiation-induced activation of reaper. In contrast, DLP is involved in the transcriptional regulation of Ark, because Ark mRNA level is decreased in DLP mutants and increased upon ectopic overexpression of DLP. Interestingly, DLP mutants have reduced longevity and reduced female fertility. Altogether, our data suggest complex functions for DLP, which include an anti-apoptotic effect exerted through repression of some Dmp53 functions, and activation of some proapoptotic genes.

Mammalian p53 is a transcription factor that plays a fundamental role in cellular response to genotoxic stress (1). Activated p53 induces cell cycle arrest, DNA repair, or apoptosis by both transcription-dependent and independent ways (2). The observation that p53 is the most commonly mutated gene in human cancer (3) underlines the importance of these activities in preserving genome integrity and eliminating transformed cells that pose a risk at the organism level. Although a great body of knowledge has accumulated about p53, its regulation and activity is still not understood in full detail. Furthermore, the existence of two paralogs of p53 (p63 and p73) in mammalian cells with partly overlapping functions, makes the dissection of the cellular role of p53 difficult, and underlines the importance of simple models for studying p53 function. p53 and its only *Drosophila* homolog Dmp53 share limited conservation at the sequence level, yet the two proteins are surprisingly similar in domain structure and their residues critical for DNA binding are well preserved (4, 5). Importantly, Dmp53 is able to bind to human p53 recognition sites and activate transcription in vitro (5, 6). Although Dmp53 is nonessential under normal circumstances, null mutants show genomic instability and radiosensitivity (7, 8). In response to genotoxic stimuli, activated Dmp53 induces DNA repair or programmed cell death by activating the expression of its target genes, such as the pro-apoptotic reaper (rpr) and hid, and the DNA repair genes *Ku70* and *Ku80* (9). Loss of Dmp53 abolishes radiation-induced apoptosis in larval imaginal discs (6, 8), and overexpression induces cell death (5, 9). Interestingly, a disturbed level of p53 affects aging and longevity both in mouse and *Drosophila* (7, 10–12). Although little is known about the regulation of Dmp53, the information accumulated so far suggests that Dmp53 is regulated in a partially conserved, ancestral way. MDM2 does not have a *Drosophila* homolog, and the amino acid residues critical for its binding are not preserved in Dmp53 (4). In contrast, similar to its human counterpart, Dmp53 is phosphorylated, and this modification is necessary for the induction of Dmp53-dependent apoptosis (9, 13).

The death domain-associated factor 6, Daxx, is one of the cofactors modulating p53 functions. Daxx was initially identified as a cytoplasmic Fas receptor-binding protein that potentiates apoptosis (14, 15). More recent studies found that Daxx resides primarily in the nucleus, and participates in transcriptional regulation (16–18). Loss of Daxx results in extensive apoptosis and early embryonic death in mice (19), suggesting that it may also bear anti-apoptotic functions. This view is supported by the finding that the expression of p53, similarly to that of several other proapoptotic proteins, is down-regulated by Daxx in myelocytes (20). Several groups demonstrated the binding of Daxx to p53 but the consequences of this interaction are not clearly elucidated. Tumorigenic mutant forms of p53 were found to bind Daxx and inhibit the activation of the Daxx-dependent ASK1/JNK pathway (21). Other studies showed that Daxx also binds wild-type p53 and modulates its transcriptional activator function in vitro (22–24), although these experiments provided partially conflicting results. Several recent reports...
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GST Pull-down Experiments—The C-terminal region of DLP cDNA identified in Y2H screen was inserted into pET28c and transcribed-translated in the presence of [3H]leucine using the TNT T7-coupled reticulocyte lysate system (Promega) following the manufacturer’s instructions. Dmp53-3C was cloned into pGEX-4T-1 vector using EcoRI and SalI sites. Expressed GST-Dmp53-3C was bound to glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer’s instructions. In vitro translated protein and GST-Dmp53-3C-bound beads were mixed in PD buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM EDTA pH8.0, 1 mM β-mercaptoethanol, 1% Nonidet P40) and kept at 4 °C for 2 h. Beads were washed four times with PD buffer containing 0.1% Nonidet P40, and once with PD buffer lacking Nonidet P40. Interactions were analyzed by SDS-PAGE and fluorography (using Amplify Fluorographic Reagent from Amersham Biosciences).

Quantitative RT-PCR—Developmental expression of DLP was determined by measuring DLP transcript levels in synchronized w1118 animals of different developmental stages. Dmp53-dependent transcriptional activation after DNA damage was determined by measuring rpr and Ark mRNA levels of wandering third-instar w1118 Dmp53ΔA-1–4, DLPΔ[26], DLPΔ[32], and DLPΔ[32] larvae without irradiation or 2 h after 4 krad x-ray irradiation (1 krad/min). To measure DLP and Ark transcript levels in flies overexpressing DLP P[hs-GAL4]/EP (2)2108, P[hs-GAL4]/EP (2)2193, P[hs-GAL4]/EP (2)2180, P[hs-GAL4]/P[USADLP1.7 kb], and P[hs-GAL4]/+ (control) adult females were heat-shocked for 60 min at 37 °C. 60 min after heat-shock RNA was isolated and used in Q-PCR. Total RNA was isolated with Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 2 μg of total RNA with random hexamer primers using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, CA). PCR reactions were carried out in duplicates in an ABI Prism 7500 real-time PCR system. cDNAs corresponding 18S rRNA and rpr were amplified using TaqMan Universal PCR Master Mix while SYBR-Green PCR Master Mix (27) was used for the amplification of DLP and Ark cDNA. The sequence of primers and TaqMan probes were as follows: 18S forward: GCCAGCTAGCAATT-GGGGTGA, 18S reverse: CCGGAGCCCCAAAAACGCTT, 18S probe: TATGGGTTCCTCTACGTGTTACG, rpr probe: CACGTTTGTTGATATTGGA, rpr reverse: TCGCCT-GATCGGTATGTA, rpr probe: AAGAAAGATACACAAATGCGACAGTACAGGA, Ark forward: TTCCGCAATACAA-GCTGAG, Ark reverse: CATCCAAAGGCTACCCAATG, DLP forward: TCGCCTAGCATGCTTTAAC, DLP reverse: GCCAGCAGCCA AAAAACGCTT, 18S probe: TATGGGTTCCTCTACGTGTTACG.

In Situ Hybridization—Whole mount in situ hybridization was performed using digoxigenin-labeled, hydrolyzed antisense RNA probes following the manufacturer’s recommendations (Roche Applied Science). Probes were prepared from the full-length DLP cDNA clone SD20887 (28) received through the Drosophila Genomics Resource Center. Hybridization and post-hybridization washes were done at 55 °C, RNA
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hybrids were detected with anti-digoxigenin-AP antibody followed by NBT/BCIP staining.

Drosophila Stocks and Crosses—Fly stocks were maintained at 25 °C on standard cornmeal-yeast-agar Drosophila medium. The Dmp53SA-1—4 line (29) was a generous gift from Yikang S. Rong (National Institutes of Health, Bethesda, MD). The P[UASp53] stain was a kind gift from Michael W. Young (5). The RS-element insertion lines UM-8182-3 and CB-6241-3 (30), the EP insertion lines EP (2)2108, EP (2)2193, and EP (2)2180 were kindly provided by the Szeged Drosophila Stock Centre. The RNAi strains 29374 and 29377 were obtained from Vienna Drosophila RNAi Center. P[act-GAL4] strain (BL-3954) and other stocks were obtained from the Bloomington Drosophila Stock Center. The P[ey-GAL4] strain was kindly provided by J. Mihaly (BRC, Szeged, Hungary).

To generate new DLP alleles the RS elements in the viable insertion lines UM-8182-3 and CB-6241-3 were remobilized using the TM3, ryRk Sb’ Ser’ P[Δ2-3]99B transposase source. Excision lines were identified based on the loss of the miniwhite marker gene and stocks were established using the y+ CyO balancer chromosome. From each homozygous line genomic DNA was prepared, and PCR reactions were performed with primer pairs amplifying sequences upstream and downstream of the insertion site. (Sequences of primers are available upon request.) Deletion lines were selected on the basis of loss of the PCR products. The sizes of deletions were determined by further PCR reactions and in some selected cases molecular breakpoints were determined by sequencing. In addition to the deletions, precise excision lines (PE) in which precise jumpout of the P element restored the structure of DLP, were also identified by PCR analysis. Lethal mutations were transferred over a Cy-GFP balancer chromosome, the presence of deletions inspected in GFP-negative embryos as described above and allelism tested by complementation analysis. To measure the reduction of viability after ionizing radiation wandering third-instar w1118 ; p53SA-1—4 ; DLPΔ262, DLPΔ272, and DLPΔ42 larvae were exposed to 2 krad (1 krad/min) x-ray irradiation and transferred into fresh vials, 20 larvae/vial. The number of pupae and eclosed adults were recorded and viability expressed in the percent of the number of irradiated larvae. At least six independent experiments were performed for each genotype. For the longevity assay freshly eclosed males in groups of 20 were put into vials, and the number of survivors was counted daily. For the fertility assay five 48-h-old females with 2 males were put into a vial, and the number of eggs laid in a 24-h period was recorded for 7 days in five independent experiments of each genotype. To observe the effect of DLP mutation on the phenotype resulting from Dmp53 overexpression we performed the following crosses: DLPΔ262 / P[UASp53]/T(2, 3) TSTL, Cy; Tb Hu females were mated to DLPΔ262 / P[act-GAL4]/TSTL males, and DLPΔ262 / P[UASp53]/TSTL females were crossed with PE; P[act-GAL4]/TSTL males as a control. The DLPΔ262/DLPΔ262, P[UASp53]/P[act-GAL4], and DLPΔ262/PE; P[UASp53]/P[act-GAL4] animals were selected based on the Tb+ phenotype. Three independent experiments were performed and the number of animals reaching specific developmental stages was recorded. Similar experiments were carried out using the eye-specific eyeless-GAL4 driver. For this the crosses were: DLPΔ262; P[ey-GAL4] females × DLPΔ262; P[UASp53]/TSTL, P[UASp53]/TSTL males and DLPΔ262; P[ey-GAL4] females × PE; P[UASp53]/TSTL males as a control. The DLPΔ262/DLPΔ262, P[UASp53]/P[ey-GAL4], and DLPΔ262/PE; P[UASp53]/P[ey-GAL4] animals were selected based on the Tb+ phenotype. The largest diameters of the eyes of adult flies (20 in each group in three parallels) were determined after photography. In loss-of-heterozygosity (LOH) assay DLPΔ262/DLPΔ262; mwh Dmp53SA-1—4/+ , and PE/DLPΔ262; mwh Dmp53SA-1—4/+ (control) late-third-instar (wandering) larvae were x-ray-irradiated with 250 rad (150 kV; 0.5-mm Al filter; 1,000 rad/min). Wings were dissected after eclosion, mounted in 1:1 methyl salicylate/Canada balsam (Sigma) and the number of mwh clones determined. Both genetic combinations were tested in four independent experiments, each involving 8–20 wings.

RESULTS

Daxx-like Protein Interacts with Dmp53—In an attempt to identify Dmp53-interacting proteins we employed the yeast two-hybrid (Y2H) method to screen Drosophila embryonic cDNA library using a lexA-Dmp53 fusion lacking the N-terminal transcriptional activation domain of Dmp53 (Dmp53ΔN) as bait. (Fig. 1A) Sequence analysis identified one of the positive clones as a partial cDNA of the Daxx-like protein gene (CG9537). The 810-bp long clone encodes the last 135 acids (1524–1659) of DLP indicating that the C-terminal part of the protein mediates the interaction with Dmp53. GST pulldown experiment validated the specific interaction of Dmp53 and the DLP C-terminal region encoded by the cDNA recovered in the Y2H screen (Fig. 1C).

To determine which region of Dmp53 is necessary for DLP interaction, we fused various segments of Dmp53 to lexA and tested for interaction with the identified DLP clone in Y2H experiments. These experiments revealed that the DNA binding domain of Dmp53 (Dmp53ΔN) did not, but the C-terminal part of Dmp53 (Dmp53C) showed strong interaction with DLP (Fig. 1, A and B). When we asked whether either of the two functionally distinguishable C-terminal regions alone are able to bind DLP we found that neither the oligomerization domain (Dmp53C1), nor the basic regulatory domain (Dmp53C2) alone showed interaction (Fig. 1, A and B). Consequently, the entire C-terminal region of Dmp53, containing both the oligomerization and the basic regulatory domain, is necessary and sufficient to mediate DLP binding. These results indicate that, similarly to the interaction of p53 and Daxx (22), the C-terminal region of Dmp53 is responsible for DLP binding.

The similar regions of p53 and Dmp53 involved in Daxx and DLP interaction, respectively, prompted us to test whether DLP can bind human p53. Surprisingly, in Y2H assay we detected strong interaction between the C-terminal region of DLP and human p53 (Fig. 1D). Thus, although the human and fly p53 orthologs have low sequence similarity, the protein features necessary for Daxx/DLP binding are evolutionarily conserved.

Daxx-like Proteins Are Conserved in the Drosophila Genus—The DLP gene has a coding capacity for a putative protein of 1659 amino acids, which shows partial similarity to its 740-residue-long human ortholog, Daxx. Comparison of the two proteins revealed that they share 27% sequence identity and
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Within the DLP were viable. Thus, we concluded that DLP mutations are most probably viable and selected three alleles for further analysis (Fig. 2B). In DLP^{126}, a 1.7-kbp deletion removes the downstream part of the first intron and 1296 bp of coding sequence from the second exon. DLP^{132} carries a 0.3-kbp deletion that removes the first exon-intron junction and 28 nucleotides from the first exon. In DLP^{142} a 0.4-kbp deletion removes the translational start site. We believe that the deletions in these DLP alleles interfere with the splicing and/or translation of the DLP message. Using primers located in the vicinity of original P element insertion site DLP specific mRNA in mutant animals cannot be detected. We believe that these represent null alleles of the gene, however, in the lack of DLP specific antibody, we cannot rule out the possibility that they are hypomorph alleles, in which a truncated form of the protein is produced. In addition to the deletions, by PCR analysis we also identified lines in which precise excision of the P element restored the structure of DLP. In further genetic experiments one of these

46% similarity in the Daxx-homology region (residues 1125–1472 of DLP), a region conserved in all known Daxx homologs (Fig. 2A). The Dmp53 interacting region of DLP shows 51% similarity to the part of Daxx reported to mediate p53 binding in vitro (22). Beside the regions mentioned above, DLP bears N-terminal glutamine-, proline-, and alanine-rich regions, which seem to be specific for the Drosophila Daxx homolog. Identifiable DLP-like proteins in members of the Drosophila genus are highly homologous in their Daxx homology regions and share significant sequence similarities outside this domain (Fig. 2A).

Generation of DLP Mutants—DLP is encoded on the second chromosome at the cytological position 26D8. The transposons in the P(RS3) element lines UM-8182-3 and CB-6241-3 (30) reside in the first intron of the gene, +568 bp and +638 bp downstream of the transcriptional start site, respectively. To investigate DLP functions in vivo, we generated mutant alleles of the gene by remobilization of the P element present in these lines. Excision lines obtained following transposon remobilization were identified by loss of the lines. Excision lines obtained following transposon remobilization of the P element present in these alleles of the gene, however, in the lack of DLP specific antibody, we cannot rule out the possibility that they are hypomorph alleles, in which a truncated form of the protein is produced. In addition to the deletions, by PCR analysis we also identified lines in which precise excision of the P element restored the structure of DLP. In further genetic experiments one of these

FIGURE 1. DLP interacts with the C-terminal region of Dmp53. A, schematic representation of the domain structure of Dmp53: The regions of Dmp53 used as baits in yeast two-hybrid experiments are shown below the protein diagram. TA, transcription activation domain; DBD, sequence specific DNA binding domain; O, oligomerization domain; B, basic regulatory domain. The results of His-auxotrophy reversion and β-galactosidase filter assays are indicated. B, growth ability on His plates and β-galactosidase activity of yeast cells expressing DLP fused to GAL4 activation domain and Dmp53 regions fused to lexA DB region. C, left panel: demonstration of DLP and Dmp53 interaction in pull-down experiment. The arrow points to the band corresponding to the [3H]leucine-labeled DLP C-terminal part synthesized in vitro transcription-translation reaction. Right panel, Coomassie Brilliant Blue-stained purified GST-Dmp53 and GST proteins used in the GST pull-down experiment. D, interaction of p53 and DLP is demonstrated by the growth ability on His plates and the β-galactosidase activity of yeast cells expressing a DLP-C-terminal region fused to GAL4 activation domain and human p53 (lacking the N-terminal activation region) fused to lexA DB-domain.

(PE) was used as an isogenic control of DLP mutants.

DLP Is Developmentally Regulated but Is Not Required for Normal Development—To determine whether loss of DLP has any effect on Drosophila development we crossed homozygous DLP^{126}, DLP^{132}, and DLP^{142} females to heterozygous males and determined the ratio of homozygous and heterozygous offsprings. No significant differences were found in any allele (data not shown), proving that DLP is not essential for execution of the developmental program. Lines expressing DLP siRNA obtained from the VDRC (Vienna Drosophila RNAi Center) collection are also viable and develop normally (data not shown). Therefore, we decided to determine whether DLP is expressed during development or it is silenced under normal circumstances. To measure DLP expression we performed quantitative real-time RT-PCR (Q-PCR) analysis on wild-type and homozygous embryos containing maternally deposited transcripts (Fig. 3A). Zygotic expression of DLP starts at the time of gastrulation after a nearly complete exhaustion of maternally deposited message by cellular blastoderm stage. At later stages of development, specific expression can be detected in tissues containing mitotic cells such as in the brain hemispheres and
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imaginal discs (Fig. 3, B–F). Thus, unlike murine Daxx, whose loss results in early embryonic lethality (19), DLP mutations do not interfere with the normal developmental program, even though DLP is expressed through development.

Genetic Interaction between DLP and Dmp53—Because we identified DLP as a protein binding to Dmp53, the question rose whether loss of DLP influences Dmp53 dependent responses. Radiosensitivity, reduced tolerance to ionizing radiation is a hallmark feature of Dmp53 mutants. As this phenotype is because of the inability to activate Dmp53-dependent pathways, we decided to investigate if DLP mutants have similar radiosensitive phenotype. We irradiated wild type, Dmp535A-1–4, DLP126, DLP132, and DLP142 wandering third instar larvae with 2 krad x-ray and determined the proportion of animals that eclosed as adults (Fig. 4A). As expected, loss of Dmp53 dramatically reduced the survival rate after irradiation (p < 0.001, ANOVA). However, no such change could be observed in DLP mutants indicating that DLP does not have a key role in the activation of Dmp53-dependent pathways after high dose ionizing radiation.

Next we asked whether DLP modulates genomic stability, by measuring loss of heterozygosity (LOH) provoked by low dose x-ray irradiation. Since the occurrence of mwh phenotype following x-ray irradiation reflects Dmp53 function, as low and high number of mwh clones indicate the presence of functional Dmp53 and the lack of Dmp53 function, respectively (see supplemental Fig. S1), we performed the LOH assays in a sensitized Dmp53/+ background. Late third instar DLPU26/DLPU26 or

PE/DLP126 (control) larvae heterozygous for Dmp535A-1–4 and mwh mutations were irradiated with a low-level x-ray dose (250 rad). Cells that lost the wild-type copy of mwh display the recessive mwh phenotype, which is easily recognizable and can be scored on the wings (8). The number of mwh clones obtained following x-ray treatment in the DLP126/DLP126 was significantly reduced (68%) compared with the control (Fig. 4B).

Finally, we compared the effect of Dmp53 ectopic overexpression in DLP heterozygous (control) and DLP homozygous mutant genetic background. 36% of the pupariated animals overexpressing Dmp53 ubiquitously by actin-GAL4 driver in control background eclosed. In contrast, overexpression of Dmp53 in DLP mutants caused shifted lethality, because two-thirds of the animals died in early pupal stage and only 9% of them eclosed (Fig. 4C). We observed similar enhancer effect

FIGURE 2. Schematic representation of putative Daxx-like proteins and the positions and extensions of the deletions used in the study. A, sequence similarities of putative Daxx-like proteins of Drosophila species (DsIm, D. simulans; Dana, D. ananassae; Dvir, D. virilis; Dmoj, D. mojavensis), and human Daxx (Hsap) to D. melanogaster (Dmel) DLP are shown. The lengths of the predicted proteins are given in parentheses. Protein segments showing significant homology to D. melanogaster DLP are filled, and percent sequence identities are indicated. Gaps longer than 40 amino acids are represented by lines. DHR: Daxx homology region, PBR: p53 binding region. B, genomic organization of the DLP gene, and the positions of the deletions are shown. Boxes represent exons; translated regions are filled. Primers used for mapping and RT-PCR analysis are marked by arrows.

FIGURE 3. Daxx-like protein is expressed throughout Drosophila development. A, relative DLP transcript levels determined in wild-type animals by Q-PCR are shown as percent of detected mRNA level in early embryos. (E: embryo; L1, L2, and L3: larval instars; w L3: wandering 3rd instar larva; pP: pseudopupa; P5: pupal stage 5; PhA: pharate adult.) The highest levels of expression were found in embryos, wandering L3 larvae, and adult females. B–F, detection of DLP RNA by in situ hybridization. B, early embryo; C, embryo in cellular blastoderm stage; D, gastrulating embryo; E, CN system of third instar larva; F, wing imaginal discs. Note the high level maternal mRNA in early embryo, the start of zygotic expression at gastrulation stage and the non-homogeneous expression in the larval CNS and imaginal disc.
when ectopic overexpression of Dmp53 was targeted to the eye. The eyes of DLP mutant adults overexpressing Dmp53 under the control of ey-GAL4 driver were smaller in size than in control background both in females (Fig. 4D) and males (data not shown). These data indicate genetic interaction between DLP and Dmp53, and suggest a role for DLP in repressing Dmp53-mediated responses.

DLP Background Has a Different Effect on the mRNA Level of Reaper and Ark—As Daxx is known to participate in transcriptional regulation, next we investigated whether the transcript levels of pro-apoptotic genes reaper, an inhibitor of IAP proteins (6), and Apaf-1-related-killer (Ark), a caspase activator (31), are altered in DLP mutants. To determine if DLP influences the basal level of these messages, we measured the mRNA levels of rpr and Ark in wild type, Dmp535A-1–4 mutant, and DLP mutant larvae by Q-PCR.

We found no differences in reaper mRNA levels (Fig. 5A). However, the Ark mRNA level was significantly decreased in Dmp535A-1–4 and also in DLP larvae (p = 0.003, repeated measures ANOVA; Fig. 5B) suggesting that both Dmp53 and DLP participate in the maintenance of the basal mRNA level of Ark. Next we sought to determine if loss of DLP abolishes Dmp53-dependent transcriptional activation upon radiation-induced DNA damage. We irradiated wild type, Dmp535A-1–4 mutant, and DLP mutant larvae with 4 krad x-ray and after 2 h recovery Ark and reaper mRNA levels were measured by Q-PCR. Ark mRNA level did not increase significantly after irradiation (Fig. 5B). Reaper expression was up-regulated in wild-type larvae, while its transcript level did not change significantly in Dmp53 mutant animals, as expected. In DLP mutants, similarly to the wild-type control, reaper mRNA levels were elevated suggesting that DLP is not required for Dmp53-dependent transcriptional activation at the reaper locus (Fig. 5A). Because these data indicated the involvement of DLP in the regulation of Ark we wondered whether ectopic overexpression of DLP has any effect on the Ark mRNA level. Up-regulation of DLP expression was achieved by either a hs-GAL4-driven ubiquitous expression of a DLP transgene that carries the Daxx homologous region of DLP, or by ectopic expression of the chromosomal DLP gene from a promoter present in an inserted EP transposon (EP (2)2108). DLP overexpression from either transgene resulted in an increase in Ark mRNA level (Fig. 5C). We did not observe an increased level of Ark message in those EP element carrier lines in which the transcription from the transposon promoter runs opposite to the DLP transcription unit (EP (2)2193 and EP (2)2180). Our experimental system did not permit a detailed analysis of the effect of DLP overexpression on the extent of Ark message induction since the variability in the DLP level achieved by its overexpression via the hs driver. However, in parallel experiments the overexpression of Dmp53 and DLP under the control of the same UAS-GAL4 driver combinations resulted in similar levels of Ark RNA, and no synergistic effect was observed upon co-expression of Dmp53 and DLP (see supplemental Fig. S2).

DLP Mutant Animals Have Reduced Fertility and Longevity—Finally, because the level of p53 affects aging both in mice and Drosophila, we were interested to see whether DLP mutation also has an effect on the fertility and on the lifespan of adult flies. The effect of the loss of DLP function on female fertility was determined by measuring the egg laying capacity of DLP mutant and control flies (Fig. 6A). In repeated experiments, the average number of eggs laid by groups of 5 females in 24-h

![FIGURE 4. Genetic interaction between DLP and Dmp53. A, chart shows the proportion (mean ± S.E.) of wild type, p535A-1–4 DLP520, DLP152, and DLP152 animals that eclosed as adults after irradiation with 2 krad x-ray in the third larval instar. B, number of mwh clones on the wing of DLP homozygous (U26/U26) and DLP heterozygous (PE-U26) control animals (p = 0.006, Student’s t test). To sensitise the LOH assay the occurrence of mwh phenotype was determined in Dmp53 heterozygotes. C and D, effect of DLP mutation on the phenotypes resulting from Dmp53 overexpression. C, graph shows the proportion of DLP homo- (U26/U26) and heterozygous (PE-U26) animals expressing a Pact-GAL4-driven P(UASDmp53) transgene arrested at different stages of development. D, average eye size of DLP homo- (U26/U26) and heterozygous (PE-U26) females that express P(UASDmp53) under the control of P(ey-GAL4), p < 0.001, Student’s t test. Size of the eyes is given in arbitrary units.

![FIGURE 5. The effect of DLP on the mRNA level of proapoptotic genes. The rpr (A) and Ark (B) mRNA levels in untreated (open) and x-ray irradiated (black) wild type, Dmp53, and DLP mutant L3 larvae determined by Q-PCR and normalized to 18S rRNA levels (means ± S.E.). C, levels of Ark (open) and DLP (black) mRNAs after heat shock in adult wild-type females (controls), in females carrying P(UASDLP1.7kb) transgene (DLP), and in females which carry EP (2)2108, EP (2)2193, and EP (2)2180 insertions as indicated. Each category shown contains the P(hs-GAL4) driver. The transcription direction from EP (2)2108 is toward DLP while from EP (2)2193 and EP (2)2180 it is to the opposite.
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FIGURE 6. Loss of DLP reduces longevity and female fertility. A, DLP mutant females have decreased fertility as indicated by the number of eggs laid by control (DLP<sup>+/+</sup>/PE) and DLP mutant (DLP<sup>+/−</sup>/DLP<sup>−/−</sup>) females (5 females/vial) in 24-h intervals (means ± S.E.). B, percent survival of control (DLP<sup>+/+</sup>/PE) and DLP mutants (DLP<sup>+/−</sup>/DLP<sup>−/−</sup>, DLP<sup>+/2</sup>/DLP<sup>+/2</sup>, and DLP<sup>+/4</sup>/DLP<sup>+/4</sup>) animals. To measure longevity freshly eclosed males were collected, placed into vials in groups of 20, and the number of surviving animals counted daily.

intervals was consistently significantly decreased in DLP transheterozygotes compared with heterozygous controls (Fig.6A).

To determine the longevity of DLP mutant animals, we put equal numbers of homozygous DL<sup>P<sub>1226</sub>/</sup>, DL<sup>P<sub>1232</sub>/</sup>, DL<sup>P<sub>1428</sub>/</sup>, mutant or isogenic heterozygous control males into vials (20 males/vial) and counted the number of survivors daily (Fig. 6B). We found that the median of the lifespan of DLP mutant animals significantly decreased compared with that of control (p < 0.001, ANOVA). A particularly strong effect could be observed in the case of DL<sup>P<sub>1226</sub></sup> animals whose numbers dramatically dropped after 40 days. Similar results were obtained in experiments using different controls to compare their lifespan whith that of DLP mutant animals (see supplemental Fig. S3).

DISCUSSION

Mammalian Daxx is a regulator of apoptotic response, it controls both transcripition and signal transduction by interacting with key regulatory factors. We found that the Drosophila DLP and Dmp53 proteins, similarly to their mammalian orthologs, interact directly. Despite the weak conservation at the sequence level, the protein regions responsible for mediating the interac-

tion fairly correspond to those found in mammals. The Daxx binding domain of p53 was mapped to its C terminus (21–24).

Accordingly, we found that the entire C-terminal region, containing both the oligomerization and the basic regulatory domain of Dmp53, is required for DLP binding in flies. In the case of Daxx, two p53 binding regions were identified. One of them is a 140-residue-long C-terminal region involved in mediating interactions with several proteins (21–23), the other one is a less well-defined central region harboring the acidic domain of Daxx (21, 22, 24). In flies we found that the C-terminal 135 residues of DLP, corresponding to the C-terminal p53 binding region of Daxx, are required for interaction with Dmp53. Furthermore, we found that the C terminus of DLP is also able to bind human p53. These findings indicate that the molecular details of the interactions are well preserved and suggest the importance of Daxx/DLP in the regulation of p53 activity.

Mammalian cell culture experiments provided evidence that Daxx regulates p53 function by modulating its transactivating activity, although the results gained from these experimental systems are often contradictory. Daxx was found to inhibit the activity of p53-responsive reporter constructs directed by the synthetic G5p53 element, the p21<sup>WAF1</sup> promoter (23, 24) or the Mdm2 promoter (22). On the other hand, Daxx stimulated p53-dependent transcription from the Bax and PUMA promoters (22) and from a p53-responsive sequence derived from the Mdm2 promoter (32).

To investigate whether DLP is required for Dmp53 functions in Drosophila, we generated mutant alleles of DLP carrying internal deletion in the gene. DLP mutants are homozygous viable and show a decrease in longevity and fertility similarly to mutants of other factors involved in apoptotic response, e.g. Dmp53 (7). We measured reaper transcription levels after x-ray irradiation in DLP mutant and wild-type larvae to determine whether DLP modulates Dmp53-dependent transcriptional activation after DNA damage. We did not find significant changes in rpr activation suggesting that DLP is not required for the Dmp53 activated transcriptional response at the rpr enhancer. In concert with the lack of influence on rpr activation, DLP mutants did not show a radiosensitive phenotype. However, we found that loss of either Dmp53 or DLP reduced the basal level of Ark mRNA suggesting that both proteins are required for the maintenance of the basal activity of some proapoptotic genes. We observed genetic interaction between Dmp53 and DLP, as phenotypes resulting from ectopic overexpression of Dmp53 were enhanced in the DLP mutant background, and in LOH assays the number of clones indicating failure in repair following DNA damage and/or failure in the elimination of damaged cells was lower in DLP mutants. These data suggest a suppressing effect of DLP in some Dmp53-mediated functions. At first, this might seem surprising in the light that the expression of reaper, one of the major proapoptotic targets of Dmp53, is not influenced by DLP. However, apoptosis occurring independently of reaper activation has been observed in the Drosophila system at several cases. Thus, our in vivo data together with the observed mRNA levels, suggest that DLP is involved in a Dmp53-mediated apoptosis pathway different from those including reaper. Dmp53 mutations are known to affect longevity. We observed a similar effect of DLP...
mutations in repeated experiments comparing the lifespan of DLP mutant combinations to different controls, among them isogenic lines. However, from our data, we cannot determine whether the effect of DLP on lifespan is dependent on its interaction with Dmp53. We note here, that in this respect Dmp53 and p53 seem to behave partly differently because in Drosophila the loss of Dmp53 results in a reduced lifespan while in mice overproduction of a short isoform of p53 accelerates aging (7, 11).

Several distinguishing features of the p53 regulatory networks should be taken into consideration if one would like to explain the differences found between the mammalian and insect systems. The regulation of mammalian p53 is based on its interaction with the repressor protein MDM2. As MDM2 does not have a Drosophila homolog, this control mechanism seems to be a new evolutionary invention and might have fundamental effect on other interactions p53 involved in. Similarly to MDM2, promyelocytic leukemia protein (PML), a critical component of the PML nuclear bodies (PODs), have not been identified in flies. Both Daxx (17) and p53 (33) are recruited to PODs where PML3 relieves p53 from Daxx-mediated repression by competing with Daxx for p53 binding (23). Finally, based on the analyses of predicted protein sequences from five Drosophila species we found that DLP proteins also share conserved sequences outside the Daxx homology region. Although these regions do not contain any described functional domains, they may influence the interactions DLP proteins are involved in.

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