Daxx Cooperates with the Axin/HIPK2/p53 Complex to Induce Cell Death

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Abstract

Daxx, a death domain–associated protein, has been implicated in proapoptosis, antiapoptosis, and transcriptional regulation. Many factors known to play critically important roles in controlling apoptosis and gene transcription have been shown to associate with Daxx, including the Ser/Thr protein kinase HIPK2, promyelocytic leukemia protein, histone deacetylases, and the chromatin remodeling protein ATRX. Although it is clear that Daxx may exert multiple functions, the underlying mechanisms remain far from clear. Here, we show that Axin, originally identified for its scaffolding role to control β-catenin levels in Wnt signaling, strongly associates with Daxx at endogenous levels. The Daxx/Axin complex formation is enhanced by UV irradiation. Axin tethers Daxx to the tumor suppressor p53, and cooperates with Daxx, but not DaxxΔAxin, which is unable to interact with Axin, to stimulate HIPK2-mediated Ser46 phosphorylation and transcriptional activity of p53. Interestingly, Axin and Daxx seem to selectively activate p53 target genes, with strong activation of PUMA, but not p21 or Bax. Daxx-stimulated p53 transcriptional activity was significantly diminished by small interfering RNA against Axin; Daxx fails to inhibit colony formation in Axin−/− cells. Moreover, UV-induced cell death was attenuated by the knockdown of Axin and Daxx. All these results show that Daxx cooperates with Axin to stimulate p53, and implicate a direct role for Axin, HIPK2, and p53 in the proapoptotic function of Daxx. We have hence unraveled a novel aspect of p53 activation and shed new light on the ultimate understanding of the Daxx protein, perhaps most pertinently, in relation to stress-induced cell death. [Cancer Res 2007;67(1):66–74]

Introduction

Daxx was initially identified as a protein that binds to the Fas death domain and has been implicated in a Fas-mediated apoptotic pathway by serving as an adaptor protein linking Fas signaling to c-Jun-NH₂-kinase (JNK) pathways via apoptosis signal–regulating kinase 1 (1, 2). Genetic studies showed that mouse embryos deficient in Daxx cannot survive beyond embryonic days 8.5 to 9.5, indicating that Daxx is necessary in the early development of mouse embryos. However, results from TUNEL assays gave evidence that embryos with disrupted Daxx genes displayed extensive apoptosis, suggesting that Daxx may play a protective role in preventing apoptosis in the early embryo. Similarly, Daxx silencing by small interfering RNA (siRNA) was reported to sensitize cells to multiple apoptotic pathways, implying an antiapoptotic role for Daxx (3, 4).

Nevertheless, several lines of evidence have shown that Daxx may indeed exert proapoptotic functions, and that Daxx may play opposing roles with respect to apoptosis depending on the context (5–13). In Daxx−/− cells, the apoptosis rates in response to serum starvation were only slightly increased, which makes it difficult to conclude that Daxx deficiency–caused apoptosis in mutant mouse tissues is a direct consequence of a loss of antiapoptotic function of Daxx. In addition, it is clear that Daxx is required to potentiate stress-induced cell death in cell lines. It has been shown that Daxx induces apoptosis by interacting with several nuclear proteins, such as PML and HIPK2 in the nucleus (14, 15). It was also shown that Daxx interacted with p53 and promoted p53-dependent apoptosis (11). However, another study contradicted such an observation, showing that Daxx does not communoprecipitate with wild-type p53, but only with tumorigenic mutant forms of p53. Intriguingly, only the Daxx-interacting mutants of p53 could inhibit stress-induced Daxx-mediated cell death (10). Although it remains unclear how Daxx modulates p53 function, the existing evidence clearly points to a functional linkage between Daxx and p53. A recent finding that Daxx interacts with, and inhibits, the transcriptional activity of Tcf4, which plays a critical role in maintaining the proliferative status of the stem cells in the crypts of the intestine (16, 17), suggests that Daxx may inhibit cell proliferation via multiple mechanisms.

Axin is a negative regulator of Axis formation in the development of mouse embryos; its deficiency leads to axis duplication (18). It acts as an architectural platform for the degradation of the oncogenic protein β-catenin (19–23). Axin has, in fact, emerged as a major scaffold for many other pathways, including JNK mitogen-activated protein kinase signaling, p53 signaling, and transforming growth factor β (TGF-β) signaling (24–28). Recently, heterotrimeric Gα subunits activated upon prostaglandin E2 stimulation were shown to interact with Axin, thereby disrupting the Axin/GSK3β degradation complex and leading to stabilization of β-catenin (22, 23). Most relevantly, we previously found that Axin forms a complex with p53 and its regulatory kinase HIPK2. Knockdown of Axin by siRNA reduced UV-induced p53 Ser46 phosphorylation and p53-mediated apoptosis (28). In addition, HIPK2 has been shown to interact with Daxx (15). All these observations prompted us to reevaluate a then seemingly unlikely clone identified by a yeast
two-hybrid screen using full-length Axin (which encoded Daxx) as bait many years ago. Here, we show that Daxx interacts strongly with Axin both in vivo and in vitro, in that Axin serves as a scaffold for the assembly of the Axin/Daxx/HIPK2/p53 complex to promote the phosphorylation of p53 at Ser46 by HIPK2. The results provide an important mechanistic link for Daxx to tumor suppressors, p53 and Axin.

Materials and Methods

Plasmids. The full-length cDNA encoding Daxx was amplified using cDNA generated from mRNA of HeLa cells using Pfu polymerase, and was inserted into PCMV5 expression vector after sequence verification. Deletion mutants were generated using standard techniques as previously described (28).

Preparation of antibodies. Mouse anti-HA (F-7), anti-Myc (9E10), anti-Hsp60 (H-1), and rabbit anti-p53 (DO-1), and rabbit anti-p53 (FL393) antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Mouse anti-FLAG (M2) and anti-β-actin were purchased from Sigma. Mouse anti-cytochrome c monoclonal antibody was a product of BD Biosciences. Rabbit anti–actin-p53-Lys382 and anti–actin-p53-Lys383 antibodies were purchased from Upstate Biotechnology, Inc. Anti-phospho-p53-Ser15, anti-phospho-p53-Ser20, anti-phospho-p53-Ser39, and anti-phospho-p53-Ser46, and anti-acetylated-p53-Lys38 antibody bodies were all purchased from Cell Signaling Technology (Sigma, Saint Louis, MO). The polyclonal antibody against Axin (C2b) has been previously described (28), and rabbit polyclonal antibody against Daxx was prepared by injecting the protein region of amino acids 625 to 740.

Cell culture, transient transfection, immunoprecipitation, and Western blotting. HEK293, HEK293T, H1299, U2OS, SaOS-2, HeLa, MCF-7, and SNU-475 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU of penicillin, and 100 mg/mL of streptomycin. Transient transfections were carried out using Dsper (Roche, Penzberg, Germany). LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), or calcium phosphate precipitation method. Cell lysate preparation and immunoprecipitation were carried out as detailed previously (24). To determine whether Axin, Daxx, and p53 form a ternary complex, a two-step coimmunoprecipitation was done as previously described (28).

Immunokinase assay. H1299 cells were transfected with p53, Daxx, Axin, HIPK2, p300, or their mutants as indicated. At 30 h posttransfection, cells were harvested with lysis buffer. p53 was immunoprecipitated with anti-FLAG or anti-HA antibody; phosphorylated or acetylated p53 was detected with their corresponding antibodies.

Cell apoptosis. HEK 293 cells, H1299, and SNU-475 cells grown on glass coverslips in six-well tissue culture plate. When the cells were confluent, a transient transfection was done with 0.5 μg of green fluorescent protein–expressing vector pEGFP-C3 (Clontech, Palo Alto, CA) together with a total of 3 μg of other plasmids including Myc-Daxx, Myc-DaxxΔAxin, HA-Axin, HA-AxinΔDaxx, Myc-p53, Myc-p53-R175H, pSUPER-Daxx, pSUPER-Axin, pSUPER-p53, and pSUPER-HIPK2 in different combinations. Cells were then stained with Hoechst 33342 and examined as previously described (28), and the remaining cells were scraped and lysed for Western blotting. For SNU-475 cells, Axin was introduced by using lentivirus infection. Briefly, 10 μg of pGIBI vector or pGBOI-Axin together with 10 μg of PMLΔ, 6 μg of VSV-G, and 4 μg of RSV-REV were transfected into 293T cells by using the calcium phosphate precipitation method. The lentiviral products were harvested thrice every 24 h, and were used to infect SNU-475 cells after concentration by centrifugation (29).

Immunofluorescent staining. HeLa cells were grown on glass coverslips in the cell culture medium described above for 16 h. Expression plasmids of Myc-Axin, HA-HIPK2, HA-Daxx, and Myc-Daxx were transfected into HeLa cells in different combinations as indicated where necessary. Approximately 24 h after transfection, cells were left untreated or irradiated with UV (80 J/m²), then cultured for another 6 h, and fixed with 3.7% formaldehyde-PBS for 10 min. The staining procedures were subsequently carried out as previously described (28), and visualized under a confocal laser scanning microscope (TCS SP2; Leica Microsystems, Inc., Bannockburn, IL).

Transcriptional reporter assay. p53-luc reporter (Stratagene, La Jolla, CA) was as described previously (28). PUMA-FRAG1-Luc and PUMA-FRAG2-Luc (30) were gifts from Dr. Vogelstein (The Johns Hopkins University, Baltimore, MD). HEK293 or H1299 cells were transfected in six-well dishes at 90% confluence with different reporters, 0.5 μg of LacZ expression plasmid and 0.5 μg of pEGFPNI, together with 2 μg of other plasmids including empty vector, Daxx, DaxxΔAxin, Axin, Axin-Daxx, p53, pSUPER-Axin, pSUPER-p53, and pSUPER-HIPK2 in different combinations as indicated. All transfections were carried out in triplicate for at least five times, and error bars represent SD of the means.

Colony formation assays. HEK 293, SNU-475 (Axin-/-), U2OS, and SaOS-2 (p53+/–) cell lines were employed for colony formation assays. Cells were plated onto 60 mm dishes. When grown to 60% confluence, cells were transfected with empty pcDNA6 vector, pcDNA6-Daxx, or pcDNA6-DaxxΔAxin individually. Approximately 48 h after transfection, drug-resistant cells were selected with fresh medium supplemented with 10 μg/mL of blasticidin for 3 weeks. Surviving colonies were fixed with 3.7% formaldehyde-PBS for 20 min at room temperature. After rinsing thrice with PBS, colonies were stained with 1% crystal violet in 20% ethanol.

Subcellular fractionation. Cell fractionation was done according to protocols as previously described (31, 32). Briefly, cells were collected and homogenized by 75 strokes in a 2 mL Kontes Douncer with the B-type pestle (Kontes Glass Company, Vineland, NJ) in an ice-cold homogenization buffer [250 mmol/L sucrose, 20 mmol/L HEPES-KOH (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin]. Afterwards, cell lysates were centrifuged at 1,900 × g for 5 min at 4°C to remove the nuclei. The supernatant was then centrifuged at 17,000 × g for 15 min at 4°C and the resulting pellet was the mitochondrial fraction. Then, the supernatant was subjected to a second round of centrifugation at 16,000 × g for 20 min at 4°C and the remaining supernatant was the cytosolic fraction. The protein levels were measured by using the Bio-Rad Protein Array (Bio-Rad, Richmond, CA) and equal amounts of protein were analyzed by SDS-PAGE.

Results

Daxx interacts with Axin in vivo and in vitro. To identify proteins that interact with Axin, we carried out yeast two-hybrid screen with a mouse fetal brain cDNA library using full-length Axin as bait in pGBK7 vector (Clontech). Sequence analysis showed that one of the fish clones in pACT2 vector encoded the NH2-terminal 359 amino acid residues of Daxx as shown in Fig. 1A. To confirm the interaction between Axin and Daxx in mammalian cells, we cloned the full-length coding sequence of Daxx and did coimmunoprecipitation of Axin and Daxx in HEK 293T cells. HA-Daxx and Myc-Axin proteins were overexpressed in 293T cells, followed by reciprocal immunoprecipitation with anti-HA for Daxx and anti-Myc for Axin. The immunoblotting results showed that Daxx and Axin were coprecipitated with each other (Fig. 1B). We then tested whether endogenous Axin and Daxx could interact with each other using lysates of untransfected 293T cells with anti-Axin C2b (28) and anti-Daxx rabbit polyclonal antibody (for characterization of the antibody, see Supplementary Fig. S1). As shown in Fig. 1C, endogenous Axin and Daxx were also coimmunoprecipitated with each other, indicating that Axin forms a strong complex with Daxx in the cell.
(amino acids 1–197) for interaction with Axin, consistent with the presence of the NH2-terminal region of Daxx in the clone identified by a yeast two-hybrid screen (Supplementary Fig. S2B). These results show that there exist concrete domains in Axin and Daxx for their mutual interaction.

**Axin tethers Daxx to p53 in a ternary complex.** We previously showed that Axin possesses a domain for direct interaction with p53, in addition to association with p53 through HIPK2 (see ref. 28). In particular, one study showed that Daxx interacts with p53 (11), although another study contradicted that finding (10). We asked whether the complex of Axin and Daxx also contains p53. Myc-Axin and HA-Daxx were transfected into HEK293 cells, from which endogenous p53 was immunoprecipitated with the DO-1 anti-p53 antibody. When increasing amounts of Myc-Axin (0.5, 1.0, and 2.0 μg, respectively) were transfected, Daxx coimmunoprecipitated with p53 gradually increased (Fig. 2A). These data suggested that Axin, Daxx, and p53 were copresent in the same complex. To formally establish that they actually form a ternary complex, we carried out a two-step coimmunoprecipitation (Fig. 2B). In this experiment, anti-HA was used to precipitate HA-Axin in the lysates from 293 cells with HA-Axin or untagged Axin (as control). The first immunoprecipitation was done using anti-HA. The complex was eluted with HA peptide (Santa Cruz Biotechnology), followed by the second step of coimmunoprecipitation with anti-Myc for Daxx or control IgG. Immunoprecipitates from each step were blotted with anti-Myc, anti-Axin, and DO-1 for protein levels, respectively.

**Figure 1.** Daxx interacts with Axin in vivo and in vitro. A, yeast two-hybrid screening using full-length Axin as bait was done according to instructions from the manufacturer (Clontech). The “fish” clone contains a cDNA insert corresponding to amino acids 1 to 359 of Daxx as shown beneath the schema of full-length Daxx. B, 293T cells were cotransfected with HA-Daxx and Myc-Axin, and reciprocal coimmunoprecipulation was done with anti-HA (left) and anti-Myc (right). Daxx and Axin were detected in the corresponding immunoprecipitates by Western blotting with anti-HA and anti-Myc, respectively. C, immunoprecipulation of endogenous proteins from HEK293 cells was done separately with control IgG, rabbit anti-Axin C2b, and rabbit anti-Daxx polyclonal antibody, and immunoprecipitates along with total cell lysates (TCL) were analyzed separately by Western blotting with anti-Axin and anti-Daxx antibodies.

**Figure 2.** Axin tethers Daxx to p53 in a ternary complex. A, Axin enhances the interaction between Daxx and p53 in a dose-dependent manner. HA-Daxx (1.0 μg) was cotransfected with increasing amounts of Myc-Axin (0.5, 1.0, and 2.0 μg, respectively) into HEK293 cells. Cell lysates were immunoprecipitated with anti-p53 (DO-1) for endogenous p53. Precipitates and total cell lysates were then immunoblotted with anti-Myc for Axin, anti-HA for Daxx, and DO-1 for p53, individually. B, two-step coimmunoprecipitation of the complex containing Axin, Daxx, and p53. Top, procedures of the two-step coimmunoprecipitation. Briefly, Myc-Daxx was transfected into 293 cells with HA-Axin or untagged Axin (as control). The first immunoprecipitation was done using anti-HA. The complex was eluted with HA peptide (Santa Cruz Biotechnology), followed by the second step of coimmunoprecipitation with anti-Myc for Daxx or control IgG. Immunoprecipitates from each step were blotted with anti-Myc, anti-Axin, and DO-1 for Daxx, Axin, and p53 protein levels, respectively. C, Axin mutants defective in binding to p53 or Daxx cannot enhance the interaction between p53 and Daxx. Blank vector, HA-Axin, HA-Axin-Daxx, and HA-Axin-M9 (lacking binding sites for p53 association, see ref. 28) were separately transfected with Myc-Daxx into 293 cells. Cell lysates were immunoprecipitated with DO-1 for endogenous p53, and samples were then analyzed by Western blot with anti-Myc, anti-HA, and DO-1 for protein levels of Daxx, Axin, and p53, respectively.
of 293 cells that were cotransfected with Myc-Daxx and contain endogenous p53. Untagged Axin was transfected separately as a control. The precipitates were eluted with HA peptide. The eluates were then precipitated with the second antibody, anti-Myc (for Daxx), with IgG as a negative control. After the second round of immunoprecipitation, the components were analyzed by Western blotting using antibodies respectively for Axin, Daxx, and p53. From the total cell lysates expressing untagged Axin, no specific signal was detected in the final precipitate. HA-tagged Axin could coprecipitate both Daxx and p53, showing that Axin forms a ternary complex with Daxx and p53.

However, further characterization of the complex formation indicated that Daxx does not directly interact with p53, but through a bridge by Axin. As shown in Fig. 2C, Daxx was coprecipitated with p53 only in the presence of full-length Axin but not AxinΔDaxx or Axin-M9 (lacking both sites for p53 association, see ref. 28). Similarly, in the presence of Axin, Daxx could coprecipitate only with wild-type p53 but not with the p53 mutant that is defective in Axin-binding (p53ΔAxin) (Supplementary Fig. S3A). In parallel, we found that p53 only associates with wild-type Daxx, but not with DaxxΔAxin, which lacks an Axin-binding domain when cotransfected with Axin (Supplementary Fig. S3B). These results indicate that Daxx does not form a direct contact with p53, but associates with p53 through Axin, in accordance with the previous assertion that Daxx does not directly interact with wild-type p53 (10).

Daxx enhances p53 phosphorylation at Ser\(^{46}\) that requires Axin. We next examined whether Daxx also contributes to enhancement of p53 phosphorylation at Ser\(^{46}\) catalyzed by HIPK2 (33, 34). First, we found that Daxx indeed activated Ser\(^{46}\) phosphorylation of p53, but not Ser\(^{15}\) or Ser\(^{20}\), and that Axin and Daxx had an additive effect on p53 phosphorylation (Fig. 3A). DaxxΔAxin, which is defective in Axin binding, reduced approximately by half its ability to induce Ser\(^{46}\) phosphorylation compared with wild-type Daxx (Fig. 3B, left). Similarly, AxinΔDaxx defective in association with Daxx exhibited reduced ability to stimulate p53 phosphorylation, indicating that maximal p53 phosphorylation requires both Axin and Daxx (Fig. 3B, right). We then tested whether Daxx-stimulated p53 phosphorylation at Ser\(^{46}\) was indeed mediated by HIPK2. The kinase-dead mutant HIPK2-K221R drastically attenuated Daxx-induced p53 phosphorylation (Fig. 3C). In addition, we generated a mutant HIPK2, HIPK2-Δp53/ΔAxin, which lacks binding sites for both p53 and Axin but retains the binding site for Daxx. When coexpressed with Daxx, HIPK2-Δp53/ΔAxin also abolished Daxx-induced phosphorylation of p53 (Fig. 3C). Consistently, the Axin mutant that is defective in binding to both p53 and HIPK2 greatly retarded Daxx-induced p53 phosphorylation, whereas single removal of the binding sites of Axin for p53 and HIPK2 (AxinΔp53 or AxinΔHIPK2) gave rise to lesser reduction of the Daxx-induced p53 phosphorylation (Fig. 3D). The above results indicate that Daxx-induced p53 phosphorylation at Ser\(^{46}\) is...
mediated by HIPK2, and that the substrate p53 is bound by Axin and HIPK2. We also examined other posttranslational modifications of p53, such as acetylation of its COOH-terminal lysine residues. It was found that although p300 robustly enhanced p53 acetylation, Axin or Daxx was unable to induce acetylation of COOH-terminal lysine residues of p53, Lys320, Lys373, and Lys382 (Supplementary Fig. S4).

To further delineate the intricate complex formation and individual contribution of the Axin/Daxx/HIPK2 complex to p53 phosphorylation, we conducted pSUPER-based siRNA knockdown experiments. pSUPER-Axin (28) reduced Daxx-induced p53 phosphorylation (Supplementary Fig. S4B); pSUPER-Daxx diminished Axin-induced p53 phosphorylation (Supplementary Fig. S4C). pSUPER-HIPK2 (28) also attenuated Daxx-induced p53 phosphorylation (Supplementary Fig. S4D). These results again point to a requirement of HIPK2 for Daxx-mediated p53 phosphorylation and Axin contributes to the maximal activation of p53 by Daxx. Characterization of the efficiency and specificity of these pSUPERs was shown in Supplementary Fig. S5.

UV induces colocalization of Axin, Daxx, and HIPK2 in the nucleus. To visualize whether Axin, Daxx, and HIPK2 are subcellularly colocalized in the cell, we cotransfected Axin, Daxx, and HIPK2 alone or in combination into HeLa cells and carried out immunostaining. Axin is largely distributed in the cytoplasm, with Daxx and HIPK2 being exclusively present in the nucleus, regardless of single-transfection (data not shown) or cotransfection (Fig. 4). However, when the cells were exposed to UV irradiation, Axin was partially translocated into the nucleus and is overlapped with Daxx (Fig. 4A), and with HIPK2 (Fig. 4B). Daxx and HIPK2 are colocalized in the nucleus before or after UV treatment (Fig. 4C), in agreement with the previous report (14). Notably, when Axin was cotransfected with Daxx M1 mutant that is localized in the cytoplasm, Axin was also found colocalized with the mutant Daxx protein in the cytoplasm (Supplementary Fig. S6). Importantly, UV treatment seems to strengthen the interaction between Axin and Daxx, as determined by coimmunoprecipitation assay (Fig. 4D). All of these observations strongly indicate that Axin interacts with Daxx in the cell and form a ternary complex with HIPK2.

Daxx cooperates with Axin to stimulate p53 transcriptional activity. Because Daxx has been shown to differentially modulate transcription from different p53-responsive promoters (11), we first tested whether Daxx could regulate the p300-activated p53 reporter which carries the p53-specific enhancer element. When Daxx was coexpressed into 293 cells with the p53-Luc reporter, the luciferase activities were increased by ~6-fold, slightly less than the stimulation by Axin (Fig. 5A, left). Cotransfection of Daxx and Axin further enhanced the transcriptional activity of the p53-Luc reporter (Fig. 5A, left), indicative of an additive effect by the two proteins. DaxxΔAxin (mutant M5 as diagrammed in Supplementary Fig. S2) and AxinΔDaxx (D7) exhibited a diminished ability to stimulate p53-dependent transcriptional activity (Supplementary Fig. S7A). Accordingly, siRNA against Axin, but not the control siRNA, attenuated the Daxx-mediated p53 transcriptional activity (Fig. 5A, right), showing that Daxx depends on Axin to activate p53 transcriptional activity. The siRNA against HIPK2, but not control siRNA, diminished the Daxx-induced transcriptional activity of p53 (Supplementary Fig. S7B), suggesting that HIPK2 is also required for the activation of p53 reporter stimulated by Daxx. Specific knockdown of p53 by pSUPER-p53 in 293 cells abolished the Daxx-induced activation of p53-dependent transcriptional activity (Supplementary Fig. S7C). Consistently, overexpressed Daxx failed to activate p53-Luc reporter in p53 null H1299 cells or H1299 cells overexpressing p53S46A (Supplementary Fig. S7E). However, in H1299 cells reintroduced with wild-type p53, Daxx exhibited a stimulatory effect on the reporter gene activity in a dose-dependent manner.
manner (Supplementary Fig. S7D), indicating that the Daxx-stimulated p53-Luc reporter activity was indeed mediated by p53.

To further explore the exact p53 target genes regulated by Daxx and Axin, a luciferase activity assay was done by using three additional reporter genes, PUMA, p21, and Bax reporters (28, 30). It was found that Daxx and Axin cooperatively stimulated the PUMA-FRAG1-Luc transcription activity (Fig. 5B), although they did not activate PUMA-FRAG2-Luc reporter that lacks the p53 binding sites in FRAG1-Luc (Supplementary Fig. S7F). However, as for p21 and Bax-Luc reporters, neither Daxx nor Axin could induce the luciferase activity (Supplementary Fig. S7G and H), suggesting that Daxx and Axin selectively enhance the transcriptional activity of p53 target genes.

**Daxx and Axin induce cell apoptosis through cytochrome c release.** It has been reported that the PUMA gene encodes two BH3 domain-containing proteins that are localized in the mitochondria (30, 35). In response to transactivation by p53, PUMA proteins are induced, which then form complex with Bcl-2 or Bcl-x<sub>L</sub> to induce cytochrome c release and cell apoptosis. Given that Axin and Daxx could stimulate PUMA gene transcription, we tested if they could induce cytochrome c release. The results showed that Daxx and Axin alone induced cytochrome c release when overexpressed in 293 cells (Fig. 5C). Moreover, when the two proteins were cotransfected, they showed a synergistic effect on cytochrome c release, indicating that Daxx and Axin cooperatively induce cell apoptosis through induction of PUMA, and subsequently, of cytochrome c release. On the other hand, transcription-independent induction of cell death by p53 has gained increasing attention (36, 37). In this way, p53 directly induce cytochrome c through translocation into the mitochondria, in which it forms inhibitory complexes with protective Bcl-2 and Bcl-x<sub>L</sub>. To address whether Axin and Daxx could also induce cell apoptosis through p53 transcription-independent pathways, we carried out experiments by isolating mitochondria from cells transfected with Axin, Daxx, or both, untreated or treated with camptothecin, and followed the detection of p53 by Western blot. Whereas camptothecin could effectively induce entry of p53 into the mitochondria, Axin or Daxx did not have such an effect. These results are shown in Fig. 5D, and indicate that Axin and Daxx most likely activate cell death through transcription-independent pathways.

**Inhibition of cell survival by Daxx requires endogenous Axin** and **p53.** Daxx was shown to sensitize apoptosis induced by a variety of stimuli including UV (8), TGF-β (9), arsenite trioxide, and IFN-γ (38), and up-regulation of Daxx also mediates apoptosis in.
response to oxidative stress (39). We went on to assess any effect of Daxx on cell growth by performing clonogenic formation assay. For this assay, HEK293, SNU-475 (Axin/–), U2OS, and SaOS-2 cells were used. In 293 and U2OS cells which contained functional p53, overexpression of DaxxΔAxin that lacks the interaction domain for Axin did not inhibit cell growth compared with the wild-type Daxx that showed a strong inhibitory effect on colony formation, emphasizing that interaction of Daxx with Axin is important for Daxx-dependent inhibition of cell growth (Fig. 6A). In SNU-475 cells lacking endogenous Axin and in p53-null SaOS-2 cells, Daxx failed to inhibit the clonogenic survival (Fig. 6A), consistent with the data from apoptosis assays which showed that both Axin and p53 are each crucial for Daxx-induced apoptosis (Supplementary Fig. S8).

In the apoptosis assay, it was shown that specific knockdown of Axin, p53, or HIPK2 diminished Daxx-induced apoptosis in HEK293 cells (Supplementary Fig. S8A–C). Daxx displayed an attenuated ability to induce apoptosis in HEK293 cells expressing a dominant-negative form of p53 (R175H; Supplementary Fig. S8D), and failed to cause apoptosis in H1299 cells (Supplementary Fig. S8E), confirming our conclusion that Daxx-induced apoptosis depends on p53. Conversely, in Axin-null SNU-475 cells, p53 induction of apoptosis was severely compromised unless Axin was reintroduced by lentivirus infection, whereas siRNA against Daxx reduced p53-dependent cell death even in cells with reintroduced Axin (Fig. 6B, left). Similarly, p53 also needs both endogenous Axin and Daxx to gain maximal ability to induce cell death in H1299 cells as...
parallel routes and a convergent meansto activate p53 (Fig. 6)

Moreover, when Axin or Daxx were
knocked down by its specific siRNA, fewer cells were found to undergo apoptosis after UV treatment, and when both Axin and Daxx were knocked down, UV-induced cell death was further decreased (Fig. 6C).

#### Discussion

In the present study, we have provided strong evidence for a physical and functional interaction of Axin, Daxx, HIPK2, and p53. We show that Daxx does not form direct contact with p53, but that Axin serves as a bridge to tether Daxx to p53. Importantly, the association between Axin and Daxx is enhanced in cells after UV treatment, which indicates that the complex formation is physiologically regulated. Whereas Daxx alone can activate p53 that is presumably bound with HIPK2, Axin significantly augments such an effect, suggesting that Axin/Daxx/HIPK2/p53 complex formation leads to maximal stimulation of p53 activities, at least with respect to transcriptional activation and cell growth inhibition. The physiologic relevance of our novel finding can be further manifested by the observation that Axin and Daxx interact with each other at their endogenous levels. Moreover, Daxx-mediated inhibition of cell growth depends on the presence of both functional Axin and p53, as Daxx fails to induce cell death in Axin−/− cells or p53-deficient SaOS-2 cells in clonogenic assays. Knockdown of Axin or Daxx severely compromise UV- or p53-induced apoptosis, consistent with the notion that Axin/Daxx complex formation is needed to induce maximal activation of p53.

It was previously shown that Daxx interacts with HIPK2, and upon TGF-β1 treatment, HIPK2 phosphorylates Daxx which, in turn, leads to JNK activation (15). Our results clearly established that Axin, Daxx, and p53 form a ternary complex that promotes HIPK2 phosphorylation of p53 at Ser46. Knockdown of Axin by siRNA significantly reduced Daxx-induced p53 phosphorylation; DaxxxAxin defective in Axin-binding displays a much compromised ability to induce p53 phosphorylation. Similarly, when Daxx was knocked down or when its Daxx-binding domain was deleted, Axin exhibited reduced activity towards activation of phosphorylation or enhancement of transcriptional activity of p53. Based on all these observations, it is legitimate to suggest that Axin and Daxx seem to adopt both parallel routes and a convergent means to activate p53 (Fig. 6D). In either case, HIPK2 seems to be the protein kinase that catalyzes the Ser46 phosphorylation. Daxx alone can interact with, and activate, HIPK2 leading to increased phosphorylation of p53. Under certain physiological conditions or in the presence of stress stimuli such as UV, Axin is translocated into the nucleus to form Axin/Daxx/HIPK2/p53 complex that yields a higher stimulation of p53 than Daxx/HIPK2/p53 or Axin/HIPK2/p53. It is therefore conceivable that cellular context with regard to Axin abundance in different cell lines can be an important factor when assaying for the ability of Daxx to induce cell death.

Our current work has also established that Axin and Daxx stimulates the transcriptional activation of proapoptotic p53 target genes. Interestingly, Axin and Daxx display strong selectivity in boosting p53-dependent genes. Among the reporter genes tested, including PUMA, p21, and Bax, only the PUMA reporter gene is activated. We also found that Axin and Daxx could induce cytochrome c release, in accordance with the induction of the PUMA gene by the two proteins. However, we did not see a direct translocation of p53 into the mitochondria to cause the release of cytochrome c, in contrast to several reports showing that gamma irradiation can induce translocation of p53 into mitochondria to permeabilize the outer membrane. Rather, Axin/Daxx-induced apoptosis seems to adopt a transcription-dependent route, by activating proapoptotic genes such as PUMA that are mitochondrial proteins and inhibit antiapoptotic Bel-2 or Bcl-XL. Complex formation of PUMA with Bel-2, in turn, causes the release of cytochrome c to initiate the activation of the apoptotic cascade which involves Apaf-1 (37). In sum, our results have provided a mechanistic insight into how Daxx cooperates with other cellular factors to stimulate the multifaceted function of p53 as a tumor suppressor.

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