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Negative Regulation of p53 Functions by Daxx and the Involvement of MDM2*

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In normal cells p53 activity is tightly controlled and MDM2 is a known negative regulator. Here we show that via its acidic domain, Daxx binds to the COOH-terminal domain of p53, whose positive charges are critical for this interaction, as Lys to Arg mutations preserved, but Lys to Ala or Ser to Glu mutations abolished Daxx-p53 interaction. These results thus implicate acetylation and phosphorylation of p53 in regulating its binding to Daxx. Interestingly, whereas Daxx did not bind to p53 in cells as assessed by immunoprecipitation, MDM2 expression restored p53-Daxx interaction, and this correlated with deacetylation of p53. In p53/MDM2-null mouse embryonic fibroblasts (DKO MEF), Daxx repressed p53 target promoters whose p53-binding elements were required for the repression. Coexpression of Daxx and MDM2 led to further repression. p53 expression in DKO MEF induced apoptosis and Daxx expression relieved this effect. Similarly, in HCT116 cells, Daxx conferred striking resistance to 5-fluorouracil-induced apoptosis. As p53 is required for 5-fluorouracil-induced cell death, our data show that Daxx can suppress cell death induced by p53 overexpression and p53-dependent stress response. Collectively, our data reveal Daxx as a novel negative regulator of p53. Importantly, post-translational modifications of p53 inhibit Daxx-p53 interaction, thereby relieving negative regulation of p53 by Daxx.

The critical role of p53 in tumor suppression is manifested in frequent mutations of the *p53* gene in cancers (~50% of all human cancers) and in its inactivation in many other cancers by cellular or viral oncogenes and other epigenetic alterations (1). p53-deficient mice develop normally, although such mice exhibit higher incidence of tumors than their wild-type counterparts, demonstrating a critical role for p53 in suppressing tumors (2). p53 exerts its tumor suppression function by activating expression of genes involved in growth arrest and apoptosis (3, 4), and it can also induce apoptosis directly by binding to Bcl-2 family proteins and triggering cytochrome *c* release (5). Inducing growth arrest and cell death by p53 can impact negatively on normal cell growth and organismal development. Indeed, deletion of the *mdm2* gene, whose product is a negative regulator of p53, results in embryonic lethality, but deletion of both *p53* and *mdm2* simultaneously completely rescues such lethal phenotype (6, 7). Thus, p53 activity must be tightly controlled under physiological conditions. In addition to MDM2, numerous cellular and viral proteins interact with p53 and these proteins can positively or negatively modulate p53-mediated biological effects. Recently, we and others demonstrated that the transcriptional corepressor Daxx interacts with p53 (8–10), but the biological significance of this interaction remains to be explored.

Daxx was initially identified as a binding protein of Fas death domain and was shown to potentiate Fas-mediated apoptosis (11). Subsequent studies implicate Daxx in promoting apoptosis in diverse stress conditions (12–15). Paradoxically, homozygous deletion of the *Daxx* gene in mice results in embryonic lethality and widespread apoptosis was observed in Daxx-deficient embryos and cells (16), indicating that Daxx has anti-apoptotic function and is critical for organismal development. The anti-apoptotic property of Daxx was confirmed in a recent study. Reducing Daxx expression by small interfering RNA sensitized apoptosis by multiple death stimuli (17).

Biochemically, Daxx may act as a transcription regulator that can repress or activate transcription (18–21). Thus, Daxx might regulate cellular processes by modulating transcription of specific genes under different conditions. Daxx has been found to partition in large multiprotein complexes that include core histones and histone deacetylases (HDACs)[1] (22) and a chromatin-remodeling complex that includes ATRX syndrome protein and displays ATP-dependent activities (23, 24). As Daxx associates directly with a number of DNA-binding transcription factors, including Pax3 and -5 (18, 21), ETS1 (20), and p53 and its family members p73 and p63 (8–10), it is likely that Daxx represents a crucial link between DNA-binding factors and transcription regulatory complexes. Whereas this model might provide a framework for better understanding the roles of Daxx in apoptosis and other cellular mechanisms, it remains

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[1] The abbreviations used are: HDAC, histone deacetylases; DKO MEF, $p53^{-/-}$ $mdm2^{-/-}$ double knockout mouse embryonic fibroblast; E3, ubiquitin-protein isopeptide ligase; IP, immunoprecipitation; CBP, CREB-binding protein; PCAF, p300/CBP associated protein; TAD, transactivation domain; DBD, DNA-binding domain; SD, synthetic dropout medium; aa, amino acid(s); GFP, green fluorescent protein; WT, wild-type; 5-FU, 5-fluorouracil; FACS, fluorescence-activated cell sorter; CREB, cAMP-response element-binding protein.

contentious regarding the biological significance of Daxx-p53 interaction. In particular, it was shown that tumor-derived p53 mutants, but not the wild-type protein, interacted with Daxx (10). We report here that both wild-type p53 and its mutants interacted effectively with Daxx *in vitro*. Daxx-p53 interaction was strongly influenced by posttranslational modifications of the p53 COOH-terminal regulatory domain. Cellular oncoprotein MDM2 enhances Daxx-p53 interaction *in vivo*. We also provide evidence that Daxx inhibited both p53 transcriptional and apoptotic activities.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—Various DNA fragments spanning different regions of the Daxx open reading frame were fused either to the yeast Gal4 activation domain (TAD) in plasmid pGAD-C(x) or to Gal4 DNA-binding domain (DBD) in plasmid pGBDU-C(x). Similarly, various WT and mutant p53 constructs were fused with Gal4 TAD. Yeast two-hybrid assays were conducted as described (25).

Cell Culture and Transfection—The tumor cell lines HCT116, HCT116 p53−/− (26), Saos2, and the p53 and MDM2 double knockout mouse embryonic fibroblast (DKO MEF) (7) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected with various DNA constructs using the Effectene transfection reagents (Qiagen).

Expression of Daxx in Insect Cells—The Daxx-coding sequence was cloned into the EcoRI site of pFastBacHTa (donor vector, Invitrogen). Recombinant baculovirus expressing Daxx was obtained using the Bac-to-Bac kit according to the manufacturer's protocol as described previously (25). Sf9 insect cells were infected with Daxx recombinant virus and harvested at 72 h postinfection. Daxx with the amino-terminal His_6 tag was purified from the infected insect cells using nickel-nitrilotriacetic acid-agarose beads (Qiagen) with binding and washing buffers containing 5 and 10 mM imidazole (Sigma), respectively. His-Daxx was eluted with buffer containing 0.2 M imidazole.

Immunoprecipitation (IP)—Cells were trypsinized and resuspended in Buffer B (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10% glycerol, 0.1% Nonidet P-40 (Sigma Igepal CA-630), 150 mM KCl) supplemented with 1× protease inhibitor mixture (16 μg of benzamidin HCl/ml, 10 μg of phenanthroline/ml, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 10 μg of pepstatin A/ml, 1 mM phenylmethylsulfonyl fluoride). Cell suspension was frozen at −80 °C and thawed at room temperature. Cell lysates were cleared by centrifugation at 4 °C for 1 h at 15,000 × *g*. The supernatant was precleared with Protein G-agarose beads for 1 h at 4 °C and then incubated with primary antibody at 4 °C overnight, followed by a 2-h incubation with Protein G-agarose. The antibody-conjugated beads were washed 4 times with Buffer B and then 1 time with RIPA (1% (w/w) Nonidet P-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate), and finally resuspended in SDS gel loading buffer. In some experiments, FLAG-tagged proteins were precipitated using anti-FLAG antibody (M2) conjugated to agarose beads and the precipitated proteins were eluted using buffer B containing 0.2 mg/ml FLAG peptide (Sigma).

Immunofluorescence Microscopy—Previously published protocols were used (8, 27). Briefly, DKO MEF cells grown on glass coverslips were transfected with the indicated plasmids and 24 h later were fixed with 3% paraformaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. The slides were then incubated with blocking buffer (2% fetal bovine serum, 0.1% sodium azide, and 0.1% Tween 20 in phosphate-buffered saline). After incubation with primary antibodies (anti-p53 (DO-1) or anti-MDM2 (SMP14 or N20), Santa Cruz, all 1:200 dilution), the cells were washed with phosphate-buffered saline containing 0.1% Tween 20 and then incubated with appropriate secondary antibodies conjugated with fluorescent dyes. The cells were washed and mounted in medium VectorShields containing 4′,6-diamidino-2-phenylindole.

Luciferase Reporter Gene Assays—DKO MEF (p53- and MDM2-deficient) cells were cultured in a 24-well plate. The luciferase reporter constructs pWAF1-Luc and pWAF1mut-Luc were described previously (28). p53AIP1-Luc contains the p53 DNA-binding elements within the intron 1 of the human *p53AIP1* gene as described (29). The control reporter carries the luciferase gene from the sea pansy (Promega). Reporter plasmids were transiently transfected into cells alone or with other plasmids as indicated in the figures. Transfected cells were harvested 24 h posttransfection and processed for dual luciferase assays (Promega). Firefly luciferase activity was normalized against the sea pansy luciferase activity.

Apoptosis Assay—HCT116 cells grown on glass coverslips were transfected with various combinations of expression plasmids for GFP-CRM1 (marker), FLAG-Daxx, and MDM2. The amount of DNA in each transfection was balanced with empty expression plasmid (pcDNA3.1). Cell cultures were then treated with vehicle (Me_2SO) or 5-fluorouracil (5-FU) (0.4 mM, final concentration) 12 h after transfection. The cells were fixed with 3% paraformaldehyde and mounted on antifade medium containing 4′,6-diamidino-2-phenylindole 24 h after transfection. Cells were examined under a fluorescence microscope and were considered apoptotic if they had condensed or fragmented nuclei that also exhibited rounded and shrunken morphology as revealed by the GFP-CRM1 marker. Five random fields under a ×20 objective were photographed. The green cells (total transfected cells) and apoptotic green cells were counted and percent of apoptotic cells was calculated.

Stable Transfections and Flow Cytometry Analysis—HCT116 cells were transfected with FLAG-Daxx expression vector or empty vector (pcDNA3.1). Twenty-four hours after transfection, cells were split and grown under G418 (0.6 mg/ml) selection until visible colonies appeared. Colony isolation and expansion were done as described previously (30). Several independent clones were grown and either treated with Me_2SO or 5-FU. Cells (both floating and adherent) were collected at specific time points after treatment and washed with phosphate-buffered saline. The cells in suspension were fixed by adding an equal volume of cold (−20 °C) 100% ethanol for at least 1 h with intermittent pipetting for suspending the cells. The cells were pelleted by centrifugation for 3 min at 300 × *g* and then resuspended in a solution with RNase A (0.5 mg/ml, final concentration) and incubated for 15 min at 37 °C. The cells were then stained with propidium iodide (50 mg/ml) for 30 min at room temperature in the dark. Samples were analyzed with a FACSort instrument (BD Biosciences).

RESULTS

Daxx Binds to p53 in Vitro—Because we have identified an interaction between Daxx and E1B 55-kDa oncoprotein and that Daxx and p53 colocalized in the nucleus in 293 cells (8), we wondered whether Daxx could also bind directly to p53. To detect a potential interaction between Daxx and p53, we first tested their interaction *in vitro*. When the extract of insect Sf9 cells expressing Daxx was mixed with purified WT p53, Daxx was precipitated along with p53 in an IP assay using DO-1 (Fig. 1*A*, *lane 3*). Reciprocally, p53 was precipitated from the same mixture using anti-Daxx antibody M112 (Fig. 1*A*, *lane 6*). Yeast two-hybrid assays were used to map the regions required for Daxx-p53 interactions. As shown in Fig. 1, *B–E*, full-length Daxx (*panel B*, sector 1) and Daxx fragments lacking the COOH terminus (aa 1–501 and 1–574, sectors 7 and 10) specifically interacted with p53, but not other Daxx fragments (aa 516–740 (sector 4), 1–130, 129–396, and 621–740 (data not shown)). Deletion of the acidic domain (aa 434–496) from Daxx abolished its interaction with p53 (Fig. 1*B*, *sector 15*). Thus, Daxx sequence between 400 and 501 encompassing the acidic domain is required for binding to p53 (summarized in *panel C*). Conversely, p53 constructs with progressive deletions from the NH_2 terminus up to residue 253 were able to bind to Daxx (*sectors 1–4* in *panel D*). Further deletion from the NH_2 terminus abolished p53-Daxx interaction (*sectors 5* and *6*, *panel D*). These deletion mutants were all well expressed in yeast (Fig. 1*H*, *lanes 1–5*). p53 NH_2-terminal TAD (aa 1–145) and the DBD (aa 76–315) did not interact with Daxx (data not shown, see *panel E*). Point mutations in the p53 NH_2 terminus (L22Q/W23S and K24T) did not affect Daxx-p53 interaction (*panel F*, *sectors 6* and *7*). Likewise, mutations in the core domain (V143A, R175H, R273H, "hot spots" of tumor-derived mutations, *sectors 2*, *3*, and *5*, *panel F*), except hot spot mutation R248W (*sector 4*), did not affect binding of p53 to Daxx (Fig. 1*F*). These p53 mutants were expressed at similar levels in yeast (Fig. 1*H*, *lanes 13–18*). The results contrast that from a recent report indicating that tumor-derived mutants, but not wild-type p53, interacted with Daxx (10). However, COOH-

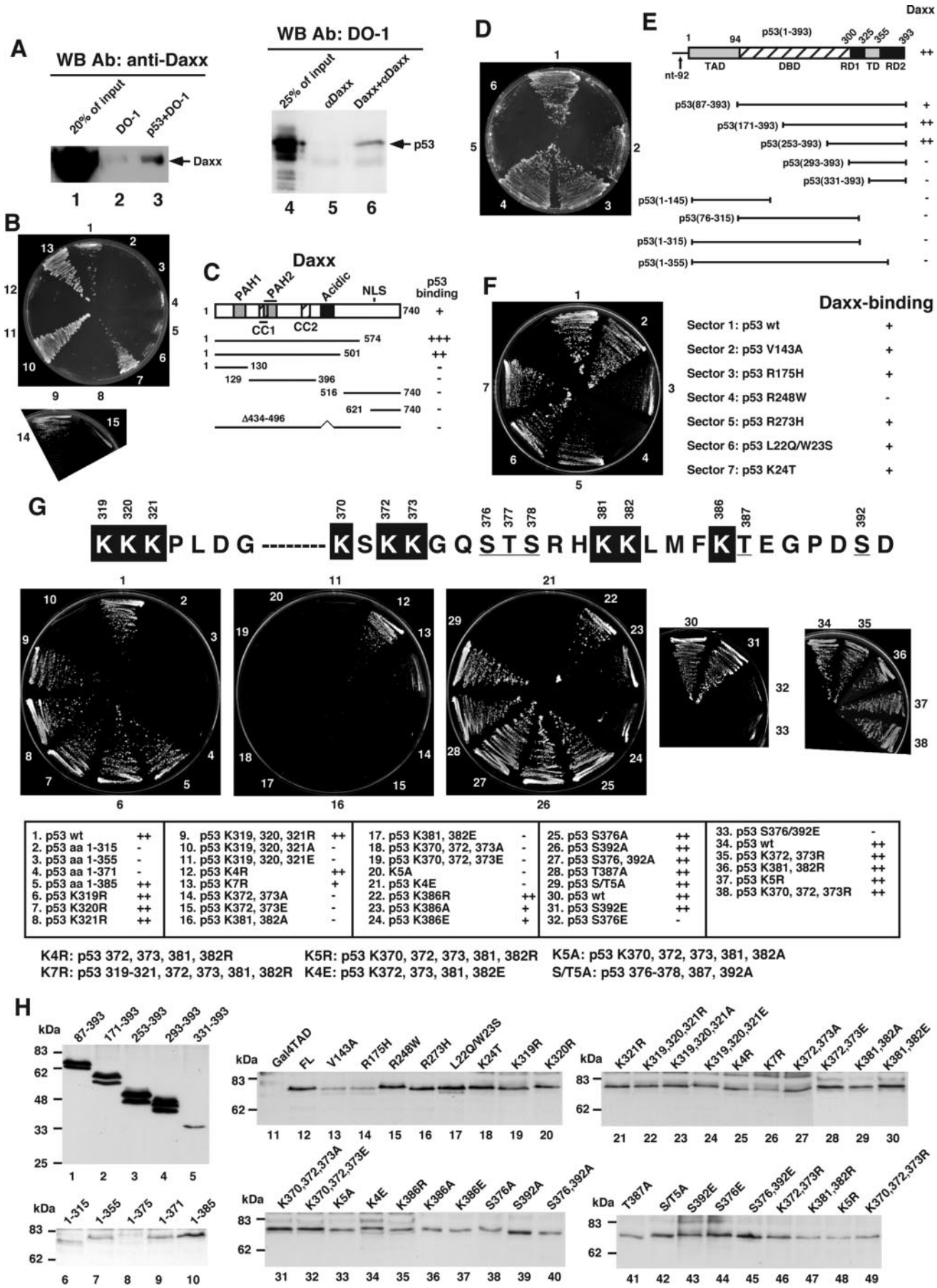

FIG. 1. **Daxx binds to p53 *in vitro* and in yeast.** *A*, Daxx and p53 interact *in vitro*. Extracts from Sf9 cells expressing human Daxx were incubated with purified human p53. The mixture was subjected to IP with either an anti-p53 antibody (DO-1, *lanes 2* and *3*) or anti-Daxx M112 (*lanes 5* and *6*). The precipitates were analyzed by Western blot using M112 (*lanes 1–3*) or DO-1 (*lanes 4–6*). *B*, mapping of the p53-interacting

terminal truncations of p53 abolished p53-Daxx interaction (aa 1–315 and 1–355, see Fig. 1*G*). Therefore, the p53 COOH-terminal regulatory domain is required for binding to Daxx, and mutations in the NH_2 terminus or core domain do not affect binding of p53 to Daxx in yeast. The data also indicate that some part of the DBD of p53 may also be involved in binding to Daxx, as the Daxx-binding region maps to residues 253–385 in p53; this region includes part of the DBD and most of the COOH-terminal domain of p53. In the case of the p53 R248W mutant, it is possible that this mutation might alter the conformation of the COOH-terminal domain so that Daxx can no longer bind to p53.

Positive Charges of the COOH-terminal Domain of p53 Are Critical for Daxx-p53 Interaction—The Daxx-binding region of p53 is important in regulating p53 activity. It was shown that this domain might negatively regulate the p53 transactivation function (31). In addition, it contains sites of acetylation by PCAF and p300/CBP as well as phosphorylation (32, 33). To assess potential roles of acetylation and phosphorylation in regulating Daxx-p53 interaction, we mutated known and potential acetylation and phosphorylation sites within the Daxx-binding region of p53 and assayed the interaction of Daxx with such p53 mutants. As shown in Fig. 1*G*, any COOH-terminal truncation before residue 371 abolished Daxx-p53 interaction, whereas residues after 385 were dispensable for binding (*sectors 2–5* in Fig. 1*G*). Mutation of any block of lysines surrounding PCAF (K320) and p300/CBP (Lys-372 and -381) acetylation sites (34–36) into positively charged arginine did not affect interaction (*sectors 6–9*, *12*, and *35–38*). Nonetheless, when all seven lysines at 319–321, 372, 373, 381, and 382 were changed into arginine (K7R), p53-Daxx interaction was notably reduced (*sector 13*). Significantly, Daxx-p53 interaction was abolished when any block of lysines were mutated into neutral residue alanine, which presumably mimics acetylated lysine (*sectors 10*, *14*, *16*, *18*, and *20*). Moreover, mutation of lysine residues into negatively charged glutamic acid also abolished Daxx-p53 interaction (*sectors 11*, *15*, *17*, *19*, and *21*). However, mutation of the potential phosphorylation sites into alanine did not affect Daxx-p53 interaction (*sectors 25–29* in Fig. 1*G*). Strikingly, mutation of Ser-376 to glutamic acid that mimics phosphorylated residues severely inhibited the Daxx-p53 interaction (*sectors 32* and *33*), whereas similarly mutated Ser-392 did not affect p53-Daxx interaction (*sector 31*) (Ser-376 and Ser-392 are documented phosphorylation sites of p53 (32)). All of the p53 constructs shown in Fig. 1*G* exhibited interaction with p300 in yeast two-hybrid assays (data not shown). In addition, we have examined expression of these p53 mutants in yeast and found that they were all expressed at similar levels (Fig. 1*H*). Thus, negative interaction results of some p53 mutants were because of specific mutations, not because of protein instability in yeast. Collectively, these results indicate that positive charges in the p53 COOH terminus play a critical role in mediating Daxx-p53 interaction, but lysine is preferred over arginine for this binding, indicating that Daxx-p53 interaction is specific and is not purely mediated by opposite static electric charges. Because mutation of either PCAF or p300/CBP acetylation sites into alanine is sufficient to inhibit Daxx-p53 interaction, acetylation by either acetylase could potentially block the binding of Daxx to p53. In addition, phosphorylation within the Daxx-binding region of p53 may also inhibit binding of Daxx to p53. Therefore both acetylation and phosphorylation might dissociate Daxx from p53.

MDM2 Restored Daxx-p53 Interaction in the Cell—Previous attempts by us and others to recover Daxx-p53 interaction in cell extracts by immunoprecipitation were unsuccessful (10).[2] Because Daxx-p53 interaction was readily detected using recombinant Daxx and p53 purified from insect cells as well as in yeast (Fig. 1), posttranslationally modified p53 in the cell might prevent p53 from binding to Daxx. Indeed, Daxx purified from insect Sf9 cells does not bind to p53 purified from cultured human cells (data not shown).

If acetylation of p53 inhibits Daxx-p53 interaction, overexpression of proteins that influence acetylation of p53 might affect binding of Daxx to p53. HDAC1 has been found to deacetylate p53 (37). Likewise, MDM2 inhibits p53 acetylation (38) and also facilitates HDAC1-mediated deacetylation of p53 (39). To assess whether these proteins might affect Daxx-p53 interaction, FLAG-Daxx (aa 1–574) was cotransfected into p53-deficient HCT116 cells along with various combinations of p53, MDM2, and HDAC1. Expression of p53, Daxx, HDAC1, and MDM2 in transfected cells was determined by Western blot (Fig. 2*A*). The cell extracts were then subjected to IP using anti-FLAG M2 beads. Strikingly, only in the presence of transfected MDM2 was Daxx-p53 interaction restored (*lanes 6* and *10*, Fig. 2*B*). We then examined the status of p53 acetylation in cell extracts. Approximately equal amounts of p53 protein was loaded (Fig. 2C) and the rabbit antiserum that recognizes p53 acetylated at Lys-373 and -382 was used to detect acetylated p53. As shown in Fig. 2*C*, the presence of MDM2 resulted in deacetylation of p53 (compares *lanes 2* and *4* with other lanes), suggesting that MDM2 might restore Daxx-p53 interaction at least in part by inhibiting p53 acetylation.

Sequence Determinants of Daxx-p53 Interaction in the Cell—

[2] L. Y. Zhao, J. Liu, G. S. Sidhu, Y. Niu, Y. Liu, F. Wang, and D. Liao, unpublished data.

domain of Daxx. Full-length p53-Gal4 TAD was assayed for interaction with various Daxx fragments fused with Gal4 DBD using the yeast two-hybrid system: *sectors 1–3*, full-length Daxx with p53, Gal4 TAD, or alone; *sectors 4-6*, Daxx aa 516–740 with p53, Gal4 TAD, or alone; *sectors 7–9*, Daxx aa 1–501 with p53, Gal4 TAD, or alone; *sectors 10–12*, Daxx aa 1–574 with p53, Gal4 TAD, or alone. *Sector 13* illustrates the E1B-p53 interaction (positive control). *Sectors 14* and *15* show the results of two-hybrid assays of Gal4-TAD-p53 and Gal4-DBD-Daxx (full-length, *sector 14*; and full-length with deletion of acidic domain, aa 434–496, *sector 15*). Yeast cells were plated on medium lacking adenine. *C*, summary of the Daxx-p53 interaction based on *B*, indicating that the Daxx domain around aa 400–501 is required for binding to p53. *CC*, coil-coiled domain; *PAH*, paired amphipathic helix domain; *NLS*, nuclear localization signal. *D*, mapping of the Daxx-binding domain of p53. Daxx aa 1–574 fused with Gal4 DBD was introduced into yeast with various p53-Gal4 TAD constructs: *sectors 1–6*, p53-(1–393), p53-(87–393), p53-(171–393), p53-(253–393), p53-(293–393), and p53-(331–393), and yeast growth was scored in medium lacking adenine. *E*, summary of the Daxx-p53 interaction based on *D,* and data not shown, indicating that the Daxx-binding domain resides in the p53 COOH terminus between aa 253 and 385 (also see *panel G*). *RD*, regulatory domain; *TD*, tetramerization domain. *F*, hot spot mutations of p53 did not affect its interaction with Daxx. Indicated tumor-derived and artificial p53 mutants were fused with Gal4-TAD and cotransformed into yeast with Gal4-DBD-Daxx-(1–574) hybrid. Yeast were grown on plates lacking adenine. *G*, mutations of lysine to alanine and serine to glutamic acid within the Daxx-binding region of p53 abolished p53-Daxx interaction in yeast. Lysines (*white letters in black background*) or serines/threonines (*underlined*) were mutated individually or in various combinations by the QuikChange protocol. The positions of these mutated residues within the p53 sequence are indicated. These mutants as depicted in the *bottom panel w*ere fused with Gal4 TAD, which were tested for interaction with the Gal4-DBD Daxx (aa 1–574) hybrid. Yeast cells were grown in SD medium lacking adenine. *H*, expression of various Gal4TAD-p53 fusion constructs in yeast. The yeast transformants harboring Gal4DBD-Daxx-(1–574) and an indicated Gal4TAD-p53 construct were grown in SD medium lacking lysine, leucine, and uracil. Yeast cell extracts were prepared according to a protocol described previously (25). The extracts were subjected to SDS-PAGE and Western blot using anti-p53 antibody (FL393, Santa Cruz).

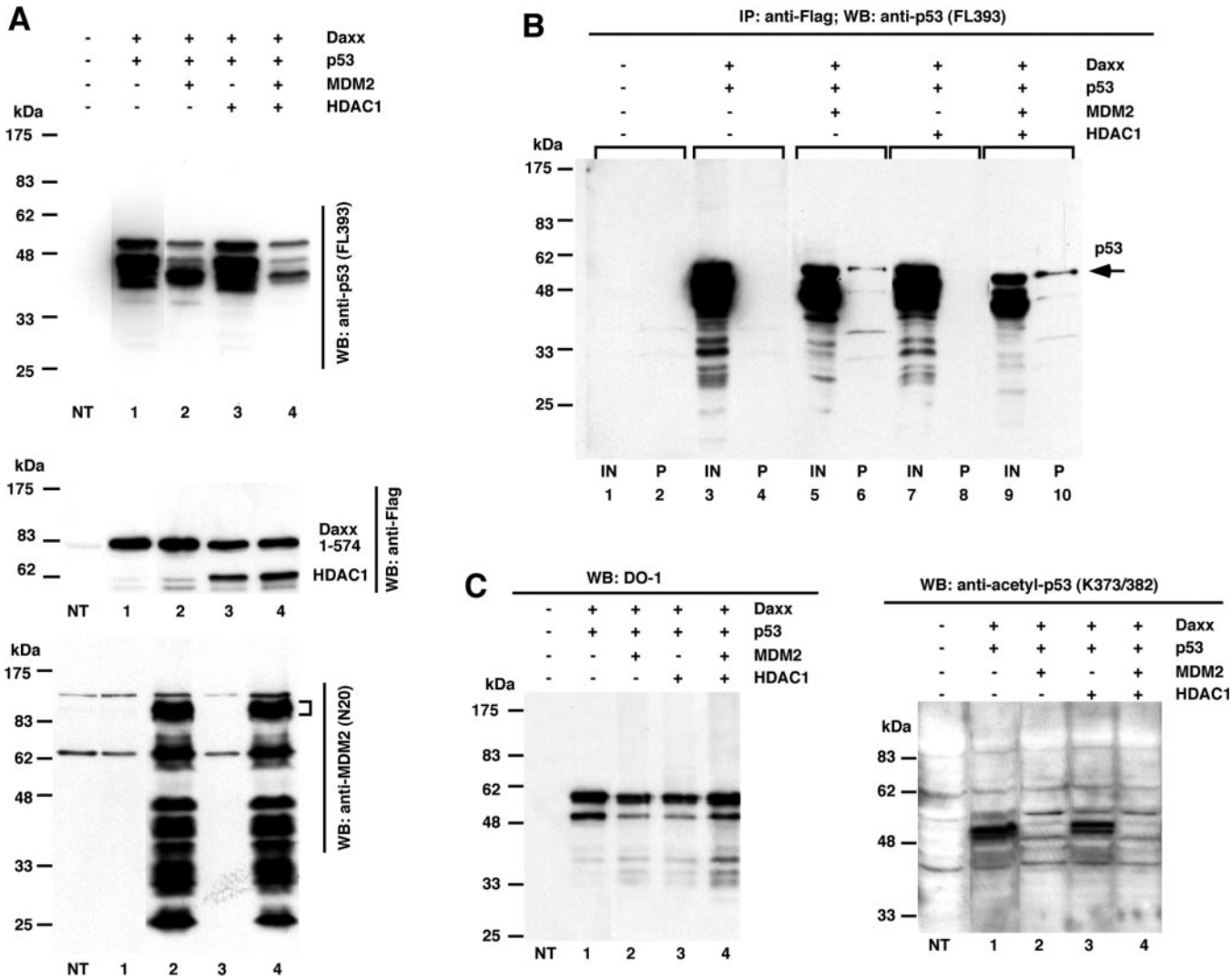

FIG. 2. **Expression of MDM2 restored Daxx-p53 interaction in human cells.** *A*, p53-null HCT116 cells were transfected with various combinations of expression plasmids as indicated and 24 h after transfection, cells were harvested and analyzed for the expression of p53 (*top*), Daxx and HDAC1 (*middle*), and MDM2 (*bottom*). *NT*, untransfected HCT116 p53−/− cells. *B*, Daxx-p53 interaction was restored upon exogenous MDM2 expression. The whole cell extracts of HCT116 p53−/− cells without transfection (*NT*) or with introduction of expression plasmids of the indicated proteins were subjected to immunoprecipitation using anti-FLAG M2 beads. The beads were washed extensively and eluted with FLAG peptide. The immunocomplexes were then analyzed in Western blot using rabbit polyclonal anti-p53 antibodies (FL393). *IN*, input whole cell extracts before immunoprecipitation (~5% of total input). *P*, immunoprecipitates. *C*, deacetylation of p53 upon MDM2 expression. Equal amounts of p53 based on Western blot (*WB*) with anti-p53 antibody DO-1 were analyzed in blots using antiserum specific to p53 acetylated at 373 and 382 (Upstate Biotechnology).

Having shown that MDM2 is critical for Daxx-p53 interaction in the cell, we wished to determine what sequences of Daxx are required for binding to p53 *in vivo*. Various Daxx constructs with the NH_2-terminal FLAG tag were cotransfected with vectors for p53 and MDM2 into p53-null HCT116 cells and expression of these proteins in the transfected cells was examined (Fig. 3*A*). The cell extracts were subjected to IP with anti-FLAG M2-agarose beads and the coprecipitated proteins were analyzed using anti-p53 antibody (Fig. 3*B*). Full-length Daxx led to abundant p53 coprecipitation (*lanes 1* and *2*). Daxx constructs aa 1–574, 1–501, and a construct with deletion of aa 191–242 (a putative amphipathic helix (18)) bound to p53 less effectively than wild-type Daxx (*lanes 4*, *6*, and *10*). In addition, the Daxx construct with a deletion between aa 502 and 574 exhibited weak binding to p53 (Fig. 3*B*, *lane 16*). The Daxx NH_2-terminal domain (aa 1–130) and the COOH-terminal constructs (aa 516–740 and 573–740) did not bind to p53. These results indicate that the acidic domain of Daxx is required for binding to p53, consistent with the results obtained from yeast two-hybrid assays (Fig. 1).

To examine whether MDM2 was present in the same FLAG-Daxx immunocomplexes, the same membrane shown in Fig. 3*B* was stripped and reprobed with anti-MDM2 antibody. MDM2 was coprecipitated with WT Daxx and its fragments (aa 1–574 and 1–502, Fig. 3C, *lanes 2*, *4*, and *6*). Evidently, more MDM2 was precipitated with WT Daxx than with the two Daxx fragments, in parallel with the amounts of precipitated p53. Interestingly, although p53 was precipitated with Daxx Δ191–242 and Δ502–574 constructs, little MDM2 was coprecipitated with them (*lanes 10* and *16*, Fig. 3*C*). These results suggest that MDM2 might play two roles in mediating Daxx-p53 interaction: (i) physically bridging and stabilizing the Daxx-p53 complex, and (ii) inhibiting covalent modifications of p53, thereby facilitating Daxx-p53 interaction. Alternatively, MDM2 might alter the conformation of Daxx to expose its p53-binding domain, because removing aa 191–242 or 502–574 from Daxx resulted in Daxx-p53 interaction in the absence of MDM2 coprecipitation (*lanes 10* and *16* in Fig. 3, *B* and *C*).

We showed above that several p53 mutants that mimic acetylated or phosphorylated p53 abolished binding of p53 to

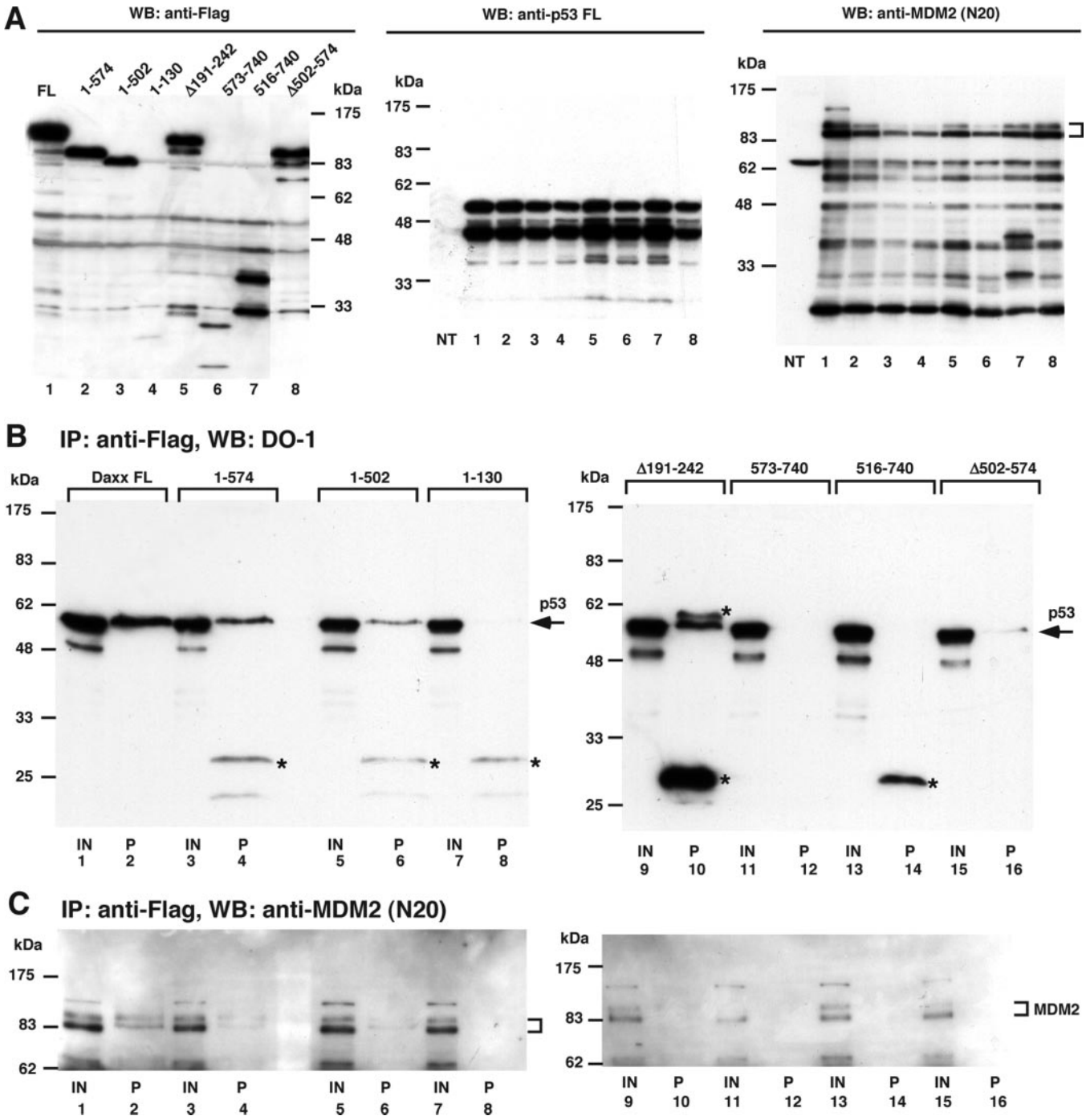

FIG. 3. **The acidic domain of Daxx is required for binding to p53 in human cell.** HCT116 p53−/− cells were transfected with vectors for p53, MDM2, and an indicated Daxx construct and the cells were harvested 24 h after transfection. Whole cell extracts were then subjected to IP using anti-FLAG M2 beads. *A*, expression of Daxx constructs, p53 and MDM2 in transfected cells. *B*, Western blot (*WB*) analysis of immunocomplexes associated with FLAG-Daxx constructs. The blots were probed with anti-p53 DO-1. *IN* and *P* were as described in the legend to Fig. 2. *Asterisks* denote mouse IgG heavy or light chains. *C*, Western blot analysis of MDM2 in FLAG-Daxx immunocomplexes. The same membranes as in *B* were striped and reprobed with rabbit anti-MDM2 polyclonal antibody (N20).

Daxx in yeast (Fig. 1*G*). To assess if these results also hold true in cultured human cells, hemagglutinin-Daxx was cotransfected with various p53 constructs and the cell extracts were subjected to IP using anti-p53 antibodies (FL393). Daxx was coprecipitated with WT p53 (Fig. 4*B*, *lane 2*) and to a lesser extent with p53 K381A/K382A (*lane 6*), K381R/K382R (*lane 8*), S376A (*lane 10*), and p53 S376E (*lane 12*). No Daxx was detectably precipitated with p53 K381E/K382E (*lane 4*) and L22Q/W23S (*lane 14*). In the absence of p53, Daxx was not precipitated (*lane 16*). Because p53 mutant L22Q/W23S does not bind to MDM2 (40), the fact that it also failed to bind to Daxx in HCT116 colon cancer cells suggests two possibilities: MDM2 is required for physically stabilizing the Daxx-p53 complex, and/or MDM2 cannot mediate deacetylation of the p53 L22Q/W23S mutant and consequently, Daxx could not bind to this mutant. However, the p53 L22Q/W23S mutant was not acetylated at Lys-373 and Lys-382 (data not shown), probably because of disruption of the p53-p300/CBP interaction by the double mutation, as it has been shown that both Leu-22 and Trp-23 are critical for p53 to bind to p300, and mutating Trp-23 completely abolished p53-p300 interaction (41). Intriguingly, although Daxx did not bind to p53 K381A/K382A and S376E

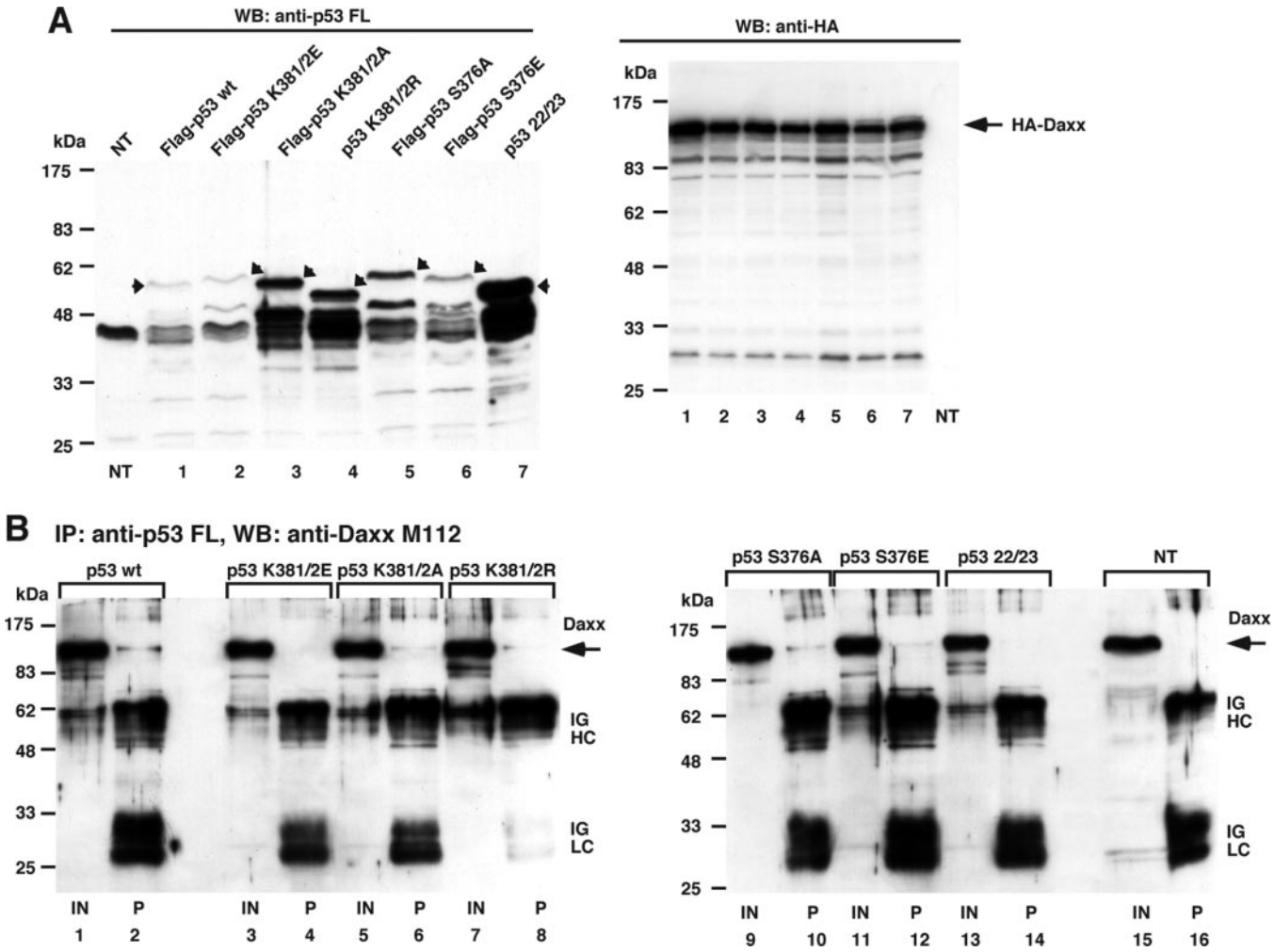

FIG. 4. **Interaction of Daxx with various p53 constructs.** HCT116 p53−/− cells were transfected with vectors for hemagglutinin-Daxx, MDM2, and an indicated p53 construct and the cells were harvested 24 h after transfection. The whole cell extracts were then subjected to IP using rabbit polyclonal anti-p53 antibody (FL393) and protein G-agarose beads. *A*, expression of p53 constructs and hemagglutinin-Daxx in transfected cells. The expected band of each p53 construct is indicated with an *arrow*. *B*, Western blot (*WB*) analysis of immunocomplexes associated with p53 constructs. The blots were probed with rabbit polyclonal anti-Daxx M112. *IN* and *P* were as described in the legend to Fig. 2. The IgG heavy or light chains are indicated. *NT*, no transfection.

mutants in yeast (Fig. 1*G*), they were still detectably coprecipitated with Daxx (Fig. 4*B*, *lanes 6* and *12*). Taken together, our data suggest that MDM2 stabilizes the interaction of p53 with Daxx in human cells.

MDM2 Enhances p53-Daxx Interaction in Primary Cells—To further examine the requirement of MDM2 for Daxx-p53 interaction, we employed p53/MDM2 DKO MEF (7). FLAG-Daxx was cotransfected with various combinations of p53, its mutants, and MDM2. All transfected constructs expressed well in DKO MEF (Fig. 5, *A* and *B*). The cell extracts were immunoprecipitated with anti-FLAG M2-beads and the immunocomplexes were analyzed with anti-p53 (FL393) and MDM2 antibodies (Fig. 5, *C* and *D*). Notably, WT p53 was very efficiently precipitated in the presence of MDM2 (Fig. 5*C*, *lane 2*). Small but detectable amounts of p53 was precipitated in the absence of MDM2 expression (*lane 4*, p53 band was visible upon longer exposure). p53 mutants K381A/K382A and L22Q/W23S were also coprecipitated with FLAG-Daxx, although to a much lesser extent than WT p53 (compare *lanes 6* and *8* with *2* in Fig. 5*C*). Interestingly, the precipitated p53 was extensively modified. In the case of WT p53, two bands with approximately equal abundance were precipitated (see Fig. 5*C*, *lower panel*): the *lower band* presumably is the unmodified p53, and the *upper band* could be monoubiquitinated. The other more slowly migrating bands may be polyubiquitinated p53. Interestingly, reprobing the same membrane with anti-ubiquitin antibody revealed both prominent bands in addition to polyubiquitinated bands (data not shown). Multiple p53 bands were also precipitated with p53 mutants K381A/K382A and L23Q/W23S (Fig. 5*C*, *lanes 6* and *8*). Notably, the precipitated WT p53 was more extensively modified (compare *lanes 6* and *8* with *2* in Fig. 5*C*), raising an intriguing possibility that Daxx might play a role in MDM2-mediated ubiquitination of p53 (see "Discussion").

MDM2 appears to constitutively associate with Daxx (Fig. 5*D*). Nonetheless, in the absence of p53 expression, the amount of precipitated MDM2 was slightly reduced (compare *lane 8* with *2*, *4*, and *6* in Fig. 5*D*). Collectively, these results suggest that the MDM2-Daxx binary complex exists constitutively and Daxx does not efficiently bind to p53 in the absence of MDM2. When all three are expressed, they form a stable complex.

Codistribution of Daxx, MDM2, and p53 in DKO MEF—We also examined subcellular distributions of these proteins. In DKO MEF cells, all three proteins were predominantly localized in the nucleus (Fig. 5*E*). Daxx appeared in nuclear body-like structures as well as in the nucleolus (*panels a*, *i*, and *m* in Fig. 5*E*), as reported previously (8), whereas both p53 and MDM2 were largely excluded from these locations (Fig. 5*E*, *b*, *f*, and *j*). Interestingly, in substantial fractions of the transfected

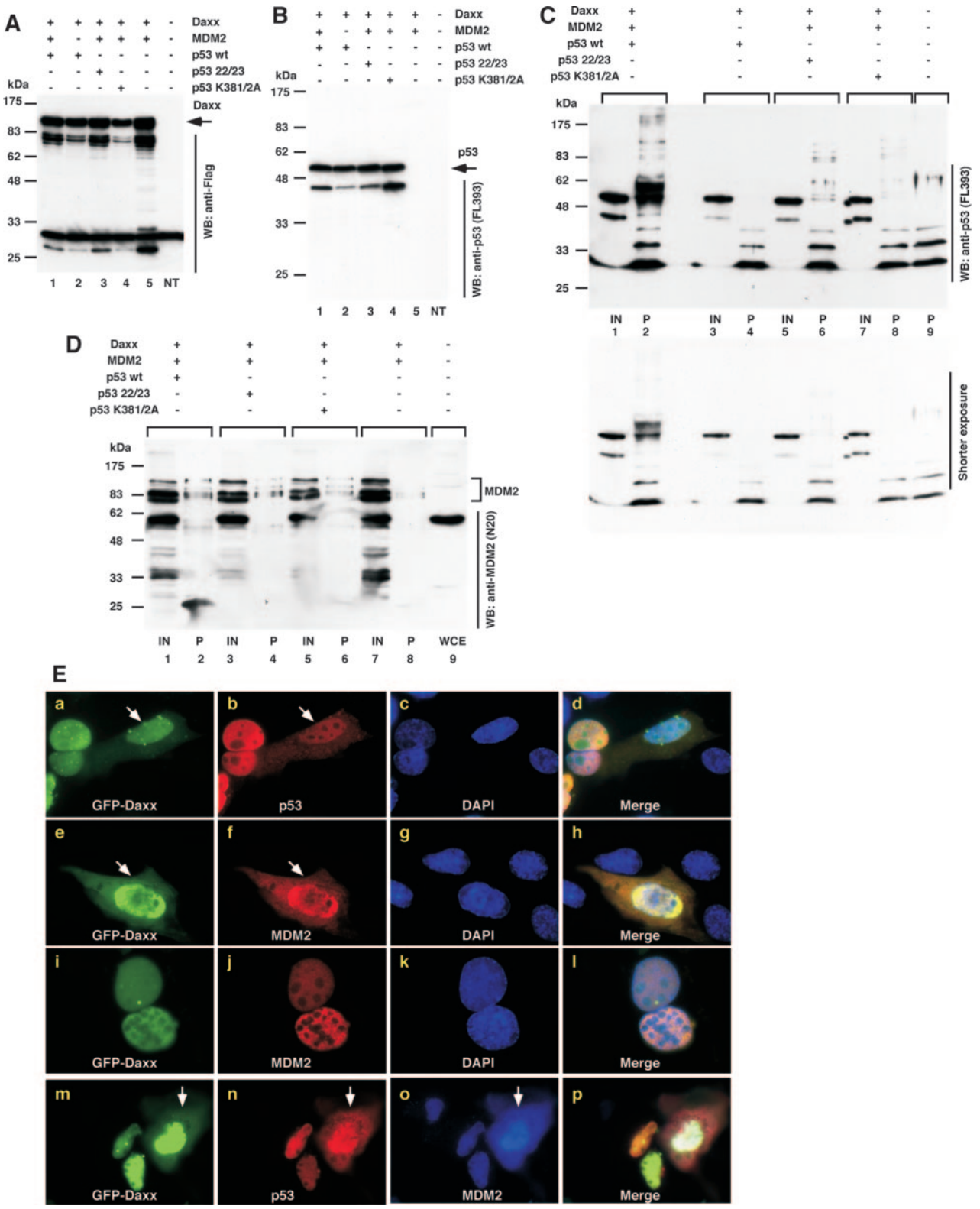

FIG. 5. **Interactions of Daxx, MDM2, and p53 in DKO MEF cells.** Cells were transfected with vectors for FLAG-Daxx (full-length), MDM2, and different p53 constructs as indicated and the cells were harvested 24 h after transfection. Whole cell extracts were then subjected to IP using anti-FLAG M2 beads. *A* and *B*, expression of FLAG-Daxx and p53 constructs in transfected cells. *C*, Western blot (*WB*) analysis of p53 associated with FLAG-Daxx immunocomplexes. The blots were probed with rabbit polyclonal anti-p53 FL393 antibodies. *Lane 9* shows the immunoprecipitates of untransfected DKO MEF. The *lower panel* shows the image of the same blot with a shorter exposure. Upon a much longer exposure, a p53 band was revealed in *lane 4* and the position of this band is the same as that shown in *lane 3*, but the slower migrating bands were not detected. *D*, Western blot analysis of MDM2 associated with FLAG-Daxx immunocomplexes. The blots were probed with rabbit polyclonal anti-MDM2 (N20) antibodies. *Lane 9* shows the whole cell extracts of DKO MEF without transfection. *IN* and *P* were as described in the legend to Fig. 2. *E*, subcellular distributions of Daxx, p53, and MDM2 in DKO MEF cells. GFP-Daxx plasmid was cotransfected with either p53 or MDM2 expression plasmid, or both, and 24 h after transfection, the cells were fixed and immunostained with anti-p53 antibody (DO-1, *panels b* and *n*) or anti-MDM2 antibody (SMP14, *panels f* and *j*). The anti-mouse IgG antibodies coupled with rhodamine were used as the secondary antibody (*panels b*, *f*, *j*, and *n*). The nuclei were revealed with 4′,6-diamidino-2-phenylindole (*DAPI*). In *panel o*, rabbit polyclonal anti-MDM2 antibody (*N20*) was used as the primary antibody and goat anti-rabbit IgG-Alexa Fluor 350 conjugate as secondary antibody.

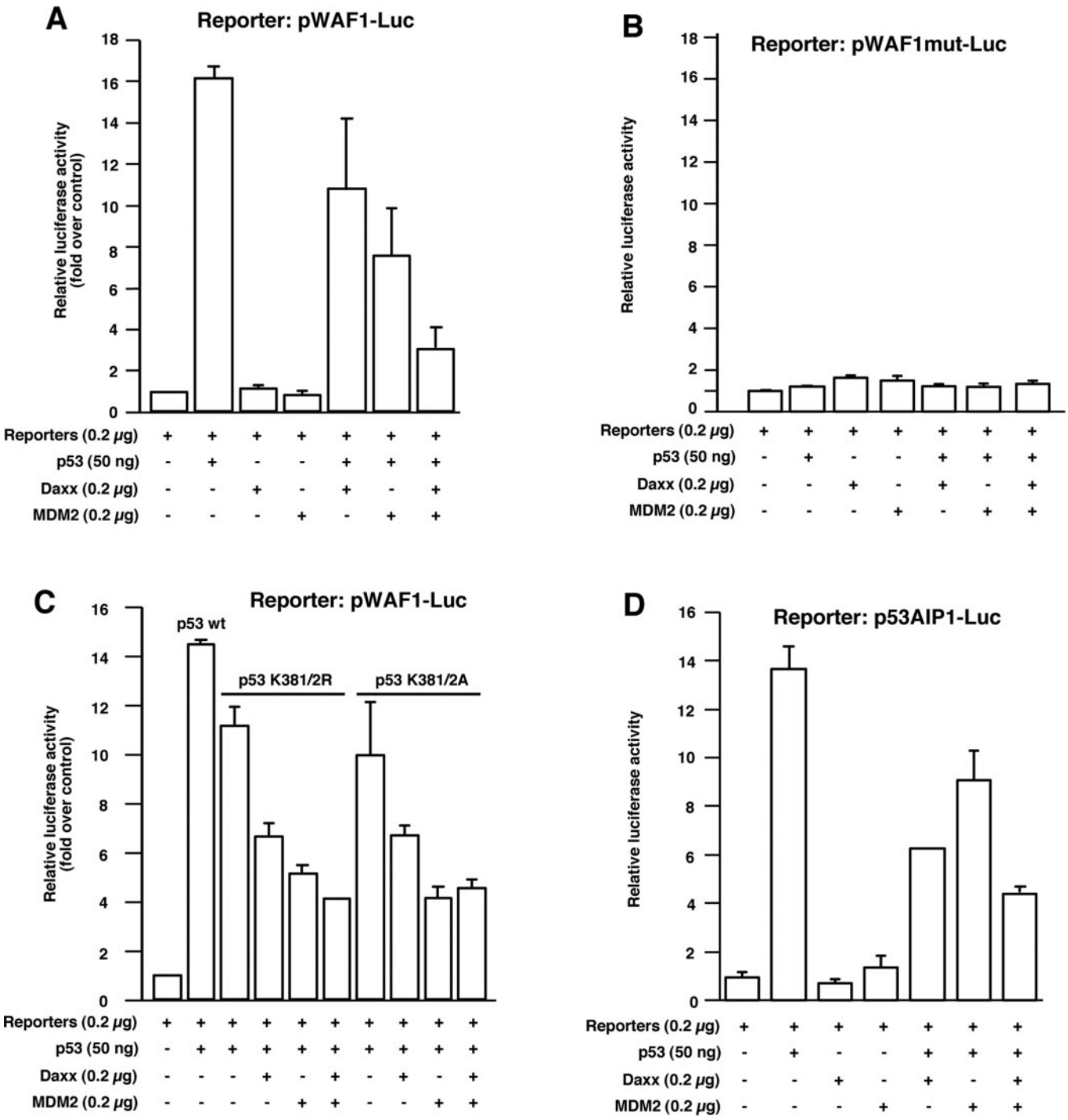

FIG. 6. **Daxx represses the promoters of the p53 target genes.** The indicated p53-responsive luciferase reporter along with pRL-SV40 (internal control) was transfected with various combinations of expression vectors into DKO MEF as indicated. Dual luciferase assays were performed 24 h after transfection. The values shown are the average of two independent experiments, and the *error bars* represent 1 S.D. *A*, Daxx represses the $p21^{WAF1/cip1}$ promoter in DKO MEF cells. The reporter carries the 2.4-kb region of the $p21^{WAF1/cip1}$ promoter that contains both p53-binding elements. *B*, p53 binding sequences in the $p21^{WAF1/cip1}$ are required for Daxx-mediated repression. Both p53 binding elements of the $p21^{WAF1/cip1}$ promoter were mutated in the pWAF1mut-Luc reporter as shown previously (8). *C*, effects of Daxx on transactivation mediated by p53 mutants. The p53 mutants K381R/K382R or K381A/K382A carry point mutations that convert lysines at 381 and 382 to arginine or alanine, respectively (see Fig. 1). *D*, Daxx represses p53-mediated transcription on the *p53AIP1* promoter. The p53AIP1 reporter carries the intron 1 of the human *p53AIP1* gene that harbors the p53 binding elements as described (29).

cells, all three proteins were seen simultaneously in both the cytoplasm and nucleus (*white arrows* in Fig. 5*E*). The immunostaining patterns of p53 or MDM2 were essentially identical whether both together (*panels m–p*) or each individually (*panels a–i*) were coexpressed with GFP-Daxx (Fig. 5*E*).

Daxx Represses the Promoters of p53 Target Genes—To investigate the biological significance of the Daxx-p53 interaction, we examined whether Daxx affects p53-mediated transcription. DKO MEF cells were transfected with the $p21^{WAF1}$ luciferase reporter that contains the promoter of the $p21^{WAF1}$ gene. Expression of p53 led to a dramatic increase of reporter activity (~16-fold induction, Fig. 6*A*). Expression of Daxx or MDM2 alone in the absence of p53 expression had no obvious effects on the *p21* promoter. Expression of p53 together with

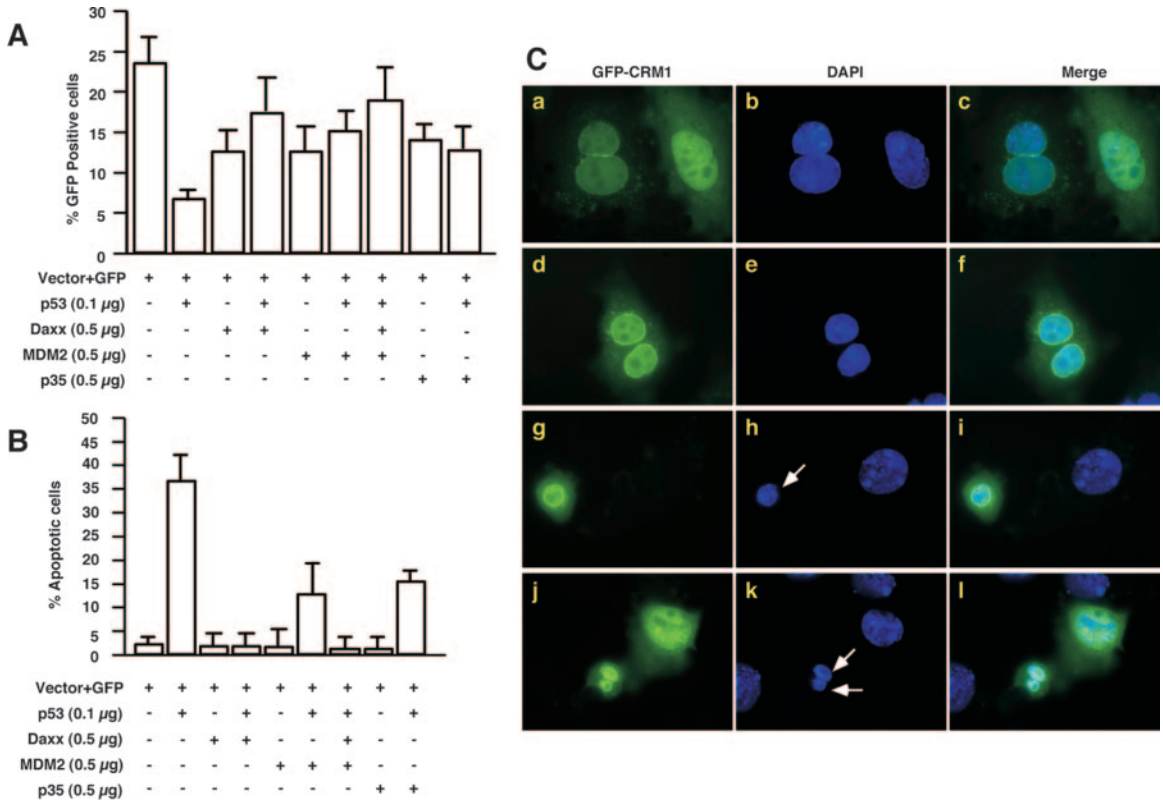

FIG. 7. **Daxx inhibits p53-mediated apoptosis.** Indicated expression vectors were transfected into DKO MEF cells grown on glass coverslips, and 24 h after transfection, cells were fixed with paraformaldehyde and mounted on antifade medium containing 4′,6-diamidino-2-phenylindole (*DAPI*) (VectorShields). The amount of plasmid DNA in each transfection was strictly balanced with the empty vector plasmid (pcDNA3.1). The GFP-CRM1 fusion was used as the transfection marker. *A*, Daxx relieved p53-mediated cell killing. The percentage of transfected cells in random microscopic fields is shown from each transfection experiment. *Error bars* are 1 S.D. *B*, Daxx inhibits p53-induced cell death. The percentage of cells with condensed nuclei (apoptotic) out of total GFP-positive cells is shown. *C*, shown are representative micrographs of transfected GFP-positive cells with normal nuclear morphology (*panels a–f*) or condensed nuclei (denoted with *white arrow*, *panels g–l*).

Daxx or MDM2 resulted in significant reduction of the reporter activity (induction was reduced from 16- to ~11- or 8-fold, respectively, Fig. 6*A*). Expression of all three proteins led to a further reduction of the reporter activity (to ~3-fold induction). As expected, p53 did not activate the *p21* promoter in the absence of p53-binding sequences in the *p21* promoter (Fig. 6*B*). Similarly, Daxx and MDM2 had no effects on *p21* promoter in the absence of p53-binding sequences (Fig. 6*B*). Thus, Daxx-mediated repression of *p21* promoter requires binding of p53 to the promoter.

We also examined whether Daxx affects transcription mediated by p53 mutants K381R/K382R or K381A/K382A. In yeast, the K381R/K382R, but not K381A/K382A mutant, binds to Daxx (Fig. 1*G*), whereas both bind to p53 in mammalian cells (Figs. 4 and 5). As shown in Fig. 6*C*, both mutants exhibited a reduced ability to activate the *p21* promoter, consistent with a positive role of acetylation at these two sites for p53-mediated transcription (42). Interestingly, Daxx or MDM2 could still repress the *p21* promoter when co-expressed with either of these two p53 mutants (Fig. 6*C*). Coexpression of Daxx and MDM2 with p53 K381R/K382R led to further, albeit small, reduction of the reporter activity, but no additional repression was observed when they were expressed with the p53 K381A/K382A mutant (Fig. 6*C*). Finally, Daxx also exerted obvious repression of the reporter construct containing the *p53AIP1* intron that harbors the p53-binding sites, and coexpression of both Daxx and MDM2 resulted in further reduction of p53-dependent transcription (Fig. 6*D*).

Daxx Relieved p53-mediated Apoptosis—In examining subcellular distribution of p53, Daxx, and MDM2 in DKO MEF, we noticed that a few transfected cells were observed when p53 alone was expressed, but many more transfected cells were seen when p53 was cotransfected with either Daxx or MDM2 or both together. This suggested to us that Daxx might inhibit p53-induced apoptosis. We then quantitatively investigated this initial observation. We used the GFP-CRM1 fusion construct as a transfection marker, which, unlike GFP, exhibited no cytotoxic effects. The marker was transfected alone or together with other expression plasmids as indicated in Fig. 7. In each transfection, about 20 random microscopic fields were examined. The total number of cells and transfected cells in these fields were tallied. As shown in Fig. 7*A*, about 22% of cells were transfected when GFP-CRM1 was transfected alone. In p53-transfected cells, only about 7% of cells were transfected, an indication of cell death. Expression of Daxx, MDM2, or the caspase inhibitor p35 from baculovirus resulted in ~12–17% transfection efficiency. Interestingly, all three proteins, Daxx, MDM2, and p35, when coexpressed with p53, resulted in more transfected cells than when p53 was expressed alone (Fig. 7*A*). Thus, like MDM2 and p35, Daxx can protect cells from p53-mediated apoptosis. Essentially identical results were obtained when *Escherichia coli* β-galactosidase was used as a transfection marker and the cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (data not shown).

Among transfected cells, a large fraction of them exhibited condensed nuclei (*white arrow*, Fig. 7*C*) when only p53 was expressed. These cells were considered apoptotic but had not yet been cleared in the culture. When data from all transfections were tabulated, it became obvious that p53 expression resulted in elevated levels of apoptosis, and this was sup-

pressed when Daxx, MDM2, or p35 were coexpressed (Fig. 7*B*). Taken together, these data indicate that Daxx inhibits p53-dependent apoptosis.

The data shown above indicates that expression of Daxx alone can suppress p53-induced cell death in MEF DKO cells. To further explore this observation, we next examined whether p53-dependent apoptosis induced by stress, not by p53 overexpression, can also be suppressed by Daxx. We transfected HCT116 cells with expression plasmids for GFP-CRM1 alone or together with that for either Daxx or MDM2 or both. The transfected cell cultures were then treated with 5-FU, which is known to induce p53-dependent cell death in HCT116 cells (43). The apoptotic cells clearly exhibited condensed and/or fragmented nuclei. Transfected cells were revealed with the GFP-CRM1 marker. For transfected cells that underwent apoptosis, in addition to condensed chromatin, they also displayed an intense GFP signal along with rounded and shrunken morphology (indicated with *arrows* in Fig. 8*A*, *panels m–r* are enlarged images for better visualization of apoptotic cells). By contrast, live cells showed normal distribution of GFP-CRM1 that is characteristic of CRM1 including localization in the nuclear rim (Fig. 8*A*, *panels n* and *o*, also see Fig. 7). Five random fields with ~500 transfected cells in each transfection were quantitatively analyzed. As shown in Fig. 8*B*, ~20% of GFP-CRM1-transfected cells were apoptotic. Coexpression of either Daxx or MDM2 or both together suppressed 5-FU-induced apoptosis to ~7%. As expected, the p53 protein level was markedly elevated in response to 5-FU treatment, in comparison with similarly transfected but not treated cells (Fig. 8*C*). Thus, Daxx can effectively inhibit p53-dependent apoptosis induced by stress.

We have carried out further experiments to independently assess inhibition of 5-FU-induced cell death by Daxx in HCT116 cells. Daxx overexpression clones were generated by stable transfection of the Daxx-expression plasmid into HCT116 cells. Several independent clones expressing exogenous Daxx along with a clone transfected with vector were mock-treated (Me_2SO) or treated with 0.4 mM 5-FU and the cells were collected at different time points after treatment and processed for flow cytometry (FACS) analysis. As shown in Fig. 9*A*, the protein level of Daxx was higher in a clone called Daxx135 than that in the clone with vector transfection, and 5-FU markedly induced p53 protein levels in both vector clone and Daxx135 cells (Fig. 9*B*). Consistent with published results (43), HCT116 cells were very sensitive to 5-FU treatment; virtually all cells from the vector clone were killed 96 h after treatment. In striking contrast, a substantial portion of Daxx135 cells remained viable (Fig. 9*C*) after 5-FU treatment. The percentage of cells with sub-G_1 DNA content in Daxx135 was lower than that in the vector clone throughout the time course (Fig. 9, *C* and *D*). The cell cycle profiles of vector clone and Daxx135 were similar in Me_2SO-treated cells (Fig. 9*C*). Furthermore, two independent Daxx overexpression clones (Daxx145 and Daxx171) exhibited similar resistance to 5-FU-mediated apoptosis (data not shown). Thus, the observations must not be because of clonal effects. Therefore, Daxx confers marked resistance to 5-FU-induced and p53-dependent cell death in HCT116 cells, in agreement with results shown in Fig. 8.

DISCUSSION

Daxx exhibits complex biological functions ranging from modulating apoptosis to transcriptional regulation. Daxx was originally discovered as a proapoptotic protein that binds to the cytoplasmic domains of Fas receptor and transforming growth factor-β receptor type II by activating the Jun NH_2-terminal kinase pathway (11, 14). In cultured cells, Daxx localizes predominantly in the nuclei, in which it can induce apoptosis (12, 13). Our unpublished data[2] also indicates that Daxx could markedly induce apoptosis in several tumor cell lines. Thus, Daxx may participate in multiple apoptotic pathways. In contrast, deletion of the *Daxx* gene is lethal to mouse embryonic development and *Daxx*-deficient cells exhibited widespread apoptosis (16, 44). Consistent with these findings, ablation of Daxx by RNAi sensitizes cells to Fas ligand and stress-induced apoptosis (17). Therefore, Daxx may prevent cell death during animal development and in cultured cells. We described in this paper that Daxx binds to p53 *in vitro* (Fig. 1), and interestingly, MDM2 enhances Daxx-p53 interaction in cells (Figs. 2–5). We also showed that Daxx can repress p53 target genes (Fig. 6) and protects cells from p53-mediated apoptosis (Figs. 7–9). Therefore, our findings are consistent with a role for Daxx in cell survival. Specifically, Daxx can inhibit p53-regulated transcription and apoptosis and thus represents a new negative regulator of p53.

It is interesting that MDM2 appears to enhance the p53-Daxx interaction in the cell (Figs. 2 and 5). These two proteins also exhibit cooperation in repressing p53-mediated transactivation (Fig. 6). MDM2 is a well known negative regulator of p53 and it controls p53 activities at several levels. It binds to p53 TAD and inhibits p53-mediated transcription, probably by stereo hindrance so that the transcriptional machinery cannot bind to p53 efficiently. MDM2 can also specifically inhibit acetylation of p53 (39, 45, 46), which impacts negatively on p53 activities, because acetylation of p53 enhances its binding to the chromatin of its target promoters (42), and may also inhibit ubiquitination of p53, thereby protecting it from degradation (33). Perhaps the most extensively studied aspect of regulation of p53 by MDM2 is its ubiquination of p53 (47). MDM2 acts as a p53 E3 ubiquitin ligase, thereby promoting ubiquitin-dependent proteolysis of p53. Although it has been well established that MDM2 mediates ubiquitination of p53, other factors that affect MDM2-mediated p53 ubiquitination remain to be identified. Our results hint at an intriguing possibility that Daxx might also play a role in MDM2-mediated ubiquitination of p53. Data presented in Fig. 5 indicates that the p53 species associated with Daxx were extensively ubiquitinated. Nonetheless, it is also possible that ubiquitinated p53 binds to Daxx more efficiently. Further studies are required to investigate these possibilities.

Apart from a potential role in p53 ubiquitination, Daxx might also affect p53 activities in other ways. Daxx is a corepressor and binds to HDACs (19, 22). Daxx also binds to corepressors DNMT1 (DNA methyltransferase I) (16) and mSin3a.[2] Thus, Daxx could conceivably recruit various corepressors to p53 target promoters and repress the expression of p53 target genes. Noticeably, in the absence of MDM2, Daxx binds to p53 poorly as assessed in IP experiments, but their interaction remains detectable in DKO MEF upon longer exposure of the blot (Fig. 5). Thus, MDM2 enhances but is not absolutely required for Daxx to bind to p53. Consistent with this, Daxx could repress p53-mediated transcription in the absence of MDM2 (Fig. 6). Additionally, Daxx can potently inhibit p53-induced cell death in the absence of MDM2 in DKO MEF (Fig. 7). Similarly, overexpression of Daxx alone can protect HCT116 cells from 5-FU-induced cell death (Figs. 8 and 9), although IP failed to recover Daxx-p53 interaction in the absence of MDM2 overexpression (Fig. 2). There are several scenarios that may explain our observations. First, inhibition of p53-induced apoptosis does not require direct p53-Daxx interaction. For example, Daxx might repress proapoptotic p53 genes such as *PUMA*, *p53AIP1*, and *Bax* and this repression is mediated through direct interaction of Daxx with proteins that in turn contact p53. Second, Daxx might also inhibit the ability of p53 to directly trigger apoptosis from mitochondria without a direct

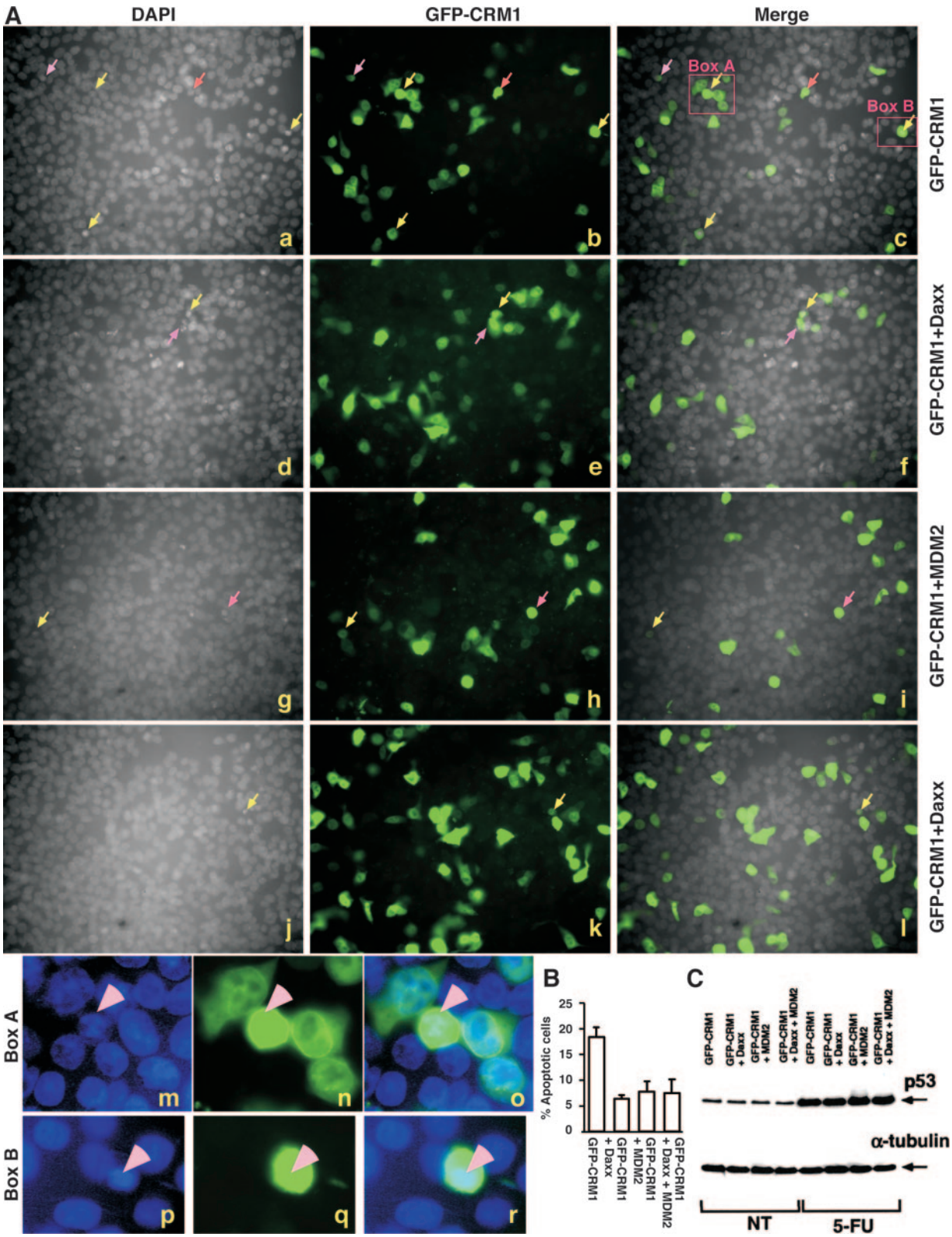

FIG. 8. **Daxx suppresses 5-FU-induced apoptosis in HCT116 cells.** *A*, the cells were grown on glass coverslips and transfected with the indicated expression plasmids and 12 h after transfection, 5-FU was added to a final concentration of 0.4 mM. Twenty-four hours after transfection, cells were fixed with paraformaldehyde and mounted on VectorShields and examined under a ×20 objective. Cells with condensed and/or fragmented nuclei are considered apoptotic. In the meantime, the GFP-CRM1 signals became intense with rounded and shrunken morphology. Representative apoptotic cells are denoted with *arrows*. Shown is one complete field from each transfection. *Panels m–r* are enlarged images of selected areas (*Box A* and *B*) of images shown in the top row (*panels a–c*) for better visualization of the features of apoptotic cells. *B*, quantification of apoptosis in transfected cells. About five microscopic fields were analyzed in each transfection. The percentage of apoptotic cells out of the total transfected cells is plotted. *C*, induction of p53 by 5-FU in HCT116 cells. Cells were grown in 6-well plates, transfected, and treated as in *A*. The cells were harvested and whole cell extracts were subjected to Western blot with anti-p53 antibody (DO-1). For comparison, cells similarly transfected but not treated with 5-FU (NT) were included in the blot. The same membrane was stripped and reprobed with antibody to α-tubulin as loading control.

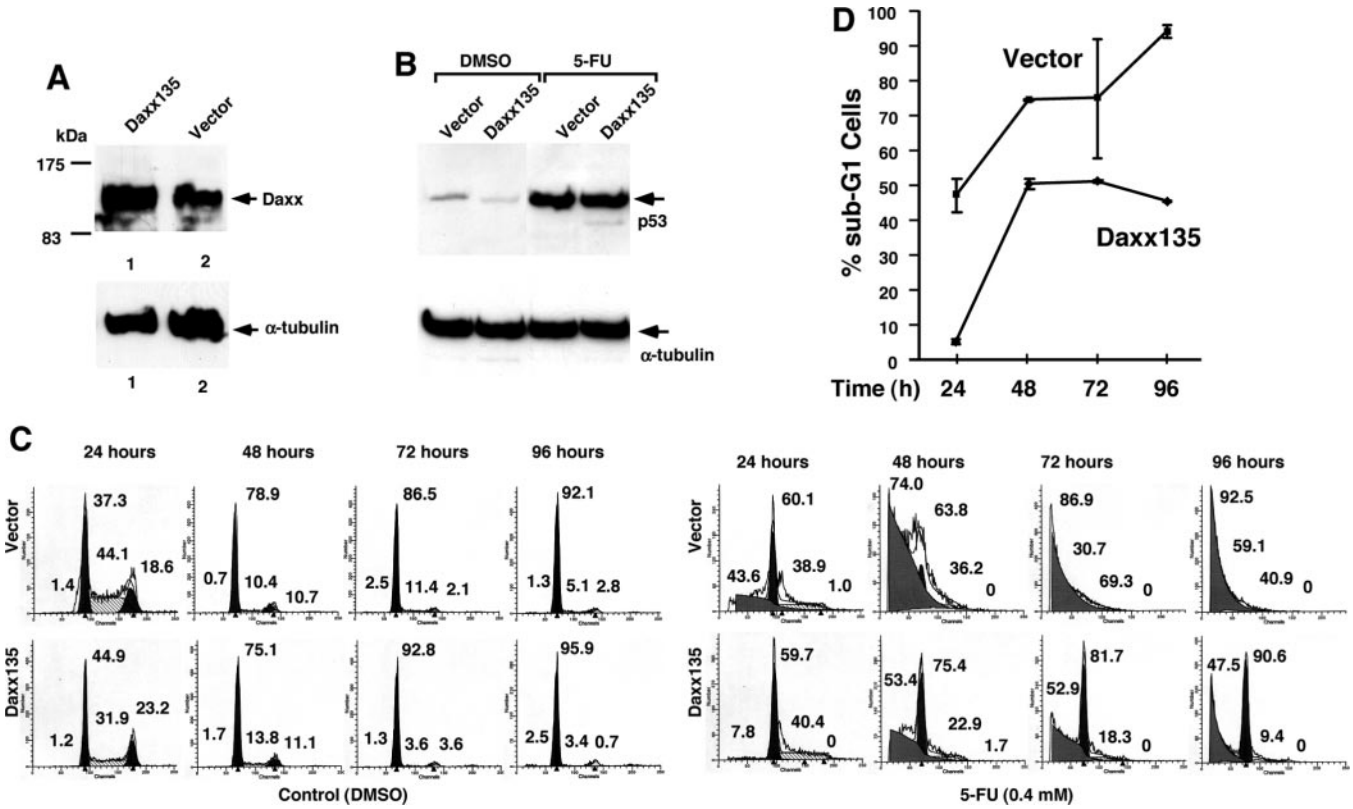

FIG. 9. **Resistance to 5-FU-mediated apoptosis in HCT116 cells with stable overexpression of Daxx.** *A*, Daxx protein levels in vector-transfected and Daxx135 cells. Daxx135 is a clone of HCT116 with stable transfection of the FLAG-Daxx expression vector, whereas *Vector* is a stable clone of the expression vector-transfected HCT116 cells. Daxx was detected with M112 in Western blot (*WB*) and equal loading was assessed with Western blot using antibodies to α-tubulin. *B*, induction of p53 by 5-FU in vector and Daxx135 cells. The cells were treated with Me_2SO (control) or 5-FU (0.4 mM) for 24 h and whole cell extracts were analyzed for p53 and α-tubulin protein levels. *C*, FACS analysis of vector and Daxx135 cells. The cells were treated with Me_2SO (*control*) or 5-FU (0.4 mM) and analyzed by flow cytometry and the cell cycle profiles of both vector and Daxx135 cells at different time points were determined by propidium iodide staining and FACS analysis. The four numbers in each cell cycle graph are percentage of cells in sub-G_1, G_0/G_1, S, or G_2/M stages, respectively. *D*, Daxx135 is resistant to 5-FU-mediated killing. The percentage of cells with sub-G_1 DNA content of 5-FU-treated cells in both vector and Daxx135 was plotted. The results are the average of two independent experiments and variations are indicated with *error bars* (1 S.D.).

p53-Daxx interaction. As shown in Fig. 5, Daxx and p53 codistribute in both the cytoplasm and nucleus. Although purely speculative, cytoplasmic Daxx might be able to suppress the ability of p53 to activate the mitochondrial mechanism of apoptosis. Finally, Daxx-p53 interaction might still occur in the absence of MDM2 (see Fig. 5). This interaction may be transient in nature and sensitive to posttranslational modifications of the COOH-terminal domain of p53 so that it is not readily detected in IP. Alternatively, Daxx and p53 might be a part of the large complexes and both p53 and Daxx might be inaccessible for anti-p53 and Daxx antibodies, so that IP is ineffective for detecting Daxx-p53 interaction. In accordance with this latter possibility, we have observed striking co-localization of Daxx and p53 in 293 cells (8).

Intriguingly, our results show that posttranslational modifications including acetylation and phosphorylation of p53 in the COOH-terminal regulatory domain can inhibit the association of Daxx with p53 (Figs. 1 and 2). On a technical note, the yeast two-hybrid data presented in this paper document the general utility of using yeast for studying the influence of p53 posttranslational modifications on its binding to other human proteins. Yeast has neither Daxx nor p53 as well as many modifying enzymes of these two proteins. Thus, although one cannot rule out that some human proteins may be nonspecifically modified in yeast, our data showed that Daxx-p53 interaction could be robustly scored in yeast. Significantly, only specific mutations of p53 affected Daxx-p53 interaction in yeast. In contrast, Daxx does not bind to p53 isolated from cultured cells in IP experiments (data not shown, also see discussion above). Importantly, expression of MDM2 restores Daxx-p53 interaction, and this apparently resulted from deacetylation of p53 upon MDM2 expression (Fig. 2). It is well known that various types of stress activate p53 through inducing phosphorylation of p53 by a number of kinases and acetylation of p53 by acetylases p300 and PCAF (33). Phosphorylation at the TAD of p53 has been implicated in dissociating MDM2 from p53 (33). We show here that both phosphorylation and acetylation at the COOH-terminal regulatory domain might disrupt the complex of p53 with Daxx, another negative regulator of p53. Our data thus further underscores the importance of posttranslational modifications of p53 in regulating p53-mediated stress responses.

Our results have some other important implications. It has been known for a long time that the COOH terminus of p53 inhibits its transactivation functions. Thus removal of the COOH terminus or binding of antibody to it can dramatically enhance the transactivation potential of p53 (31, 48). Inhibitory effects of the COOH terminus could be neutralized by its phosphorylation, acetylation, or both (31). In addition, expression of the p53 mutant containing a COOH-terminal fragment results in p53 activation (49). Recently, it was proposed that a transacting factor mediates the inhibitory effects of the p53 COOH terminus (50). Because Daxx binds to the COOH-terminal domain of p53 and both acetylation and phosphorylation can disrupt this interaction, Daxx may be an important factor that mediates the inhibitory effects by tethering corepressors to the p53 COOH-terminal domain. Thus, our findings provide

some insights into these important early observations. Finally, in light of the embryonic lethality phenotype of Daxx-deficient mice embryos (16), it is tempting to suggest that like MDM2 and related MDMX (51), Daxx might also facilitate embryonic development by negating p53-mediated effects. MDM2-deficient mice die very early before day E6.5, whereas Daxx and MDMX-deficient mice die at a similar stage during development (7, 16, 51). Thus, these proteins may control p53 activity in complementary, fail-safe mechanisms.

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REFERENCES

1. Vogelstein, B., Lane, D., and Levine, A. J. (2000) *Nature* **408,** 307–310
2. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992) *Nature* **356,** 215–221
3. Vousden, K. H. (2002) *Biochim. Biophys. Acta* **1602,** 47–59
4. Prives, C., and Hall, P. A. (1999) *J. Pathol.* **187,** 112–126
5. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) *Mol. Cell* **11,** 577–590
6. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) *Nature* **378,** 206–208
7. Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995) *Nature* **378,** 203–206
8. Zhao, L. Y., Colosimo, A. L., Liu, Y., Wan, Y., and Liao, D. (2003) *J. Virol.* **77,** 11809–11821
9. Kim, E. J., Park, J. S., and Um, S. J. (2003) *Nucleic Acids Res.* **31,** 5356–5367
10. Ohiro, Y., Usheva, A., Kobayashi, S., Duffy, S. L., Nantz, R., Gius, D., and Horikoshi, N. (2003) *Mol. Cell. Biol.* **23,** 322–334
11. Yang, X., Khosravi-Far, R., Chang, H. Y., and Baltimore, D. (1997) *Cell* **89,** 1067–1076
12. Zhong, S., Salomoni, P., Ronchetti, S., Guo, A., Ruggero, D., and Pandolfi, P. P. (2000) *J. Exp. Med.* **191,** 631–640
13. Torii, S., Egan, D. A., Evans, R. A., and Reed, J. C. (1999) *EMBO J.* **18,** 6037–6049
14. Perlman, R., Schiemann, W. P., Brooks, M. W., Lodish, H. F., and Weinberg, R. A. (2001) *Nat. Cell Biol.* **3,** 708–714
15. Gongora, R., Stephan, R. P., Zhang, Z., and Cooper, M. D. (2001) *Immunity* **14,** 727–737
16. Michaelson, J. S., Bader, D., Kuo, F., Kozak, C., and Leder, P. (1999) *Genes Dev.* **13,** 1918–1923
17. Chen, L. Y., and Chen, J. D. (2003) *Mol. Cell. Biol.* **23,** 7108–7121
18. Hollenbach, A. D., Sublett, J. E., McPherson, C. J., and Grosveld, G. (1999) *EMBO J.* **18,** 3702–3711
19. Li, H., Leo, C., Zhu, J., Wu, X., O'Neil, J., Park, E. J., and Chen, J. D. (2000) *Mol. Cell. Biol.* **20,** 1784–1796
20. Li, R., Pei, H., Watson, D. K., and Papas, T. S. (2000) *Oncogene* **19,** 745–753
21. Emelyanov, A. V., Kovac, C. R., Sepulveda, M. A., and Birshtein, B. K. (2002) *J. Biol. Chem.* **77,** 11156–11164
22. Hollenbach, A. D., McPherson, C. J., Mientjes, E. J., Iyengar, R., and Grosveld, G. (2002) *J. Cell Sci.* **115,** 3319–3330
23. Xue, Y., Gibbons, R., Yan, Z., Yang, D., McDowell, T. L., Sechi, S., Qin, J., Zhou, S., Higgs, D., and Wang, W. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10635–10640
24. Tang, J., Wu, S., Liu, H., Stratt, R., Barak, O. G., Shiekhattar, R., Picketts, D. J., and Yang, X. (2004) *J. Biol. Chem.* **279,** 20369–20377
25. Liu, Y., Colosimo, A. L., Yang, X. J., and Liao, D. (2000) *Mol. Cell. Biol.* **20,** 5540–5553
26. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) *Science* **282,** 1497–1501
27. Zhao, L. Y., and Liao, D. (2003) *J. Virol.* **77,** 13171–13181
28. Zhao, L. Y., Liu, Y., Bertos, N. R., Yang, X. J., and Liao, D. (2003) *Oncogene* **22,** 8316–8329
29. Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. (2000) *Cell* **102,** 849–862
30. Jiang, C., and Liao, D. (1999) *Genomics* **62,** 508–518
31. Ahn, J., and Prives, C. (2001) *Nat. Struct. Biol.* **8,** 730–732
32. Appella, E., and Anderson, C. W. (2001) *Eur. J. Biochem.* **268,** 2764–2772
33. Brooks, C. L., and Gu, W. (2003) *Curr. Opin. Cell Biol.* **15,** 164–171
34. Gu, W., and Roeder, R. G. (1997) *Cell* **90,** 595–606
35. Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. (1999) *Mol. Cell. Biol.* **19,** 1202–1209
36. Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998) *Genes Dev.* **12,** 2831–2841
37. Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000) *Nature* **408,** 377–381
38. Jin, Y., Zeng, S. X., Dai, M. S., Yang, X. J., and Lu, H. (2002) *J. Biol. Chem.*
39. Ito, A., Kawaguchi, Y., Lai, C. H., Kovacs, J. J., Higashimoto, Y., Appella, E., and Yao, T. P. (2002) *EMBO J.* **21,** 6236–6245
40. Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994) *Genes Dev.* **8,** 1235–1246
41. Dornan, D., Shimizu, H., Perkins, N. D., and Hupp, T. R. (2003) *J. Biol. Chem.* **278,** 13431–13441
42. Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R. G., and Gu, W. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 2259–2264
43. Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999) *J. Clin. Investig.* **104,** 263–269
44. Michaelson, J. S., and Leder, P. (2003) *J. Cell Sci.* **116,** 345–352
45. Kobet, E., Zeng, X., Zhu, Y., Keller, D., and Lu, H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12547–12552
46. Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001) *EMBO J.* **20,** 1331–1340
47. Michael, D., and Oren, M. (2002) *Curr. Opin. Genet. Dev.* **12,** 53–59
48. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71,** 875–886
49. Tyner, S. D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C., Hee Park, S., Thompson, T., Karsenty, G., Bradley, A., and Donehower, L. A. (2002) *Nature* **415,** 45–53
50. Wiederschain, D., Gu, J., and Yuan, Z. M. (2001) *J. Biol. Chem.* **276,** 27999–28005
51. Parant, J., Chavez-Reyes, A., Little, N. A., Yan, W., Reinke, V., Jochemsen, A. G., and Lozano, G. (2001) *Nat. Genet.* **29,** 92–95

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