JBC Papers in Press. Published on August 31, 2004 as Manuscript M310801200

The transcriptional repressor hDaxx potentiates p53-dependent apoptosis

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Running title: Role of hDaxx in p53-dependent apoptosis

SUMMARY

p53 and its homologues, p73 and p63, are transcription factors that play an essential role in modulating cell cycle arrest and cell death in response to several environmental stresses. The type and intensity of these responses, which can be different depending on the inducing stimulus and on the overall cellular context, is believed to rely on the activation of defined subsets of target genes. The proper activation of p53 family members requires the coordinate action of post-translational modifications and interaction with several cofactors. Here we demonstrate that the multifunctional protein hDaxx interacts with p53 and its homologues, both *in vitro* and *in vivo*, and modulates their transcriptional activity. Moreover we show that hDaxx, which has been implicated in several apoptotic pathways, increases the sensitivity to DNA damage-induced cell death and that this effect requires the presence of p53. hDaxx, while repressing p53-dependent transcription of the p21 gene, does not affect the activation of pro-apoptotic genes, and therefore acts by influencing the balance between cell cycle arrest and pro-apoptotic p53 targets. Our results therefore underline the central role of hDaxx in modulating the apoptotic threshold upon several stimuli, and identify it as a possible integrating factor that coordinates the response of p53 family members.

INTRODUCTION

The p53 protein is one of the most important cellular tumor suppressors, lying at the heart of many different though interconnected stress response pathways, whose action is required to prevent genomic instability (1). Once activated, p53 coordinates a complex cellular response ending in either reversible cell-cycle arrest, irreversible senescent-like state or apoptosis (2).

Multiple mechanisms are responsible for controlling p53 activation within cells, and most of them involve post-translational modifications of the protein, such as phosphorylation, acetylation and sumoylation (2,3). Stress-induced post-translational modifications may affect the stability and the conformation of p53 (4), its ability to interact with positive and negative regulators and its subcellular localization (3).

Much less understood are the mechanisms that control the specificity of p53 response and that allow, depending on the stress and cell type, to discriminate between growth arrest and cell death. p53 exerts its functions mostly as a transcription factor and, while induction of cell cycle arrest is mainly mediated by the CDK inhibitor p21, a number of effectors are required to coordinate the apoptotic response (2). Several experimental evidences indicate that promoter specificity may be determined by the sequence of the p53 binding site on DNA, for which the protein can have different affinities (5), and also by the interaction of p53 with co-factors responsible for directing it towards specific gene subsets (6,7).

p53 belongs to a family of proteins comprising two additional members, p63 and p73, which share extensive structural and functional homologies (7). p63 and p73, despite being able to bind to p53 consensus sites and to activate transcription of several p53-responsive genes, do not behave as classical tumour suppressors and have been implicated in control of differentiation and developmental pathways (8,9). The mechanisms governing the activation of p53 homologues as well as their functional specificity are presently still poorly understood.

To gain insights on the mechanism of regulation of p53 family members, we sought to analyse their interaction profile and to isolate proteins able to bind all three proteins or specifically to only

one of them. Among the common interactors for all p53 family members we identified hDaxx, a highly conserved protein, initially isolated in mouse as a Fas interactor (10). Daxx contains in its primary sequence two nuclear localization signals (NLS) that are conserved between murine and human proteins. Accordingly, it is predominantly nuclear and has been shown to associate with PML and to localize within PML Nuclear Bodies (NBs) (11-13).

The ability of hDaxx to interact with multiple cellular factors has resulted in its assignment to several putative functions (14). Many reports have implicated Daxx in apoptosis, but whether it functions as a pro- or anti-apoptotic molecule has not yet been clarified. Despite the lack of evidences of interaction between Daxx and Fas in human cells, hDaxx overexpression is able to potentiate Fas-induced apoptosis and a direct role of hDaxx in activation of the Apoptosis Signal-regulating Kinase 1 (ASK1)/ Jun N-terminal kinase (JNK) pathway has been demonstrated, at least in some cell types and experimental conditions (10,15-17). Recently, tumorigenic mutant p53 has been shown to interact with hDaxx and to counteract its ability to activate the JNK pathway and to induce cell death (18). Moreover, hDaxx has been involved also in TGFß-induced (19) as well as in nuclear, PML-dependent apoptotic pathways (13,20). Depletion of hDaxx by antisense RNA showed a protective effect toward TGFß-induced apoptosis (19).

In contrast with these observations, Daxx knock-out embryos showed early embryonic lethality and studies with Daxx-null murine embryonic stem cells revealed an anti-apoptotic role of this protein (21). Recently it has been shown that ablation of Daxx expression by RNA interference can increase apoptosis in different cell types and that this effect is rescued by Bcl-2 overexpression (22,23).

The pro-apoptotic function of Daxx within the nucleus has been linked with its ability to function as a transcriptional repressor, possibly due to interaction with histone deacetylases and core histones (12,24). Daxx can inhibit basal transcription when fused to a Gal4-DNA binding domain, but in more physiological conditions this effect is not generalized and has been

observed only for some promoters, where hDaxx can be recruited by binding to specific transcription factors, such as Pax3 and ETS1 (25,26). In this respect, it is well conceivable that the dual role played by Daxx in apoptosis may be at least in part explained by subtle differences in the transcriptional status of a given cell.

In this report we demonstrate that hDaxx interacts with wild type (wt) p53 and its homologues p73 and p63 both *in vitro* and *in vivo*. Binding to hDaxx causes repression of p53 family members transcriptional activity toward the p21 promoter. In the case of p53 the effect of hDaxx appears to be promoter-dependent, repressing preferentially genes involved in cell-cycle arrest rather than pro-apoptotic genes. Accordingly, hDaxx overexpression sensitizes cells to drug-induced apoptosis, while RNAi knock-down of hDaxx is protective toward p53-dependent cell death.

EXPERIMENTAL PROCEDURES

***Plasmids*.** pLexA-p53wt and deletions, pcDNA$_3$p53wt and pGEX-p53wt has been previously described (27). To generate pLexA-p73α the human p73α cDNA was PCR-amplified from amino acid 112 to the STOP codon. pLexA-p63α contains the human p63α cDNA from amino acid 132 to the STOP codon and was a kind gift of S. Piccolo. pcDNA$_3$-HAp73α and pcDNA$_3$-mycp63α have been kindly provided by G. Melino and F. McKeon respectively. pGEX-p73α and pGEX-p63α contains the whole human p73α and p63α ORF fused to the GST coding sequence and has been provided by G. Blandino. cDNA encoding hDaxx was obtained from I.M.A.G.E. consortium (RZPD, Deutschen HumanGenomeProjekt) and subcloned into pcDNA$_3$ and into the retroviral vector pLPC. To generate the HA-hDaxx-pIND construct for inducible expression of HA-tagged hDaxx, the hDaxx cDNA was first introduced into pcDNA$_3$-HA and then subcloned into pIND vector (Invitrogen). p53-responsive reporter constructs have been described previously and has been provided by M. Oren, B. Vogelstein, M. Levrero and X. Lu. Constructs encoding hDaxx deletions fused to GST have been kindly provided by H. Will. pcDNA$_3$-HATNV contains the *E. coli* Thioredoxin (Trx) cDNA cloned upstream of an HA tag and before a NLS and VSV tag. Oligonucleotides encoding for p53 peptides were cloned within the RsrII site of the Trx cDNA.

Cell lines. All cell lines were cultured at 37˚C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100mg/ml). U2OS and SaOS-2 are human osteosarcoma cells, respectively wt and null for p53. 293 is a human embryonic kidney cell line.

hDaxx-inducible cell line. To generate the ecdysone-inducible hDaxx cell line, U2OS cells were co-transfected with pVgRXR (Invitrogen), expressing the two subunits of the Drosophila ecdysone receptor and with pIND-hDaxx, expressing HA-tagged hDaxx under the control of the ecdysone-inducible promoter. Following selection of cells with Zeocin (150 μg/ml) and G418

(150 μg/ml) for 2 weeks, clones were isolated and analysed for HA-hDaxx expression by western blot. Selected positive clones were firther expanded and processed.

Antibodies, western blot and immunofluorescence analysis. The following primary antibodies were used: rabbit polyclonal anti-LexA (Invitrogen); 12CA5, mouse monoclonal anti-HA (Roche Molecular Biochemicals); M-112, rabbit polyclonal anti-Daxx (SantaCruz Biotechnology); FL-393, rabbit polyclonal anti-p53 (SantaCruz Biotechnology); DO-1, mouse monoclonal anti-p53 (SantaCruz Biotechnology); rabbit polyclonal anti-phosphorylated Ser15 (pSer15) in p53 (Cell Signaling Biotechnology); rabbit polyclonal anti-actin (Sigma); rabbit polyclonal anti-p85 PARP (Promega); 9E10, mouse monoclonal anti-myc; 2A9, 2A10 and Smp14 mouse monoclonals anti-Mdm2 (kindly provided by M. Oren); rabbit polyclonal anti-PIG3 (Oncogene Science); C19, rabbit polyclonal anti-p21 (SantaCruz Biotechnology); N20, rabbit polyclonal anti-Bax (SantaCruz Biotechnology). For detection of endogenous Daxx protein in RNA interference experiments we used an affinity-purified rabbit anti-Daxx polyclonal antibody, kindly provided by J. D. Chen (13).

Western blot was performed with standard procedures, using horse-radish peroxidase-conjugated secondary antibodies (Sigma). Bound antibodies were visualised by enhanced chemiluminescence (Pierce).

For immunofluorescence analysis cells seeded on coverslips were fixed with 3% paraformaldehyde, permeabilized by treatment with 0.1% Triton X-100 in PBS and then incubated for 1 hour at 37°C with the indicated primary antibodies. Primary antibodies were revealed by a 30 min incubation goat anti-mouse TRITC- and anti-rabbit FITC-conjugated secondary antibodies (Sigma). Images were obtained with a Leica DMLB microscope and Photometrics Coolsnap camera.

Yeast two-hybrid screening. The yeast two-hybrid screening with LexA-p53wt and LexA-p73α strains was performed as previously described (27) using a human foetal brain cDNA library

cloned into the galactose-inducible expression vector pJG4-5. At least 2 million primary clones were analysed in each screening. Positive interaction between bait and fish protein resulted in the transcription of two different reporters, lacZ and Leu2 and was evaluated by blue staining on X-Gal containing plates and growth on medium lacking leucine. For western blot analysis, cells grown in medium containing either glucose or galactose and raffinose were subjected to mechanical lysis with glass beads in SDS-containing sample buffer.

In vitro binding and immunoprecipitation assays. In all cases, cells were seeded in 10 cm plates, transfected with the indicated vectors by standard calcium phosphate method and further processed 36 hours later. GST pull-down assays cells were performed by lysing cells in 300mM NaCl-containing buffer (300mM NaCl, 50mM Tris pH7.5, 0.5% NP40, 10%glycerol) supplemented with protease inhibitors (PMSF1mM and chymostatin, leupeptin, antipain, pepstatin 10µg/ml each). Lysates were then diluted 1:2 in the same buffer without NaCl and incubated for 2 hours at 4°C with 4 µg of Sepharose-GSH-bound GST proteins. For co-immunoprecipitation analysis, cells were lysed in 150mM NaCl-containing buffer (150mM NaCl, 50mM Hepes pH 7.5, 0.1% Tween20, 10% glycerol) and incubated for 1.5 hours at 4°C with 1 µg of anti-p53 (FL-393) or anti-Daxx (M-112) polyclonal antibodies. 20 µl of Protein A-Sepharose CL-4B (Amersham Biosciences) were then added to each sample and incubation at 4°C was carried on for additional 1.5 hours. After three washes in lysis buffer, immunoprecipitated proteins were separated by SDS-PAGE and analysed by western blot with the indicated antibodies.

Retroviral infection. Phoenix packaging cells were transfected with empty pLPC or with pLPC-hDaxx by standard calcium phosphate method. After 48 hours incubation at 32°C, the supernatants containing viral particles were collected, diluted 1:2 with fresh medium and used to infect sub-confluent U2OS or SaOS-2 cells. After overnight incubation at 32°C, cells were split and kept under selection with 1µg/ml puromycin for 1 week. The polyclonal populations of infected cells were then analysed for hDaxx expression, expanded and further processed. Four

U2OS and three SaOS-2 independent polyclonal pLPC and pLPC-hDaxx lines were generated and analysed.

Reporter assays. SaOS-2 or U2OS cells were seeded in 24-wells plates and transfected with Lipofectamine2000 (Invitrogen) with 200ng of Luc reporters, 20ng of p53, p73α or p63α expression plasmids (only in the case of SaOS-2) and 400ng of $pcDNA_3$-hDaxx or empty $pcDNA_3$. In all the samples, 20ng of the reporter pRL-CMV (Promega) was included for normalization of the transfection efficiency. 24 hours after transfections, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega). For reporter assays upon silencing of Daxx expression in SaOS-2 cells, 1 picomol of either Daxx-specific siRNA or control oligonucleotides were included (see below).

Apoptosis assays and RNA interference. U2OS and SaOS-2 stable pLPC and pLPC-hDaxx lines were seeded in 6 cm plates and 24 hours later cisplatin (cis-diamminedichloroplatinum II, CDDP, prepared in PBS at the concentration of 500 µg/ml) was added to the culture medium at the final concentration of 2.5 µg/ml. After 48 or 72 hours, cells were collected by trypsinization, recovering also the supernatants, washed in PBS and fixed with 70% ethanol at –20°C. After several washes in PBS, cells were resuspended in 50 µl of PBS/0.1%NP40 containing 2µg/ml RNaseA and five minutes later 200 µl of 50µg/ml propidium iodide in PBS were added. Cells were analysed by cytofluorimeter (Biorad Bryte HS) after additional 20 min incubation. Apoptosis was evaluated by scoring the percentage of cells with sub-G1 DNA content.

For RNA interference of p53, cells seeded in 6 cm plates were transfected with Oligofectamine (Invitrogen) according to the instructions of the manufacturer, with 6 picomoles of dsRNA oligonucleotides specific for human p53 (GACUCCAGUGGUAAUCUACdTdT) or with control scrambled oligonucleotides (CCUUUUUUUUUGGGGAAAAdTdT). Cells were further processed 24 hours after transfection. For apoptosis experiments upon silencing of hDaxx, SaOS-2 cells seeded on glass coverslips in 2 cm dishes were transfected by Lipofectamine 2000 (Invitrogen) with 2 picomoles of hDaxx-specific ds RNA oligonucleotides

(GGAGUUGGACCUGUCAGAGCdTdT) or with control scrambled oligonucleotides, together with either 1 μg of p53 expression vector or with 1 μg of pEGFP. 24 hours after transfection cells were fixed with 3% PFA and stained for the expression of p53 (DO-1 monoclonal antibody, Santa Cruz). DNA was stained with Hoechst 33342 and, upon observation through UV-light microscope, nuclei exhibiting condensed chromatin were scored as apoptotic. At least 200 cells that were p53-positive (or GFP positive in control experiments) for each of 4 independent experiments were counted in randomly selected fields from each plate.

RESULTS

hDaxx interacts with p53 family members

With the aim to gain information about the interaction profile of p53 and its homologues p73 and p63, we performed a series of yeast two-hybrid screenings to identify proteins able to bind to each family member. These three proteins share a similar protein domain organization (see Figure 1A), with the highest homology in the DNA binding and oligomerization domains. In contrast to p53, however, p73 and p63 are expressed as a series of alternative splice variants, differing mostly at their C terminus, with the longest isoform (alpha) containing the SAM protein interaction motif (8).

Yeast strains expressing transactivation domain-deleted human p53 and p73α fused to the LexA DNA binding domain were transformed with a foetal brain cDNA library and screenings were performed as previously described (27). Putative p53 and p73 interactors were isolated and sequenced. The encoding cDNAs were subsequently assayed for interaction with LexA-p63α. One of the most representative clones isolated in both screenings (accounting for more than 25% of the p53-interacting and 40% of the p73-interacting clones) contained cDNAs of different lengths, all encoding the C-terminal region of hDaxx. As schematically represented in Figure 1A, hDaxx showed a strong and specific interaction with wt p53 and p73α, as well as with p63α.

Since the binding between hDaxx and p53 family members in yeast was strong and specific, we first sought to confirm the interaction by performing a glutathione-S-transferase (GST) *in vitro* binding assay. Lysates prepared from p53-null SaOS-2 cells transfected with vector expressing hDaxx were subjected to pull-down assay with beads loaded with GST-p53, GST-p73α, GST-p63α or with GST alone as negative control. Subsequent western blot analysis of the resin-

bound proteins (Figure 1B) revealed that hDaxx interacted specifically with GST-fused p53 family members.

To verify the binding in human cells, we next performed a co-immunoprecipitation assay on lysates from 293 cells transfected with hDaxx expression vector, using anti-p53 polyclonal antibody or normal rabbit serum (NRS) as a control. As shown in Figure 1C, hDaxx was clearly detectable in the p53-bound fraction, while no specific signal was observed in the negative control.

Similarly, we immunoprecipitated with anti-Daxx antibody lysates of 293 cells transfected with vector expressing HA-tagged p73α together with hDaxx expression plasmid or with empty plasmid as a control. The immunoprecipitated proteins were then analysed by western blot with anti-HA antibody (Figure 1D), demonstrating also in this case a specific interaction between hDaxx and p73.

These results therefore demonstrate that hDaxx is able to bind to all p53 family members.

hDaxx acts as a repressor of the transcriptional activity of p53 family members.

Since hDaxx has been shown to interact with several transcription factors and to modulate their activity, we sought to verify whether this binding could affect the transcriptional activity of p53 family members. To this aim, we performed reporter assays with a construct containing the p53-responsive p21 promoter cloned upstream of the firefly luciferase gene (p21-Luc). p53-null SaOS-2 cells were transfected with p21-Luc and either p53, p73α or p63α expression vectors, together with hDaxx expression vector or empty plasmid as a control. In each experiment, transfection efficiency was monitored by co-expressing a fixed amount of a second reporter construct containing the CMV promoter upstream of the *Renilla reniformis* luciferase gene (pRL-CMV). As shown in Figure 2, hDaxx overexpression was able to reduce the transcriptional activity of all p53 family members, even though the effect was more pronounced in the case of

p53. Notably, hDaxx overexpression did not affect basal p21 transcription in the absence of overexpressed p53, p73 or p63, demonstrating the specificity of the effect observed. Western blot analysis on the same lysates used for reporter assays confirmed similar levels of expression of p53, p73α and p63α, either in the absence or presence of overexpressed hDaxx (Figure 2, lower panels).

These results suggest that binding with hDaxx may influence the ability of all p53 family members to activate transcription, as previously reported for other transcription factors, with no major consequences on their expression levels and/or stability.

Distinct domains of hDaxx are responsible for the binding to p53

We decided to further characterize the interaction between hDaxx and the founder member of the family, p53. hDaxx is a protein of 740 amino acids and the shortest cDNA clone isolated form the yeast two-hybrid screening encodes its C-terminal portion, from amino acid 605 to 740. The same region has also been shown to be involved in the interaction with several other factors, such as Fas (10) and PML (11).

To verify that the C terminus of hDaxx mediates the interaction with p53 family members also in other experimental systems, we took advantage of different constructs bearing hDaxx deletions (represented in Figure 3A) fused to GST, corresponding to amino acids 1-188 (GST-hDaxx A), 189-400 (GST-hDaxx B), 410-600 (GST-hDaxx C) and 600-740 (GST-hDaxx D). *In vitro* pull-down assays performed with lysates of wt p53-containing 293 cells (Figure 3A, lower panels) clearly indicated that the C-terminal 140 amino acids of hDaxx are sufficient to mediate the interaction with p53 (Western blot lane 6). Interestingly however, binding was also detected with GST-hDaxx A and, to a lesser extent, with B (lanes 3 and 4), indicating that N-terminal regions can also interact with p53. Similar results were obtained using *in vitro* translated p53 and p73 (not shown), suggesting that multiple domains of hDaxx may be responsible for contacting p53 and its homologue p73.

The oligomerization domain of p53 mediates the interaction with hDaxx

To identify the region of p53 responsible for the binding, we first tested by yeast two hybrid assay the C-terminal part of hDaxx isolated from the screening and several p53 deletion mutants. As summarized in Figure 3B, deletion of p53 C terminus to amino acid 355 did not affect the binding, while removal of 17 more residues almost abolished the interaction. This indicates that amino acids between 338 and 355 of wild-type p53, within the oligomerization domain, are required for binding to hDaxx. Accordingly, a construct encoding only the C-terminal region of p53 (amino acids 294-393) showed strong and specific interaction with hDaxx.

To confirm these findings in mammalian cells, we used constructs encoding different peptides derived from p53 C-terminal region, inserted within the scaffold of the thioredoxin (Trx) protein. These plasmids contain a cassette where the Trx cDNA is fused with an N-terminal HA tag and with C-terminal NLS and VSV tag (HA-TNV, schematically represented in Figure 3C). SaOS-2 cells were transfected with hDaxx expression vector together with a plasmid expressing the empty HA-TNV cassette or p53 peptides corresponding to regions 322-355, 355-363 and 363-384. Lysates were then immunoprecipitated with anti-Daxx polyclonal antibody and analysed by western blot with anti-HA antibody. As presented in Figure 3C, the peptide corresponding to p53 oligomerization domain (amino acid 322-355) showed a specific interaction with hDaxx, while no binding was detectable with any of the other peptides or with the empty HA-TNV cassette.

hDaxx sensitizes cells to p53-dependent apoptosis

The above results, together with several evidences indicating a complex role for hDaxx in regulation of cell death, prompted us to test whether it could be also involved in modulating p53-dependent apoptosis. To this aim we generated cell lines stably expressing hDaxx in the background of wt p53-containing U2OS cells or in p53-null SaOS-2 cells as a control.

Cells were infected with retroviruses expressing hDaxx (pLPC-hDaxx) or with the empty pLPC vector and, after 4 days of selection, the polyclonal infected cell populations were expanded and further analysed. As can be observed in Figure 4B, a clear increase in hDaxx expression was detectable in the U2OS stable cell lines (U2OS/hDaxx) as compared to control cells (U2OS/pLPC). Similar results were obtained also in SaOS-2 cell lines (Figure 4D). hDaxx localization in the stable cell lines was comparable to the endogenous protein, as detected by immunofluorescence analysis with a polyclonal anti-Daxx antibody (Figure 4C) with clear nuclear diffuse and punctate staining. No effect of hDaxx overexpression on growth rate and doubling capacity of the cells was detected, in agreement with previously reported data indicating that simple overexpression of hDaxx is not sufficient to trigger cell death (10,13).

To analyse p53-dependent apoptosis in hDaxx-overexpressing stable lines, cells were treated for 48 hours with the chemotherapic drug cisplatin (cis-diamminedichloroplatinum II, CDDP) and then analysed for induction of apoptosis by scoring the sub-G1 DNA content by FACS analysis. As reported in Figure 4A, results obtained from several experiments performed with polyclonal populations of hDaxx-overexpressing U2OS cells derived from four independent infections clearly indicated that hDaxx overexpressing cells are sensitized to CDDP-induced cell death. Increased apoptosis was confirmed by monitoring the levels of cleaved PARP protein, a well-established caspase target, using an antibody specific for the processed p85 fragment (Figure 4B, second panel).

In search for a mechanism underlying the observed effect of hDaxx overexpression on apoptosis, we initially verified p53 protein levels in the stable cell lines, but we could not observe any significant change in p53 expression and subcellular localization in hDaxx overexpressing cells as compared to control pLPC cells, either in untreated conditions or following CDDP treatment (see Figure 4B, third panel and 4C). Also the levels of p53 phosphorylated on Ser15 were comparable in the two cell lines (Figure 4B, fourth panel).

We next performed the same experiments in p53-null SaOS-2 cells. Although CDDP treatment for up to 72 hours was able to efficiently induce cell death in these cells, no significant differences in apoptosis rates were observed in three independent polyclonal populations of hDaxx-infected SaOS-2 cells as compared with pLPC-infected (Figure 4D). These results strongly imply that the ability of hDaxx to increase CDDP-induced cell death is dependent on p53. To further confirm the involvement of p53 in the effect observed, we repeated the experiments after ablation of p53 expression by RNA interference in the U2OS/hDaxx stable lines. U2OS/hDaxx or control U2OS/pLPC were transfected with small interfering RNAs (siRNAs) specific for p53 (sip53) or with control scrambled oligonucleotides (siC). 24 hours after transfection cells were split in two plates and one of them was subjected to CDDP treatment for additional 48 hours. Efficient reduction of p53 expression levels following siRNA transfection was verified by western blotting (Figure 4E, lower panels). FACS analysis of the sub-G1 DNA content revealed that, in the absence of p53, the sensitivity of hDaxx overexpressing cells to CDDP-induced apoptosis was similar to that of control pLPC-infected cells (Figure 4E). This was also confirmed by analysing the levels of cleaved PARP in the different cell lysates (Figure 4E, lower panels).

Taken together, these results demonstrate that hDaxx sensitizes cells to p53-dependent cell death, therefore identifying a novel link between this multifunctional protein and another apoptotic pathway.

hDaxx differentially modulates p53-responsive genes

The results obtained with hDaxx-overexpressing cells are in apparent contrast with the data from reporter assays, where we observed a negative effect of hDaxx on p53 transactivation ability. In these experiments however we used only a reporter construct containing the promoter of the p53-responsive p21 gene, which is mainly involved in mediating growth arrest rather than apoptosis. We then decided to analyse in more detail the effect of hDaxx on p53 transcriptional

response, employing reporter constructs containing different p53-responsive promoters. wt p53-containing U2OS cells were transiently transfected with each reporter construct together with a vector expressing hDaxx or with empty $pcDNA_3$ as a control. Transfection efficiency was monitored by co-expressing a fixed amount of pRL-CMV. As presented in Figure 5A, and in agreement with what observed in SaOS-2 cells (Figure 2), hDaxx overexpression caused a marked reduction of the activity of p21 and Mdm2 promoters. No significant effect was however observed on several promoters of pro-apoptotic genes, such as Bax, PIG3 and AIP1, while transactivation of the PUMA promoter was increased. Immunoblotting of the same lysates confirmed that the difference in transcriptional activity was not reflecting changes in p53 protein levels (Figure 5A, lower panels).

To prove that the observed effect of hDaxx on transcription was dependent on p53, we performed similar reporter assays in p53-null SaOS-2 cells (see Figure 5B). In the absence of p53, when hDaxx was overexpressed along with the various reporter constructs, no significant changes in transcriptional activity were observed. On the contrary, when a p53 expression vector was co-transfected, hDaxx repressed transcription of the Mdm2 promoter, while it did not significantly affect PIG3 promoter and activated transcription of the PUMA reporter.

We then sought to verify the effects of hDaxx overexpression on endogenous p53 target genes. Lysates were prepared from U2OS/pLPC and U2OS/hDaxx cell lines, either untreated or treated with cisplatin for 48 hours. Western blot analysis clearly indicated that, in cells overexpressing hDaxx, CDDP-induced upregulation of p21 was impaired (Figure 5C), confirming the result obtained with reporter assays.

To further confirm and expand these findings, we generated a U2OS cell line in which hDaxx expression can be induced by treatment with PonasteroneA, a synthetic analogue of ecdysone. Upon induction of hDaxx expression and treatment with cisplatin for 48 hours, cell lysates were analysed by western blot for the expression of several p53-induced genes. As shown in Figure 5D, DNA damage-mediated upregulation of both p21 and Mdm2 was reduced upon induction of

hDaxx overexpression as compared to control, uninduced cells, while the levels of two p53 proapoptotic targets, PIG3 and Bax, were slightly increased by hDaxx overexpression. All together the above results clearly indicate that hDaxx is able to modulate p53 transcriptional activity. This effect is promoter-dependent, resulting in repression of p53-mediated induction of genes involved in cell cycle arrest (p21), while genes involved in apoptosis (Bax, PIG3, AIP1) are either stimulated or remain unaffected.

Endogenous hDaxx is required for p53 apoptotic function.

Having demonstrated that overexpression of hDaxx potentiates p53-dependent apoptosis, we then sought to investigate whether also the endogenous hDaxx protein can exert the same effect on p53 activity. To this aim we knocked down hDaxx expression by transfecting p53-null SaOS-2 cells with siRNA specific for hDaxx (sihDaxx) or control scrambled oligonucleotides (siC), together with either wt p53 or control plasmid, in order to compare the effects of hDaxx depletion in cells expressing or not p53. Twenty-four hours later cells were fixed, stained with Hoechst and nuclear morphology was analysed by epifluorescence to identify apoptotic cells. As can be seen in Figure 6A, transfection with sihDaxx oligonucleotides effectively reduced endogenous hDaxx expression, and this resulted in a moderate increase in apoptosis in the absence of p53, in agreement with previous observations (22,23). However, in cells that expressed p53, ablation of hDaxx expression significantly reduced p53-dependent apoptosis (Figure 6A). To further extend this analysis, we performed reporter assays in SaOS-2 cells transfected with p21-Luc or Bax-Luc reporter constructs together with wtp53 and either sihDaxx or siC oligonucleotides. As shown in Figure 6B, and consistent with results from overexpression experiments (Figures 2 and 5), reduction of hDaxx levels caused a twofold increase in p53-dependent transactivation of the p21 promoter, while no significant effect was observed on Bax promoter. These results therefore confirmed the evidences obtained following hDaxx ectopic

expression and demonstrate an important role of this multifunctional protein in regulation of p53 functions.

DISCUSSION

In this report we demonstrate that the multifunctional protein hDaxx binds to p53 and its homologues p73 and p63. The interaction takes place both *in vitro* and *in vivo* and requires the oligomerization domain of p53. This region is conserved also in p73 and p63 (8), allowing therefore to predict a common hDaxx interaction domain in all three proteins of the p53 family.

The exact biological function of hDaxx is still awaiting elucidation and, despite several evidences implicating this protein in control of apoptosis, its exact role in such process remains controversial. While Daxx overexpression potentiates apoptosis induced by stimuli such as Fas-ligand and TGFß treatment (13,15,19), an anti-apoptotic effect of this protein has been proposed on the basis of knock-out mice models and RNA interference studies (21,22). It seems therefore that Daxx might have a dual function in regulation of apoptosis, probably depending also on the cellular context, as underlined by the fact that no increase in Fas-induced apoptosis was observed following overexpression of hDaxx in lymphoid cells (28) and that Daxx exerted an anti-apoptotic role in myeloid precursors (29).

Our results clearly indicate that physiological levels of hDaxx protein are important in setting the threshold for p53-dependent apoptosis. We in fact showed that, while overexpression of hDaxx sensitizes wt p53-containing cells to CDDP-induced cell death, ablation of endogenous hDaxx expression resulted in a protective effect toward p53-dependent apoptosis. Such effect requires p53, since in p53-null cells we observed, as previously reported (22,23), a moderate increase in cell death following downregulation of hDaxx protein levels. These results represents one of the few demonstration of a proapoptotic role exerted by hDaxx at physiological expression levels and further underlines the complex role of this multifunctional protein in controlling the apoptotic response.

In agreement with previous observations that hDaxx binds to several transcription factors and modulates their activity (5,25,26,30), we were able to show that hDaxx represses the transcriptional ability of p53 and its homologues p73 and p63 towards the p21 promoter.

However, at least in the case of p53, this negative effect was significant with respect to promoters derived from the p21 or Mdm2 genes, involved in cell cycle arrest or in p53 regulation, while no differences were observed in the induction of promoters of pro-apoptotic genes such as bax, PIG3 and AIP1. Importantly, we observed that hDaxx overexpression can also modulate the levels of endogenous p53 target genes, with effects mirroring those observed in reporter assays. Although the exact mechanism mediating p53 promoter specificity is not clear, it is now well established that at least two classes of p53-binding sites exist within p53-responsive promoters, with p21 and Mdm2 belonging to a “high affinity” class of sites, while bax and PIG3 to a “low affinity” one (5). Moreover, specific post-translational modifications of p53 as well as interaction with cellular cofactors might differentially regulate its ability to interact with a defined subset of DNA targets. Accordingly, it has recently been shown that the p53-binding protein ASPP1 specifically stimulates the apoptotic function of p53, by promoting its binding to apoptosis-related promoters (6). Also the p53 family members p73 and p63 are required to modulate the capability of p53 to efficiently bind and transactivate promoters of pro-apoptotic genes (31). Our data are in line with these observations, and suggest that hDaxx may be another factor involved in such a complex regulation. However, the exact mechanism by which the repressive function of hDaxx operates specifically toward some p53-induced genes requires further elucidation. It should be noted that, in the case of the PUMA promoter, hDaxx overexpression causes an increase in transcriptional activity in reporter assays. Even though hDaxx was shown to behave as repressor in most cases, it has also been demonstrated that, in the case of Pax5, interaction with hDaxx may result either in transcriptional repression or activation, depending on the cell type (30). The repressive function of hDaxx has been linked to its ability to recruit histone deacetylases (HDACs) to target promoters, and indeed a specific interaction between hDaxx and several HDACs has been reported (12,24). However, hDaxx can bind also to core histones, and particularly to their acetylated forms (24), therefore its activity in a particular promoter context might be differentially regulated by association with transcription

factors and additional co-repressors/co-activators. In the case of Pax5, for example, hDaxx was also found in complex with the transcriptional co-activator CBP (30).

Although we did not observe major changes in hDaxx protein levels following DNA-damage treatment, it is possible that the interaction between hDaxx and p53 family members is regulated by other mechanisms. Several factors appear to modulate hDaxx subcellular distribution by recruiting it to specific compartments, such as the cytoplasm in the case of interaction with ASK1 (17,32) or the nucleolus in the case of the interaction with the 58-kDa microspherule protein (33). Particularly interesting in this context is the interaction with PML, since hDaxx recruitment to PML Nuclear Bodies has been reported not only to be essential for its pro-apoptotic effect but also to relieve its transcriptional repressive activity (11,12,20). PML has been implicated in transcriptional regulation and has been shown to modulate responses on different promoters via interaction and sequestration of coactivators and repressors to the NBs (12,34,35). PML also regulates the function of p53 (36-38) and recruitment of p53 to NBs has been related, at least in some experimental settings, with increased transactivation of pro-apoptotic targets and reduction of cell survival (36-38). The picture has been further complicated by the recent demonstration that PML is a direct target of p53, thus contributing to its antiproliferative effects (39). How the observed interaction between p53 and hDaxx fits in this scenario still requires elucidation, but it should be noted that a recent report demonstrated that, similarly to what we observed, hDaxx can repress p53 transcriptional activity and PML is able to counteract this effect (40). While all the PML splice variants are able to interact with hDaxx, binding to p53 is restricted to a specific isoform, PMLIV (36). Therefore the pattern of expression of PML isoforms as well as the availability of other proteins to interact with them within the NBs may account for cell-type specific regulation of both hDaxx and p53 functions.

A role can also be postulated for protein modifications. Not only p53 is subjected to a complex series of post-translational modifications, but also hDaxx is phosphorylated on several sites (25) as well as SUMO-1 modified (41). Different kinases have been shown to interact with hDaxx,

such as HIPK1 (42), HIPK2 (43), HIPK3 (44) and the ZIP kinase (45), even though their exact role in hDaxx phosphorylation is not yet clear. In the case of HIPK1, it has been shown to phosphorylate hDaxx, to relocalize it from NBs to chromatin and to modulate its transcriptional repressive functions. HIPK2 may as well modulate hDaxx localization and function, since it acts by disgregating NBs and releasing NB-associated factors (43). Interestingly, HIPK2 can also bind to and phosphorylate p53, being involved in the induction of p53-dependent apoptotic response (46,47).

The ability of hDaxx to interact with different cellular factors may depend on its phosphorylation status, as observed in the case of PML that interacts with hyperphosphorylated hDaxx or of Pax3, which instead binds to the unphosphorylated form (25). In particular, the C-terminal region of hDaxx, which is responsible for binding to several factors and as we observed also to p53 family members, contains a Ser/Thr/Pro rich domain that is a potential target for phosphorylation events. Interestingly, we observed an increased interaction between hDaxx and p53 family members upon phosphatase treatment (M.G. and G.D.S. unpublished observation). Further analysis of the posttranslational modification pattern of hDaxx will surely help in clarifying how its affinity for specific cellular factors and its functions are modulated.

The evidence that hDaxx specifically represses the p21 promoter, but not pro-apoptotic p53 targets, may also provide an explanation for the observed effect on p53-dependent apoptosis, since several reports demonstrated a protective role of p21 against apoptosis (48-50). Recently the ability of c-jun to inhibit p53-dependent induction of p21 has been shown to mediate its death-promoting effect following UV irradiation (51). In this light, hDaxx may be envisioned as a factor that influences the balance of transcription between genes that induce cell cycle arrest or apoptosis. Although further experiments are required to confirm this hypothesis, our results indicate that hDaxx can modulate the threshold of induction of the apoptotic response to p53, and clearly implicate this protein not only in Fas- and TGFß- but also in DNA damage-induced apoptosis. As a further indication of the complex role of hDaxx in such processes, expression of

mutant p53 in tumour cells has been shown to abrogate the ability of hDaxx to promote Fas-dependent apoptosis (18). Mutant p53 binds to hDaxx and inhibits hDaxx-mediated activation of ASK1/JNK pathway. Furthermore mutant p53 may also downregulate Fas expression, contributing to protection against Fas-induced cell death (52).

Of note, it has been demonstrated that mouse embryo fibroblasts lacking p73 and p63 are resistant to p53-dependent apoptosis and this correlates with the inability of p53 to efficiently transactivate pro-apoptotic targets (31). In light of these findings, the evidence that hDaxx binds to and regulates the activity of all p53 family members allows us to speculate that it might represent an integrating factor to coordinate their physiological response.

ACKNOWLEDGMENTS

We wish to acknowledge our colleagues at the LNCIB for helpful discussions and particularly P. Sandy for critical reading of the manuscript and experimental support with FACS analysis. We also thank T. Macorig for technical assistance. We thank S. Piccolo, G. Melino, G. Blandino, F. McKeon, M. Oren, M. Levrero, X. Lu, J. D. Chen and H. Will for providing reagents. This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), CNR and Telethon to G.D.S.

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FIGURE LEGENDS

FIG. 1. hDaxx interacts with p53 family members.

(A) Interaction with hDaxx was analyzed by yeast two hybrid assay using p53, p73α or p63α deleted in their transactivation domain and fused with LexA, as indicated in the upper scheme. The various domains are indicated. '+' indicates positive interaction, as judged by beta-galactosidase activity and by the ability to grow in the absence of leucine. The same strains used for the interaction assays were analyzed for the expression of the bait (anti-LexA, upper panel) and of the fish (anti-HA, lower panel) proteins, either in the presence of glucose (G) or galactose/raffinose (G/R) as carbon source.

(B) In vitro binding assay with GST-p53, GST-p73α, GST-p63α or GST, as indicated, was performed on lysates from p53-null SaOS-2 cells overexpressing hDaxx. Western blot was then performed with anti-Daxx polyclonal antibody. The band corresponding to hDaxx protein as well as running positions of molecular weight markers are indicated.

(C) Co-immunoprecipitation on lysates from 293 cells overexpressing hDaxx was performed with anti-p53 polyclonal antibody or with normal rabbit serum (NRS) as negative control. Immunoprecipitates were analyzed by western blotting with anti-Daxx antibody (upper panel). An aliquot of each total lysate was then checked for the expression of hDaxx and p53 proteins (lower panels).

(D) Co-immunoprecipitation on lysates from 293 cells overexpressing HA-tagged p73α alone or together with hDaxx was performed with anti-Daxx polyclonal antibody and immunoprecipitates were analyzed by western blotting with anti-HA monoclonal antibody (upper panel). An aliquot of each total lysate was then checked for the expression of the hDaxx and p73 proteins (lower panels).

FIG. 2. hDaxx represses transcriptional activity of p53 family members.

Luciferase activity assays were performed on lysates from p53-null SaOS-2 cells, transiently transfected with the p21-Luc reporter together with wt p53, p73α or p63α and empty pcDNA3 or pcDNA3-hDaxx, as indicated. To normalize the transfection efficiency, a fixed amount of pRL-CMV reporter, constitutively expressing the *Renilla reniformis* luciferase gene, was included in each sample. Graphs represent the mean of at least three independent experiments. Standard deviations are indicated. An aliquot of each lysate was checked by western blot to verify the expression levels of the various proteins (lower panels).

FIG. 3. Distinct domains of hDaxx mediate the interaction with p53 oligomerization domain.

(A) Upper panel: schematic representation of the hDaxx protein and the different deletions used for *in vitro* binding assay; the various hDaxx domains are indicated: 181-217, coiled-coil; 389-394 and 627-633, NLS; 434-485, acidic domain; 485-626, apoptosis activation domain; 633-740, Ser/Thr/Pro rich region.

Lower panels: *in vitro* binding assays were performed with lysates of 293 cells and different hDaxx deletions fused to GST. Samples were then separated by SDS-PAGE and analyzed by western blot with anti-p53 monoclonal antibody, and ponceau red staining of the membrane was used to verify the amounts of the different GST-fusions loaded in each sample.

(B) Interaction with hDaxx was analyzed by yeast two hybrid assay using different p53 deletions fused with LexA. '+' indicates positive interaction, '-' indicates no detectable interaction.

(C) p53-null SaOS-2 cells were transiently transfected with hDaxx together with vectors expressing peptides corresponding to different p53 regions within the Trx scaffold (HA-TNV), as indicated. Immunoprecipitation was performed with anti-Daxx polyclonal antibody and subsequently western blot was performed with anti-HA monoclonal antibody.

FIG. 4. hDaxx sensitizes cells to p53-dependent apoptosis.

(A) U2OS cell lines stably transfected with empty pLPC or with pLPC-hDaxx expression vector were treated with cisplatin (CDDP) or left untreated, as indicated. 48 hours later, the percentage of apoptotic cells was determined by FACS analysis. Graphs represent the mean of seven independent experiments, performed on 4 different polyclonal cell populations. Standard deviations are indicated. A representative cell cycle profile is shown on the left.

(B) Aliquots of the lysates used for FACS analysis were analyzed by western blot with the indicated antibodies. Anti-actin staining was used as loading control.

(C) U2OS/pLPC and U2OS/hDaxx cells, either untreated or treated with CDDP for 24 hours as indicated, were analyzed by immunofluorescence with anti-Daxx polyclonal and anti-p53 monoclonal antibodies, followed by incubation with anti-rabbit FITC- and anti-mouse RITC-conjugated antibodies, respectively.

(D) p53-null SaOS-2 cell lines stably transfected with empty pLPC or with pLPC-hDaxx expression vector were analyzed as in (A). Graphs represent the mean of four independent experiments, performed on 3 different polyclonal cell populations. Standard deviations are indicated. Aliquots of the lysates were analyzed by western blot with the indicated antibodies. Anti-actin staining was used as loading control.

(E) RNA interference was performed on U2OS/pLPC and U2OS/hDaxx cells, using ds RNA oligonucleotides specific for p53 (sip53) or control scrambled oligonucleotides (siC), as indicated. 24 hours after transfection, cells were either left untreated or treated with CDDP for 48 hours and analyzed for apoptosis induction by FACS analysis as in (A). An aliquot of the lysates was analyzed by western blot with the indicated antibodies (lower panels).

FIG. 5. hDaxx differentially modulates transcription from different p53-responsive promoters.

(A) U2OS cells expressing endogenous wt p53 were transfected with either empty $pcDNA_3$ or $pcDNA_3$-hDaxx expression vector together with reporter constructs bearing different p53-responsive promoters upstream of the luciferase gene, as indicated. 36 hours after transfection, luciferase activity assays were performed. To normalize the transfection efficiency, a fixed amount of pRL-CMV reporter was included in each sample. Graphs represent the mean of at least four independent experiments. Standard deviations are indicated. An aliquot of each sample was analyzed by western blot to verify the expression levels of endogenous p53 and overexpressed hDaxx proteins (lower panels).

(B) Luciferase activity assays were performed on p53-null SaOS-2 cells transfected with the indicated p53-responsive reporters together with empty $pcDNA_3$ or $pcDNA_3$-hDaxx, either in the presence or in the absence of wt p53 expression vector. Graphs represent the mean of three independent experiments, standard deviations are indicated.

(C) U2OS/pLPC and U2OS/hDaxx cell lines were treated with cisplatin (CDDP) or left untreated, as indicated. 48 hours later, lysates were analysed by western blotting with anti-Daxx and anti-p21 primary antibodies. Stabilization of p53 after DNA damage was verified by anti-p53 staining, while anti-actin staining was used as loading control.

(D) U2OS cells expressing inducible HA-tagged hDaxx were treated with cisplatin (CDDP) for 48 hours, either inducing Daxx expression with Ponasterone A (PonA) or not as indicated. Lysates were then analysed by western blotting with the indicated primary antibodies. Expression of HA-hDaxx as well as stabilization of p53 after DNA damage were verified by anti-Daxx and anti-p53 staining, respectively. Anti-actin staining was used as loading control.

Figure 6. Endogenous hDaxx protein is required for p53 dependent apoptosis.

(A) p53-null SaOS-2 cells were transfected with p53 expression vector or with control plasmid (pEGFP to identify transfected cells), together with either hDaxx-specific ds RNA oligonucleotides (sihDaxx) or control scrambled oligonucleotides (siC) as indicated. After 24

hours cells were fixed and subjected to immunofluorescence to identify cells expressing p53. Subsequently cells were stained with Hoechst to identify apoptotic nuclei. The histogram represents the mean results of 4 independent experiments, in which p53-positive and control-transfected apoptotic nuclei were scored. Standard deviations are indicated. Aliquots of each sample were analyzed by western blot to verify the expression levels of endogenous hDaxx and overexpressed p53 proteins (lower panels).

(B) p53-null SaOS-2 cells were transfected with reporter constructs bearing p53-responsive promoters Bax and p21 upstream of the luciferase gene, either in the presence or in the absence of wt p53 expression vector, and together with either hDaxx-specific ds RNA oligonucleotides (sihDaxx) or control scrambled oligonucleotides (siC) as indicated. 24 hours after transfection, luciferase activity assays were performed. To normalize for transfection efficiency, a fixed amount of pRL-CMV reporter was included in each sample. Graphs represent the mean of at least three independent experiments. Standard deviations are indicated.

Fig. 1A

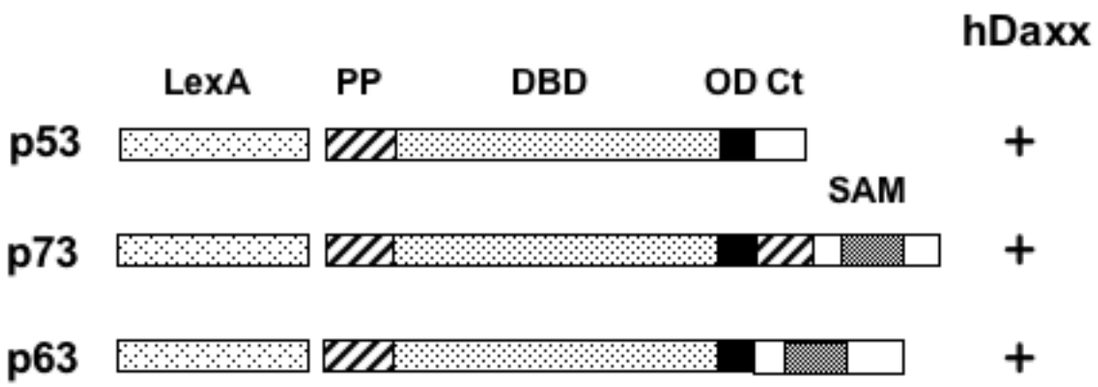
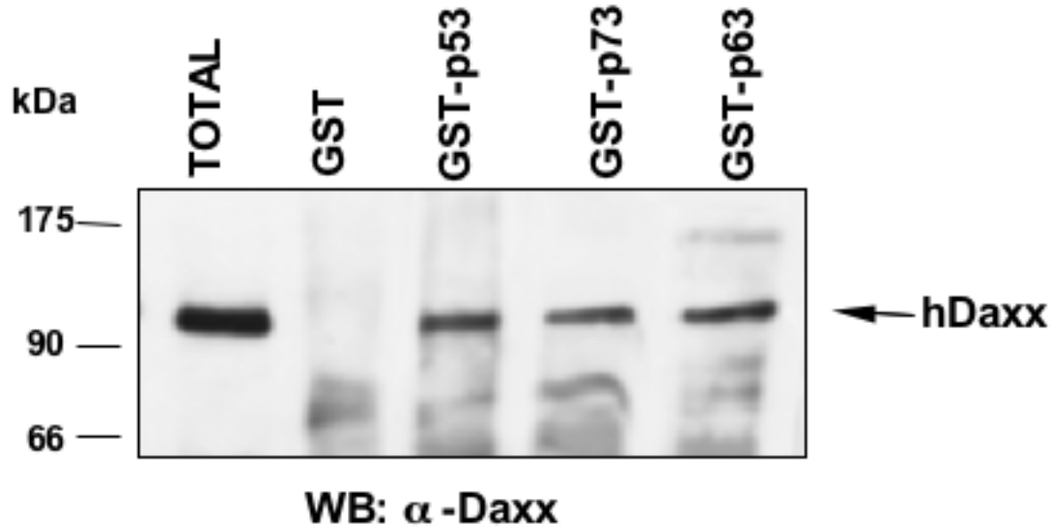


Fig. 1B



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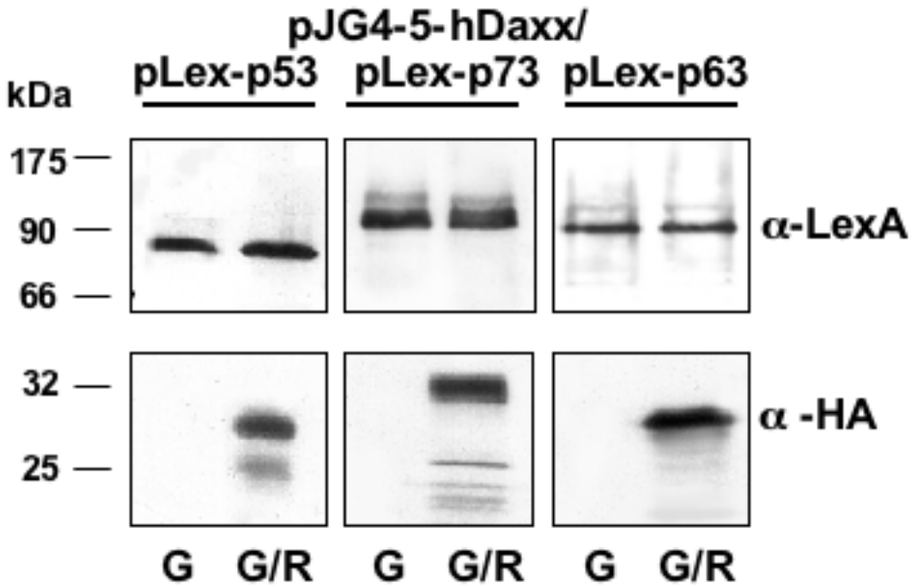


Fig. 1D

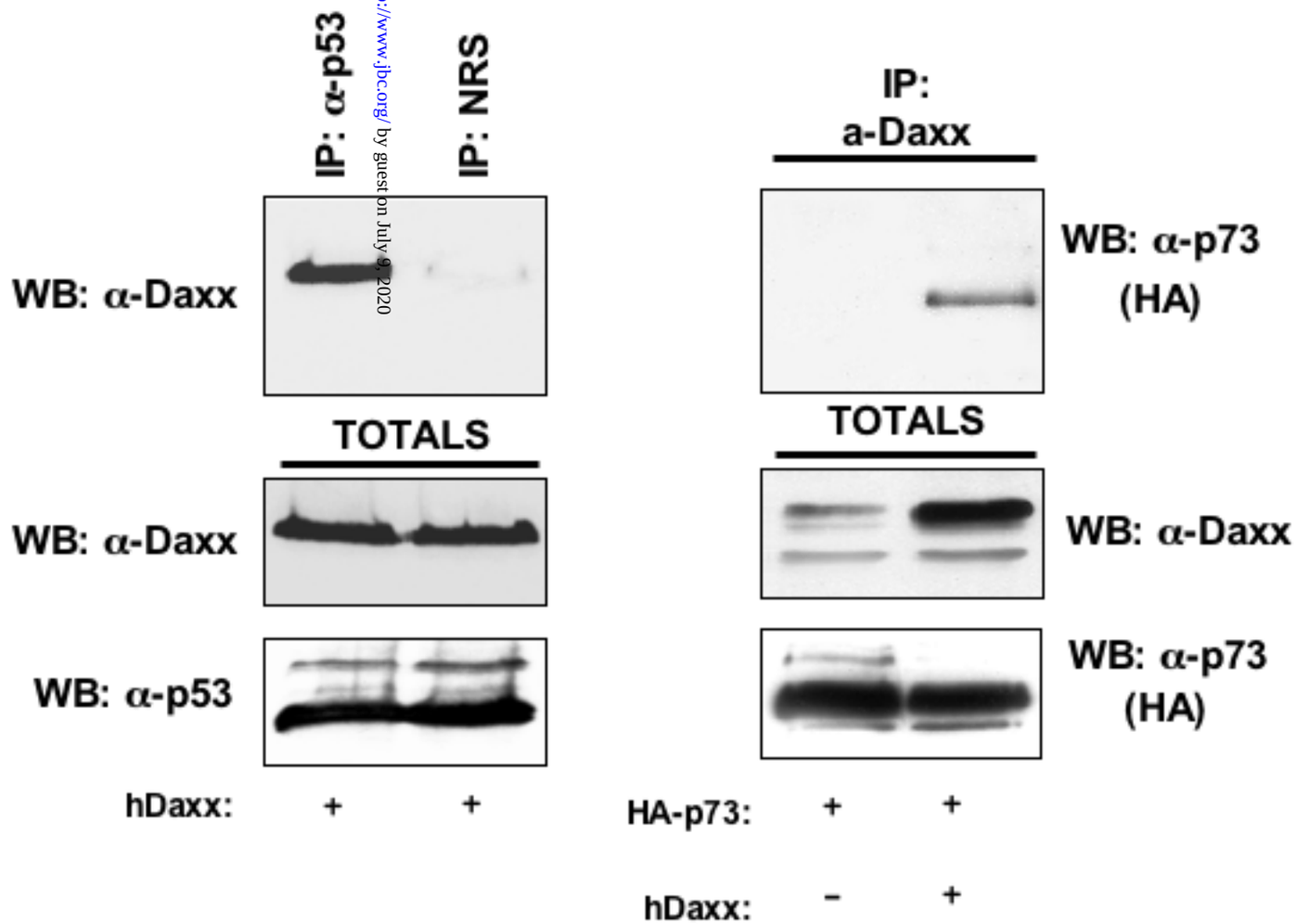


Fig. 2

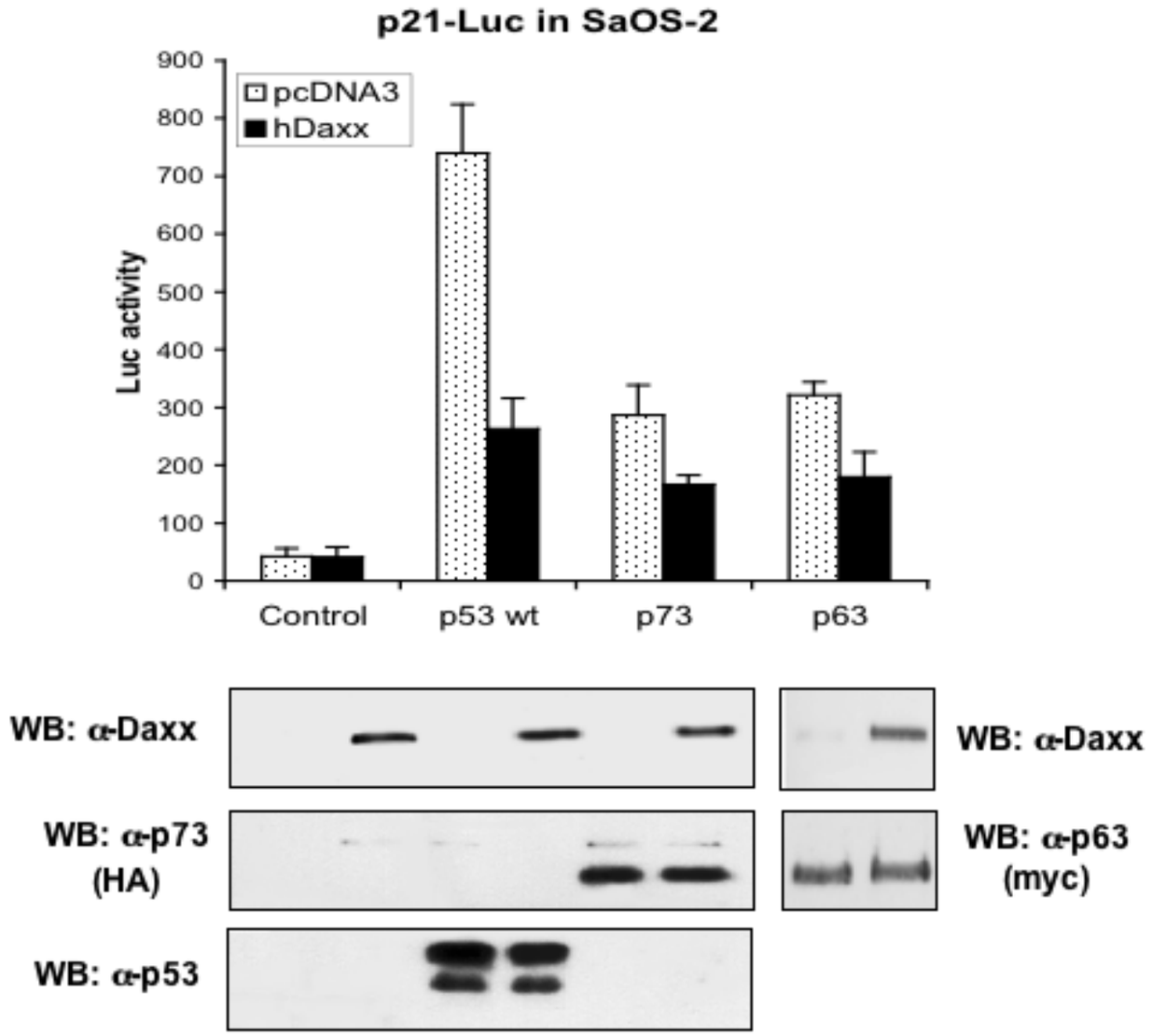
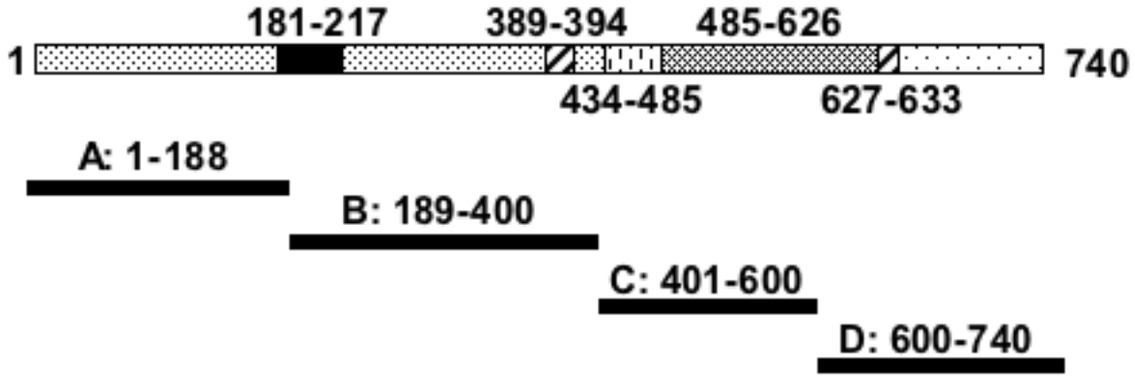


Fig. 3A



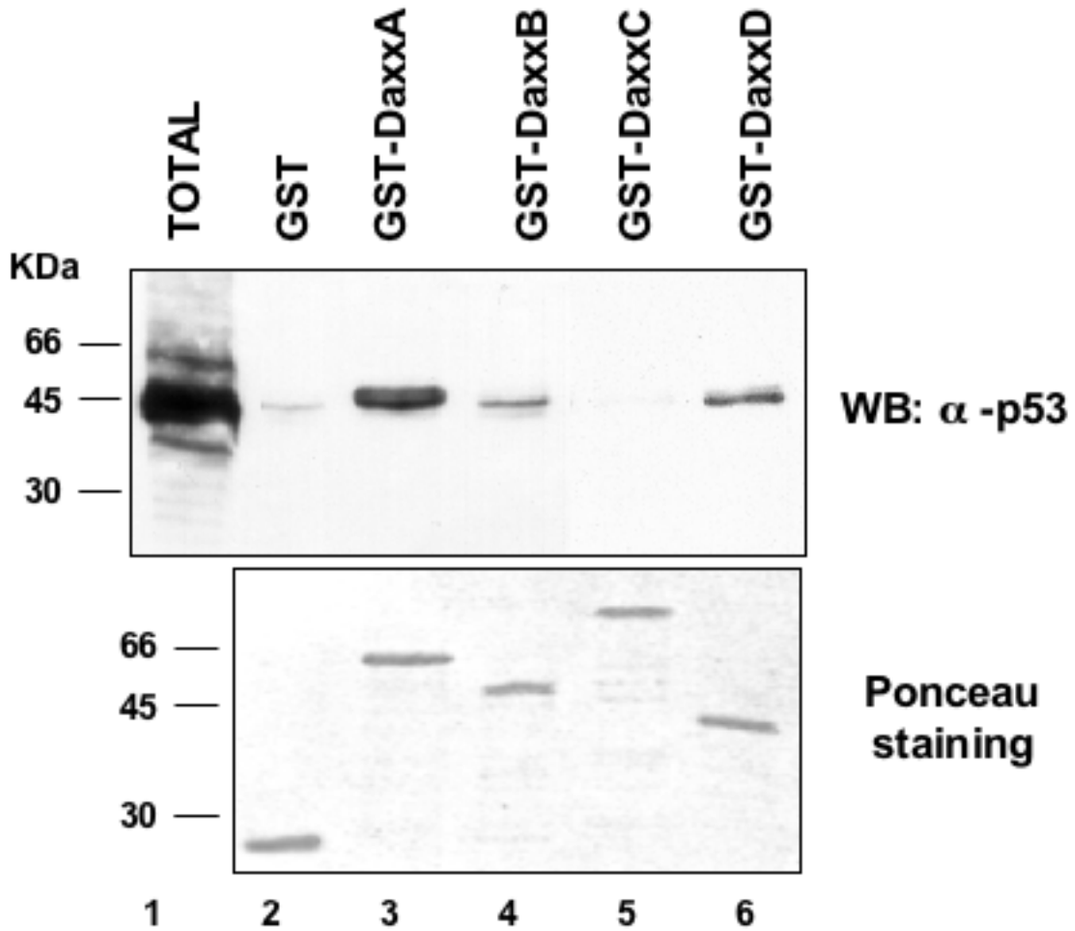


Fig. 3B

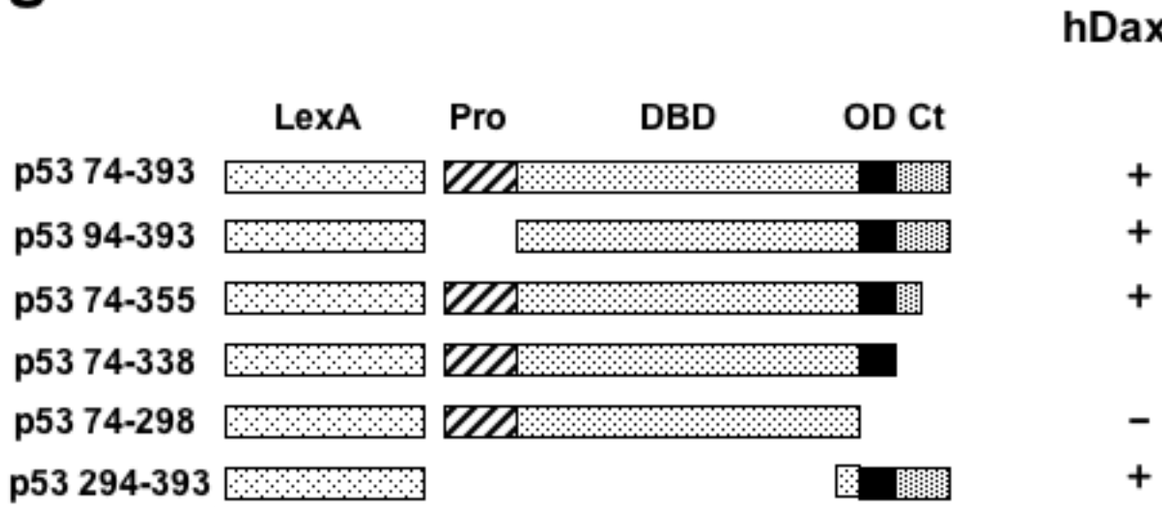


Fig. 3C

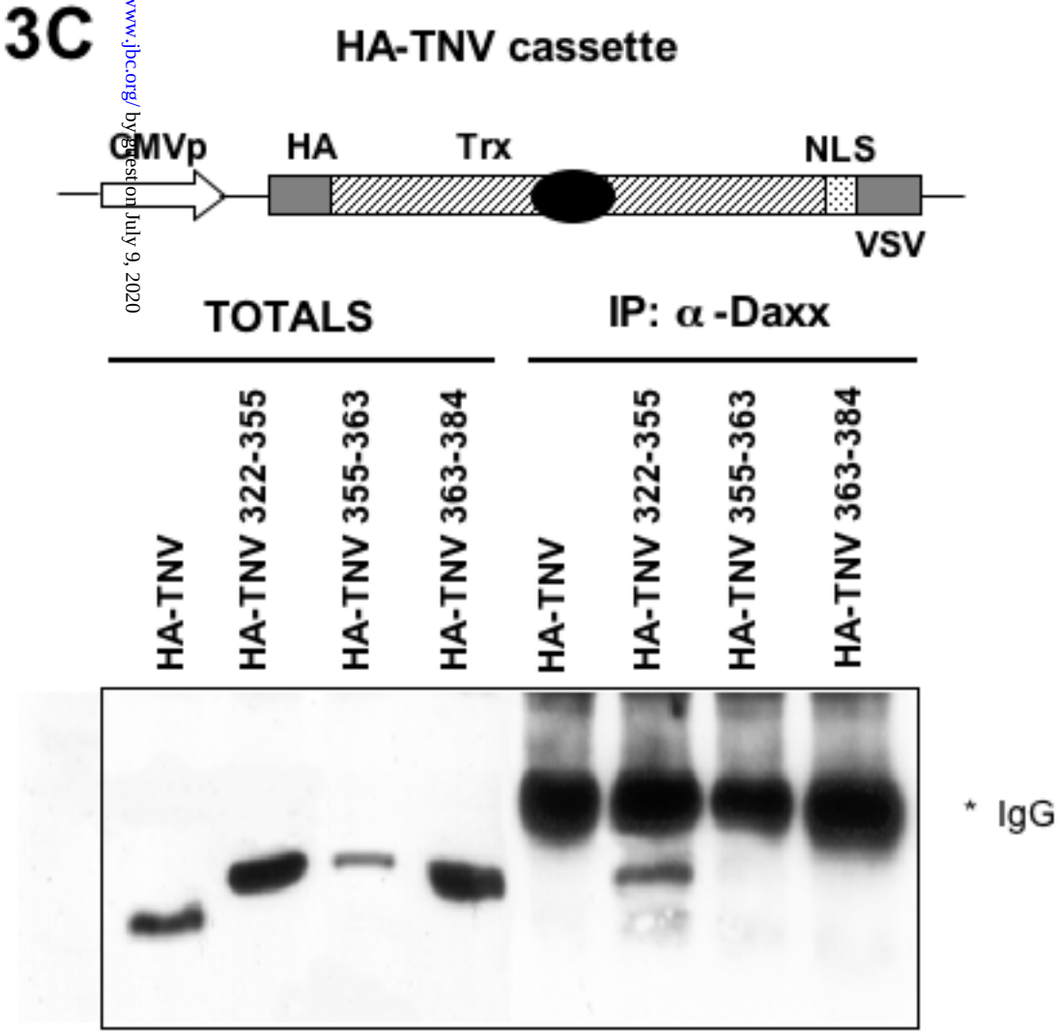


Fig. 4A

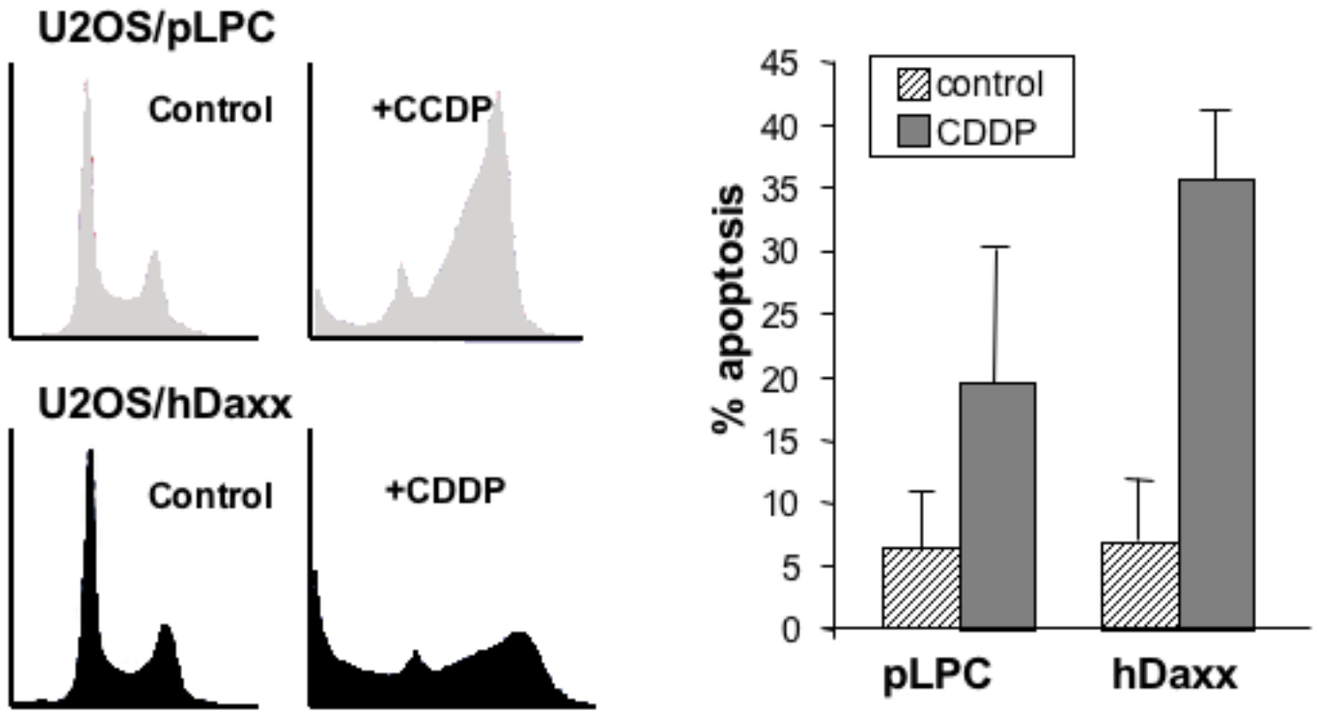


Fig. 4B

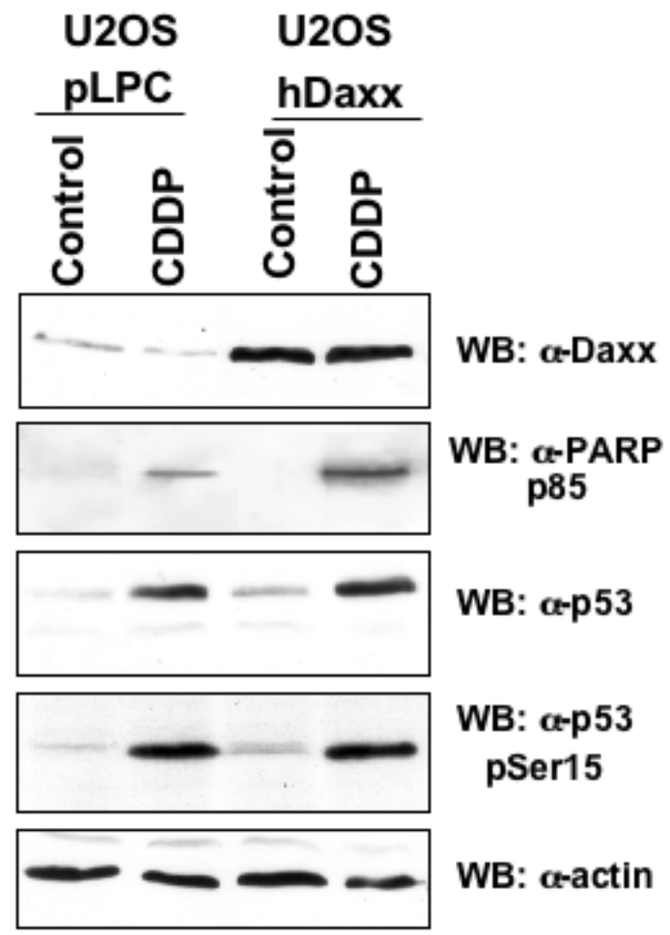


Fig. 4C

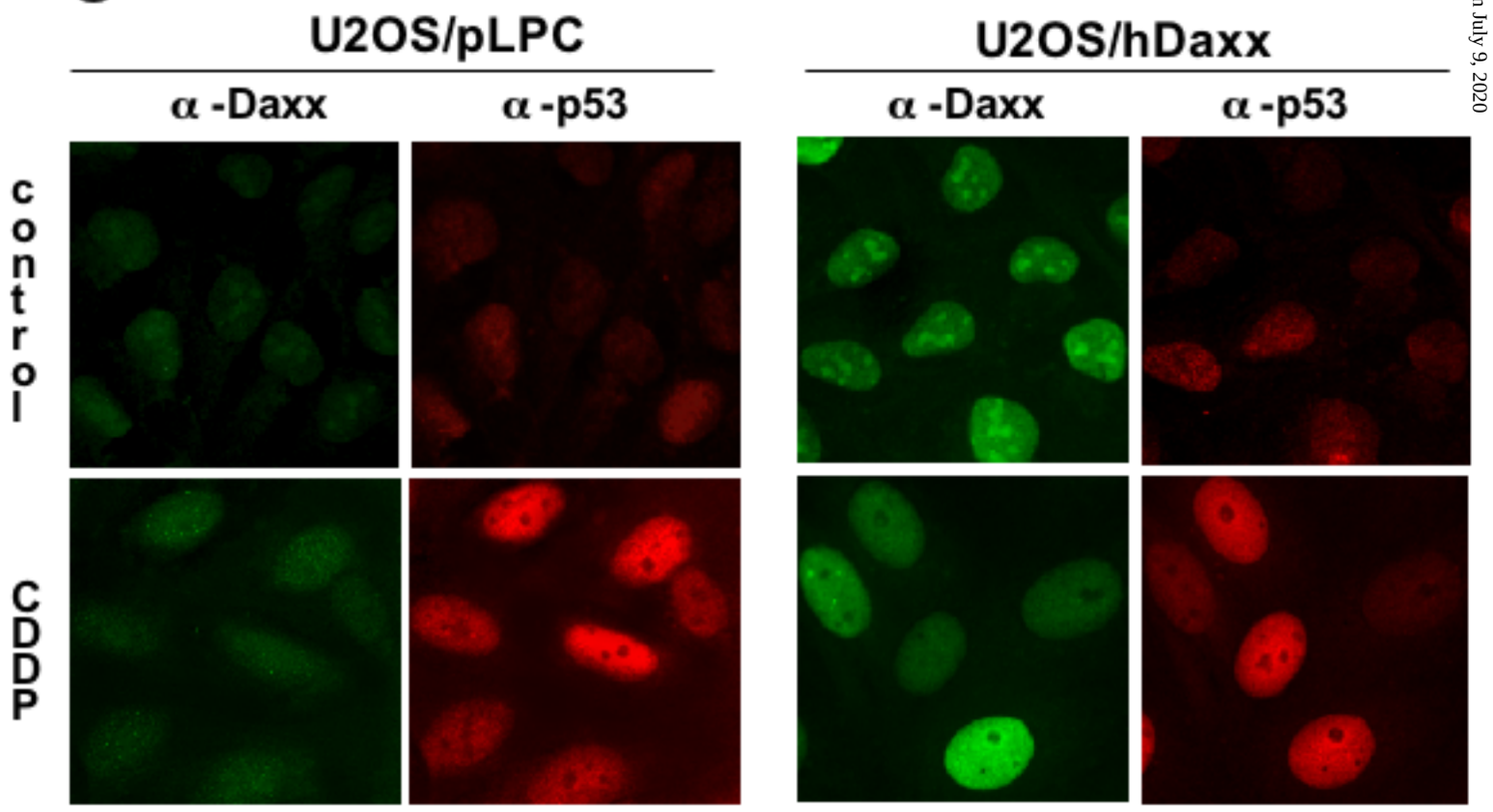


Fig. 4D

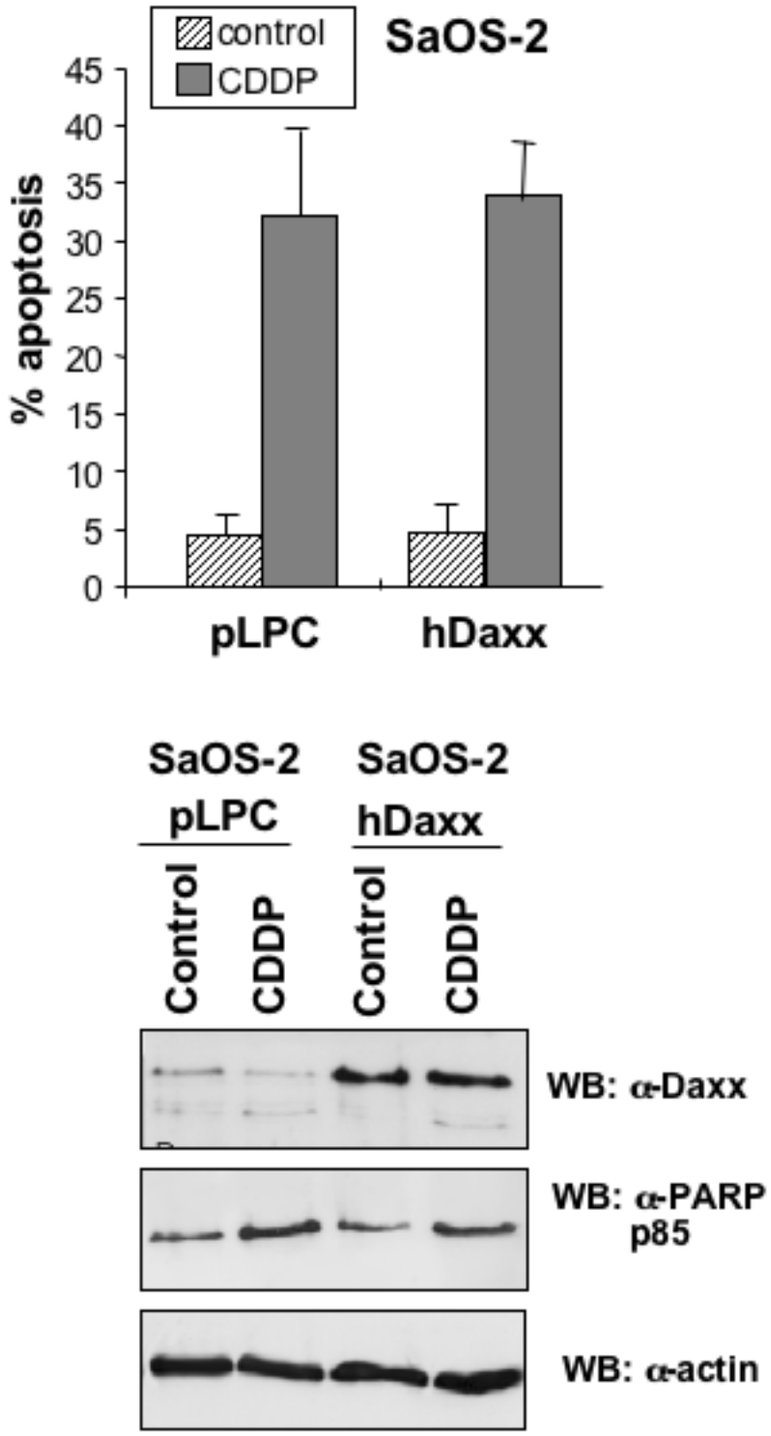


Fig. 4E

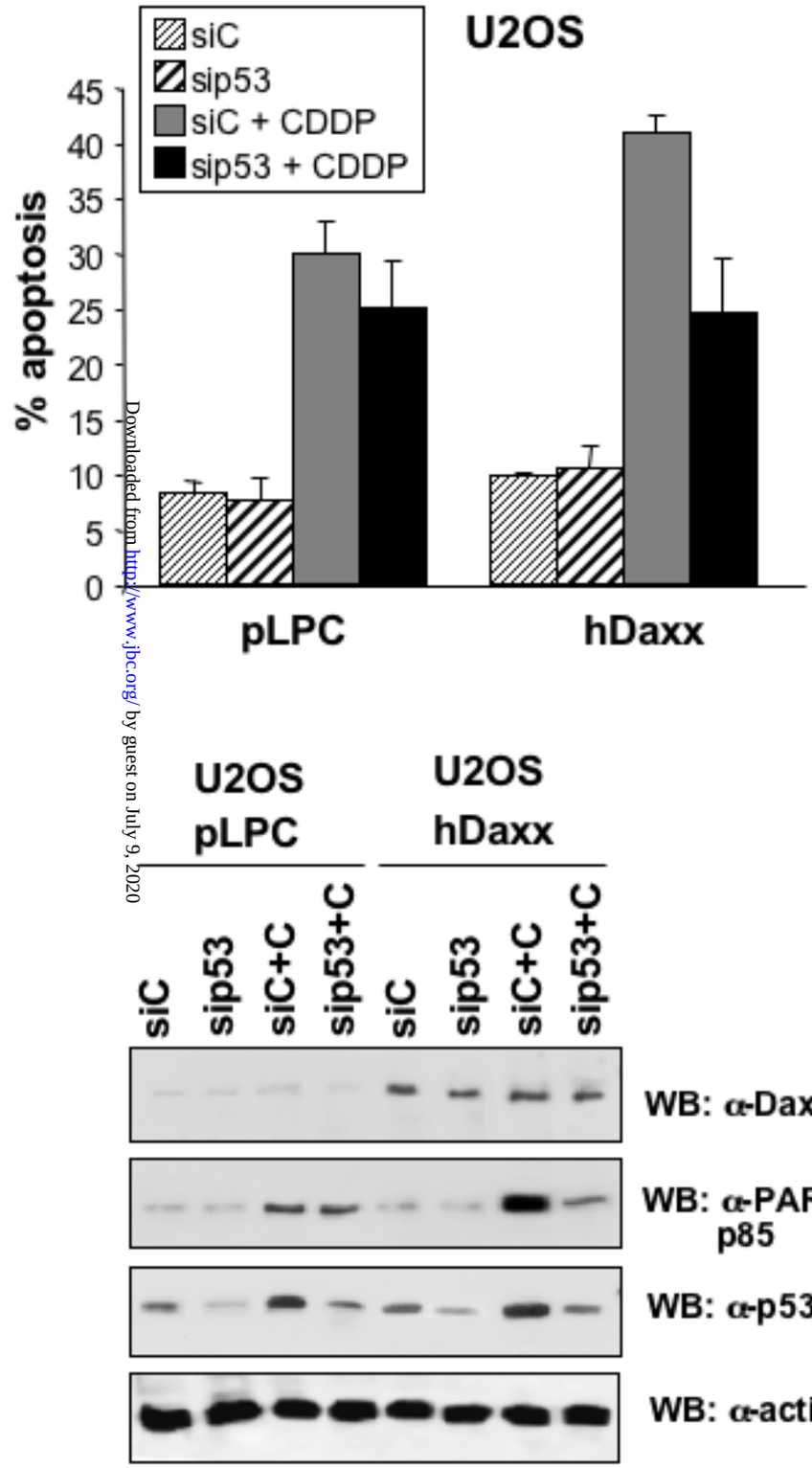


Fig. 5A

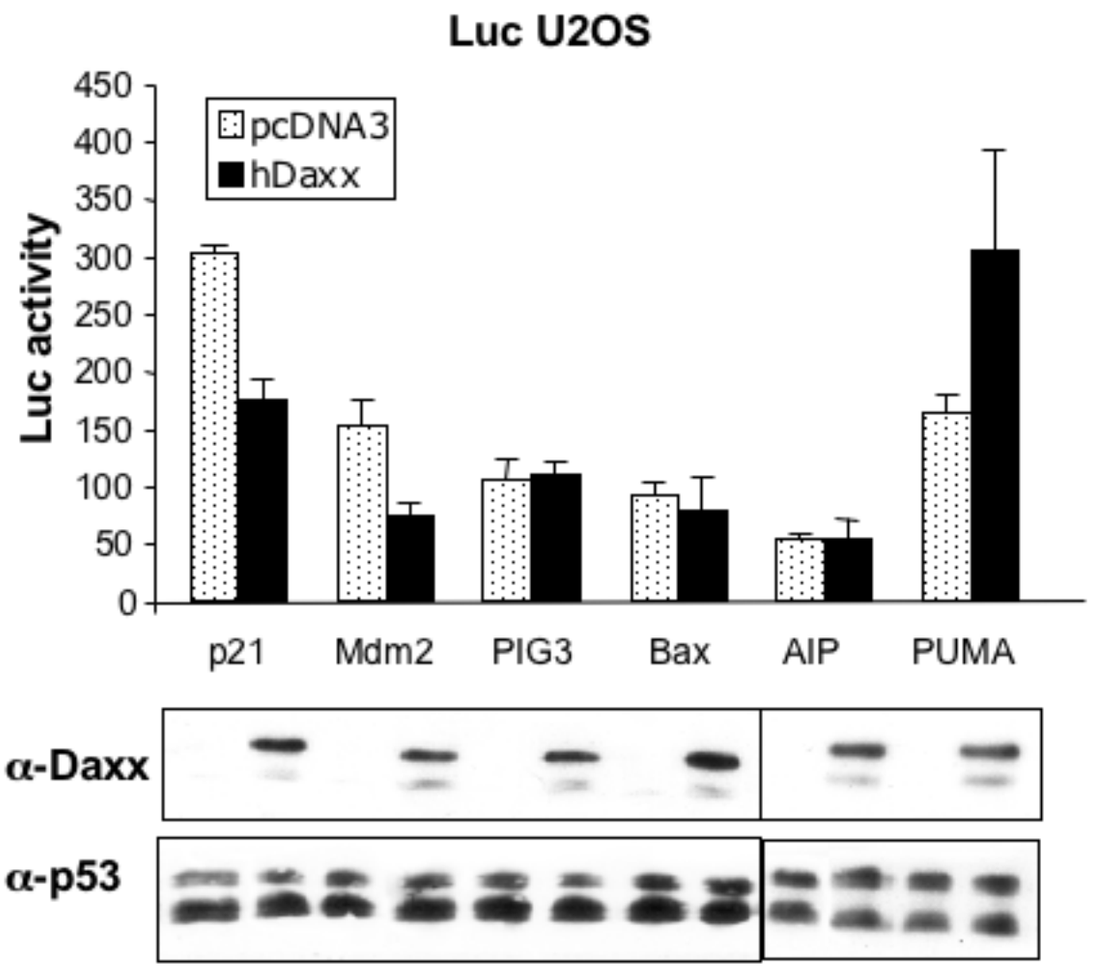


Fig. 5B

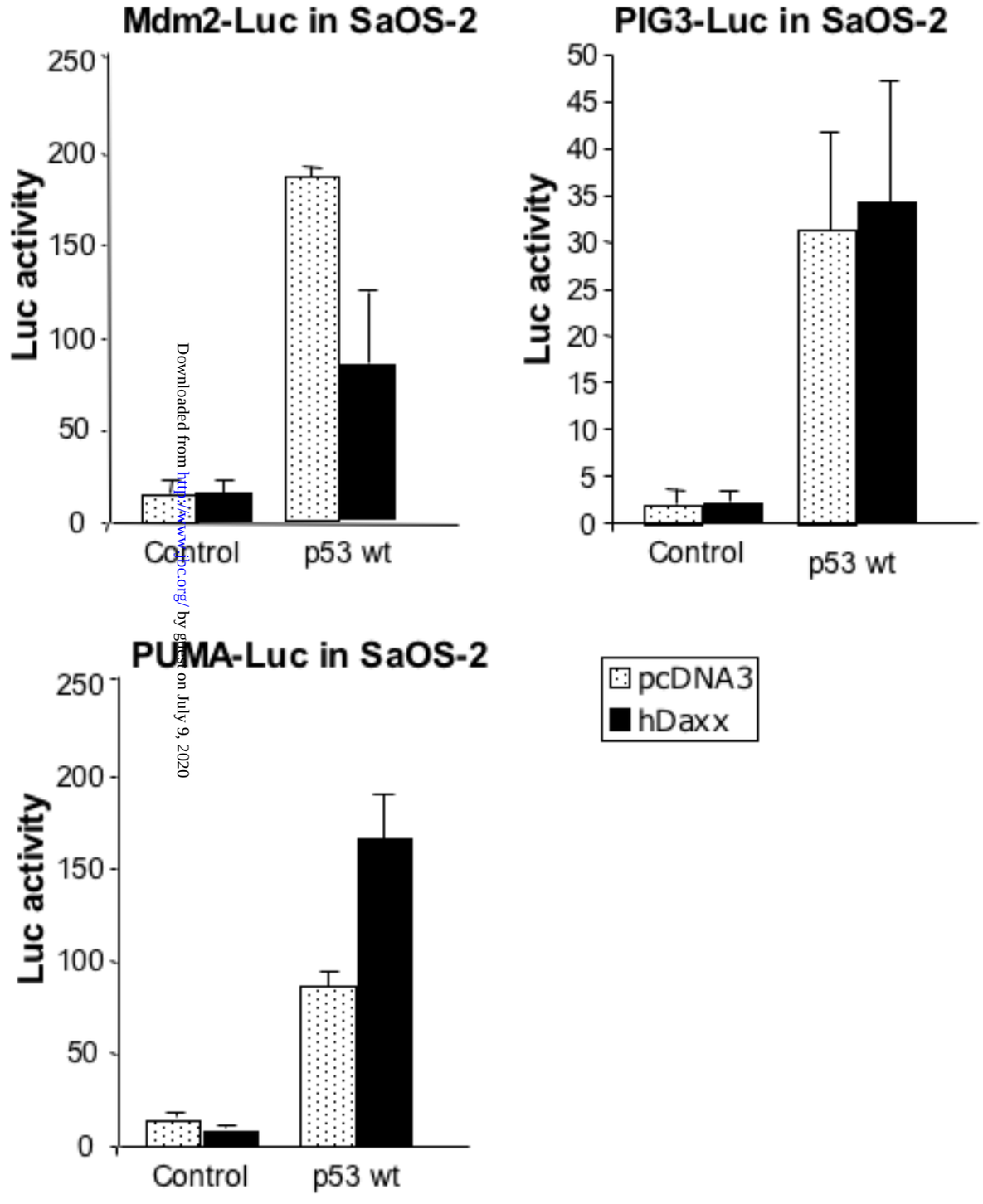


Fig. 5C

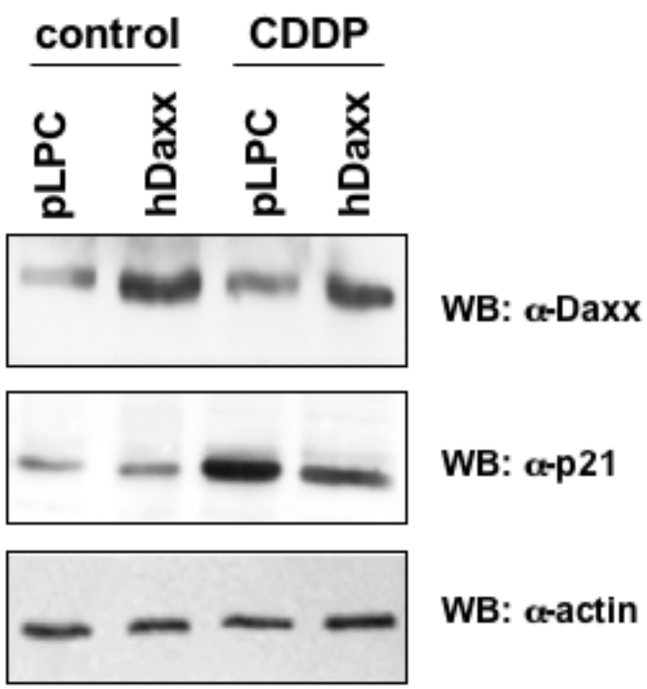


Fig. 5D

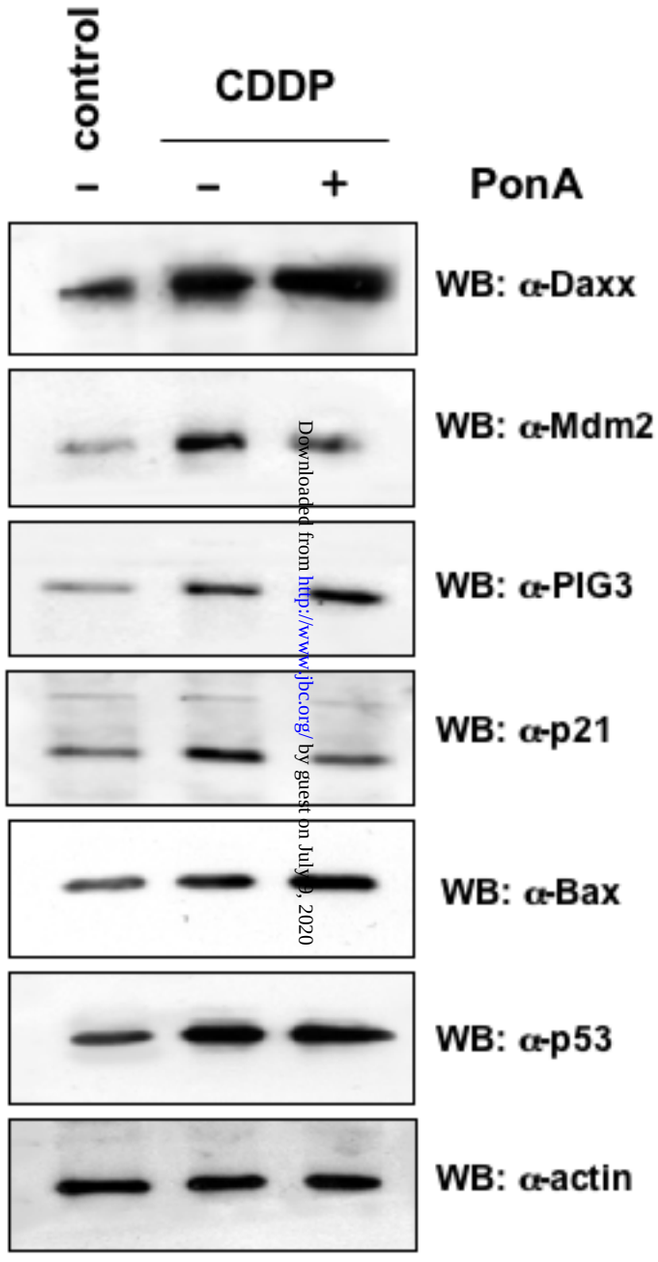


Fig. 6A

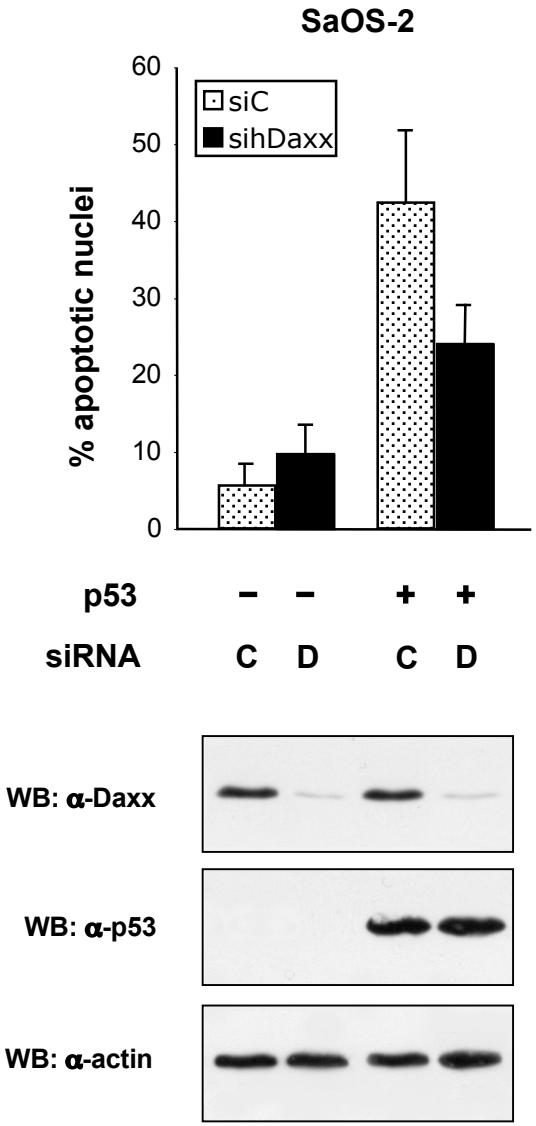
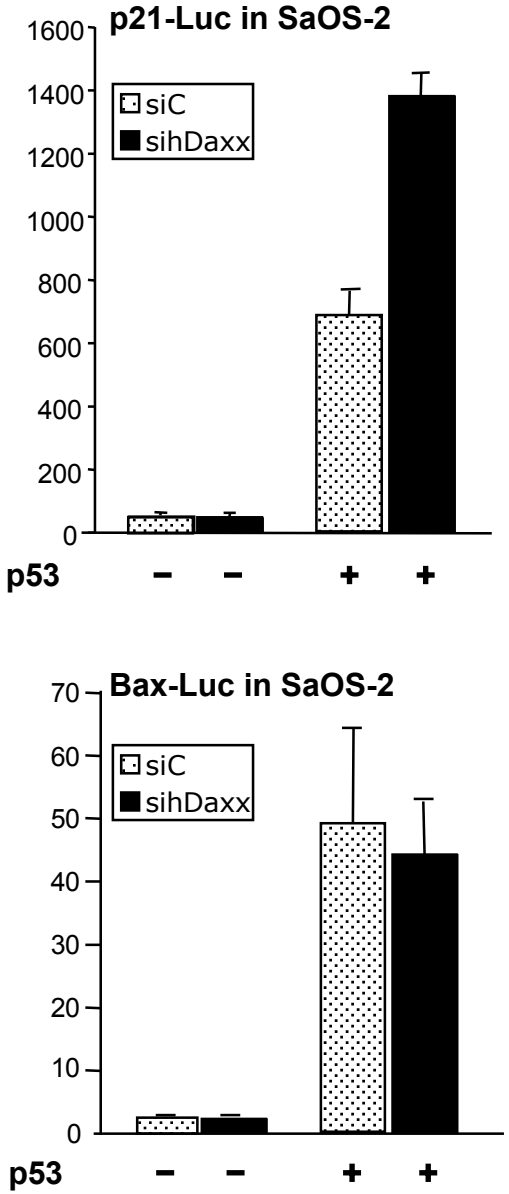


Fig. 6B



The transcriptional repressor hDaxx potentiates p53-dependent apoptosis

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J. Biol. Chem. published online August 31, 2004
