

Human T-Cell Leukemia Virus I Tax Protein Sensitizes p53-Mutant Cells to DNA Damage

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Abstract

Mutations in p53 are a common cause of resistance of cancers to standard chemotherapy and, thus, treatment failure. Reports have shown that Tax, a human T-cell leukemia virus type I encoded protein that has been associated with genomic instability and perturbation of transcription and cell cycle, sensitizes HeLa cells to UV treatment. The extent to which Tax can sensitize cells and the mechanism by which it exerts its effect are unknown. In this study, we show that Tax sensitizes p53-mutant cells to a broad range of DNA-damaging agents, including mitomycin C, a bifunctional alkylator, etoposide, a topoisomerase II drug, and UV light, but not ionizing radiation, a double-strand break agent, or vinblastine, a tubulin poison. Tax caused hypersensitivity in all p53-deleted cell lines and several, but not all, mutant-expressed p53-containing cell lines, while unexpectedly being protective in p53 wild-type (wt) cells. The effect observed in p53-deleted lines could be reversed for this by transfection of wt p53. We also show that Tax activates a p53-independent proapoptotic program through decreased expression of the retinoblastoma protein and subsequent increased E2F1 expression. The expression of several proapoptotic proteins was also induced by Tax, including Puma and Noxa, culminating in a substantial increase in Bax dimerization. Our results show that Tax can sensitize p53-mutant cells to DNA damage while protecting p53 wt cells, a side benefit that might result in reduced toxicity in normal cells. Such studies hold the promise of a novel adjunctive therapy that could make cancer chemotherapy more effective. [Cancer Res 2008;68(12):4843–52]

Introduction

Tax is a phosphoprotein encoded by human T-cell leukemia virus type I (HTLV-I) that plays important roles in viral reproduction and in the progression of adult T-cell leukemia (1, 2). Numerous studies have established that expression of Tax is sufficient for host cell transformation (3). The exact mechanism through which Tax is oncogenic is not completely understood. However, it is clear that this protein possesses potent pleiotropic functionality via protein-protein interaction and that these activities are manifest as transactivation or repression of a wide number of host cell genes

(4). Additionally, Tax expression results in loss of genomic integrity that may arise from the above transcription activities, squelching/recruitment of phosphatases, activation/inhibition of cell cycle kinases, and a variety of other mechanisms, as has been the subject of extensive review (4).

The p53 protein plays an important role in cancer biology because it is mutated in a majority of all human cancers. Such mutations account for a significant degree of clinical chemoresistance and are associated with a poor clinical outcome. Mutant forms of p53 may have dominant negative and transactivating activities with constitutively expressed p53 protein, and up to one third of cancers with mutant p53 show no p53 expression at all (5). Several groups have implicated Tax exerting its transforming effect by functional inhibition of p53 through mechanisms such as induction of p53 phosphorylation, sequestration of p300/CREB binding protein, and interaction with nuclear factor κ B (NF- κ B; ref. 6, 7). The end result has been a well-characterized inhibition of apoptosis presumably by interruption of a p53-dependent pathway (8). Interestingly, we and others have observed an enhancement of apoptosis by UV treatment in p53-null cells transfected with Tax (9, 10).

The goal of this work is to extend the findings of apoptotic enhancement to support the feasibility of using Tax as a chemosensitization tool to make cancer therapy more effective. Toward that end, we have undertaken to confirm the Tax effect in a number of different p53-null backgrounds, define the spectrum of types of DNA damage to which Tax will enhance sensitization, explore mechanistically the proapoptotic pathway triggered by Tax, and identify a minimal domain of Tax that will exert the same effect as expression of full-length protein.

Materials and Methods

Cell culture. Breast carcinoma cells MCF7 (provided by Amy Bouton, University of Virginia, Charlottesville, VA), human embryonal kidney cells 293T, HeLa, and mouse embryonic fibroblasts (MEF; provided by Peter Glazer, Yale University School of Medicine, New Haven, CT) were grown in DMEM supplemented with 10% bovine calf serum. JPX9 lymphoblasts and T47D breast cancer cells were grown in RPMI medium with 10% bovine calf serum. Cells were maintained at 37°C in a humidified 5% CO₂-containing atmosphere.

Plasmids and antibodies. Full-length Tax was cloned in His-c-myc-tagged pcDNA3 mammalian expression vector. pCMV-p53 expression vector was kindly provided by B. Vogelstein (John Hopkins University, Baltimore, MD). Deletion constructs of Tax were kindly provided by Ralph Grassmann (Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Erlangen, Germany). For E2F1 luciferase assays, we used the E2F1 reporter vector 3XE2F (Panomics).

Expression of Tax protein was detected with anti-c-myc (A-14) rabbit polyclonal antibody (Santa Cruz Biotechnology) or with direct anti-Tax, as previously described. E2F1 was detected with anti-E2F1 monoclonal antibody for human, clone KH20 (Upstate), and with mouse E2F1 protein polyclonal antibody (Cell Signaling). Expression of Puma and Noxa proteins

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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was detected with polyclonal antibodies from Assay Designs, Inc. Bax protein was detected with polyclonal rabbit antibody (N-20) from Santa Cruz Biotechnology, p53 with mouse monoclonal antibody from BD PharMingen, RB with monoclonal mouse antibody from Cell Signaling, and actin with monoclonal mouse antibody from Sigma.

Cytotoxicity and cell viability assay. Fibroblasts were seeded in six-well plates (3,000 per well). Cells were transfected with FuGene 6 transfection reagent (Boehringer Mannheim) according to the manufacturer's recommendations. Twenty-four hours after transfection, cells were treated with cytotoxic agents for different periods of time, as indicated in Results. Medium was aspirated and cells were fixed for 5 min with methanol/CH₃COOH. Survival cells were stained with 0.1% crystal violet in methanol. Colonies were extracted with methanol/SDS and absorbance was measured at 595 nm.

To measure the cytotoxicity in suspension cells, we used 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) dye reduction assay. Expression of Tax protein in JPX9 was induced with 10 μmol/L CdCl₂. Twenty-four hours later, lymphoblasts were placed in 96-well plates (300,000 per well). Several days after treatment with drugs, XTT (Diagnostic Chemicals) at 0.2 mg/mL concentration and 24 μmol/L phenazine methosulfate (Aldrich) were added to the cells followed by incubation at 37°C for 4 h; the absorbance was measured at 450 nm. The A₄₅₀ in control well (without drugs) was arbitrarily assigned a value of 100%. Mitomycin C (MMC) and etoposide were obtained from Sigma.

For cell viability assay, cells were plated in six-well plates (3,000 per well), transfected and treated with drugs as was described above. Cell viability was determined at the indicated time points using the trypan blue exclusion method. Cells were stained with trypan blue (final concentration, 0.2%) for 5 min and counted with a hemocytometer.

Statistical analysis. *P* value for each cell line was modeled separately using linear regression in SAS 9.1 and comparisons were made on the outcome measure of cell survival between control and Tax-expressing cells, both overall and at specific dose levels. In each model, the relationship between cell survival and cell types was unconstrained such that it was free to vary by dose level. Overall variation between experiments was also adjusted for in the model.

Detection of apoptosis. Apoptosis in JPX9 cells was quantified by staining with Annexin V-FITC/propidium iodide according to the manufacturer's recommendations, followed by flow cytometric analysis with FACScalibur (Becton Dickinson).

Cell cycle analysis. Cells (5×10^5) were washed with PBS and fixed in cold 95% ethanol for 30 min. After second wash, cells were resuspended in 1-mL PBS containing RNase (50 μg/mL) and incubated at 37°C for 30 min. Cells were stained with propidium iodide (50 μg/mL) in PBS for additional 30 min at 4°C. Samples were analyzed with a FACScan (Becton Dickinson).

Immunoblotting. For immunoblot analysis of protein expression, cells were lysed in NP40 buffer. One hundred micrograms of proteins were resolved by SDS-PAGE gel and transferred onto a nitrocellulose membrane. After blocking in 5% milk, membranes were incubated with the indicated primary antibody diluted in milk for 1 h at room temperature, followed by incubation with the appropriate secondary alkaline phosphatase-conjugated antibody. Immunoreactivity was detected by Western chemiluminescence protein detection (Pierce).

Luciferase reporter gene assay. For luciferase assay, cells were transfected with plasmid expressing wild-type (wt) or the indicated Tax deletions. After 24 h, the cells were cotransfected with the E2F reporter vector. The next day, luciferase assay was done according to the manufacturer's recommendation. Luciferase kit was purchased from Promega.

Results

Tax increases cytotoxicity caused by DNA-damaging agents.

Two previous reports have shown increased sensitivity induced by Tax to UV treatment in p53-null cells (9, 10). To confirm this finding, we transfected 293T cells with pcDNA3-Tax wt expressing

vector. Due to inactivation by the presence of SV40 large T antigen, 293T cells are null for p53. As a control for the efficiency of transfection, the same cell lines were transfected with pcDNA-GFP. Direct fluorescence analysis of these cells 48 hours later showed at least 60% transfection efficiency. To confirm protein expression, 100 μg of crude lysate prepared from each transfection were run by SDS-PAGE and immunoblotted for Tax (Fig. 1A). Cells expressing Tax or empty vector were exposed to a range of UV doses, and viability was determined by crystal violet assay 4 days after irradiation. 293T cells expressing Tax exhibited a significantly greater degree of sensitivity to UV than cells transfected with vector alone (Fig. 1B), consistent with the published data that Tax expression causes increased apoptosis.

To define the range of agents that show the Tax sensitization effect, we exposed the *tax* and vector transfectants to MMC, a bifunctional DNA double-strand cross-linking agent, measured after 7 days of exposure. As was the case with UV treatment, MMC induced greater cytotoxicity in the presence of Tax (Fig. 1C). Additional agents tested included etoposide, a topoisomerase II inhibitor (Supplementary Fig. S1). Cytotoxicity of etoposide was measured by crystal violet assay after 4 days of drug treatment. As in the case of UV and MMC, etoposide induced greater cytotoxicity in Tax-expressing cells. Unlike UV, MMC, and etoposide, Tax did not induce increased cytotoxicity after ionizing radiation, a relatively pure double-strand break agent, or vinblastine, a tubulin poison exerting its effect at mitosis, both measured after 4 days (Supplementary Fig. S1). These data suggest that the Tax effect is not universal; rather, Tax seems to sensitize agents that exert their effect, at least in part, due to replication fork collapse. This effect was seen in versions of non-epitope-tagged Tax as well (data not shown).

Tax chemosensitizes p53-null cells but confers resistance on p53 wt cells. Because 293T cells are immortalized and in culture for a considerable time, we wanted to assess the Tax effect in other p53-null backgrounds. First, we analyzed the MMC sensitivity of MEFs that were either wt or null for p53. We transfected both cell lines with a vector expressing Tax, and the level of expression was confirmed after 24 hours by Western blot analyses (Fig. 2A). The cells were treated with the indicated concentration of MMC. Control p53^{-/-} MEF cells were clearly more resistant to MMC than control p53^{+/+} MEFs. In contrast to the resistant p53^{-/-} MEFs transfected with vector only, the same cells expressing Tax displayed marked sensitivity to MMC. Surprisingly, the p53^{+/+} cells not only were not sensitized but in fact were more resistant in the presence of Tax. The doses of MMC that these delineated differences were relatively low (25–250 nmol/L) and in line with published reports that show serum concentrations in patients in the low nanomolar range (11, 12).

To extend our analysis to a human system, we used a p53 wt cell line, MCF7 cells. A comparison of the effect of p53 expression on mediating the Tax effect on chemosensitization was achieved using selected knockdown of p53. To simulate a p53^{-/-} background, cells were transfected with pSUPER-p53 RNAi directed for synthesis of siRNA against 19 nucleotides localized at the 3' end of the p53 transcript (13). Immunoblotting revealed loss of p53 expression even after DNA damage (Fig. 2B). These resulting p53-null cells were subjected to Tax transfection. Selective knockdown of p53 resulted in increased resistance to MMC when compared with the parental MCF7 cells or control knockdown with siRNA against lamin A. Introduction of Tax into the p53 knockdown background resulted in cells that were markedly more sensitive to MMC. As

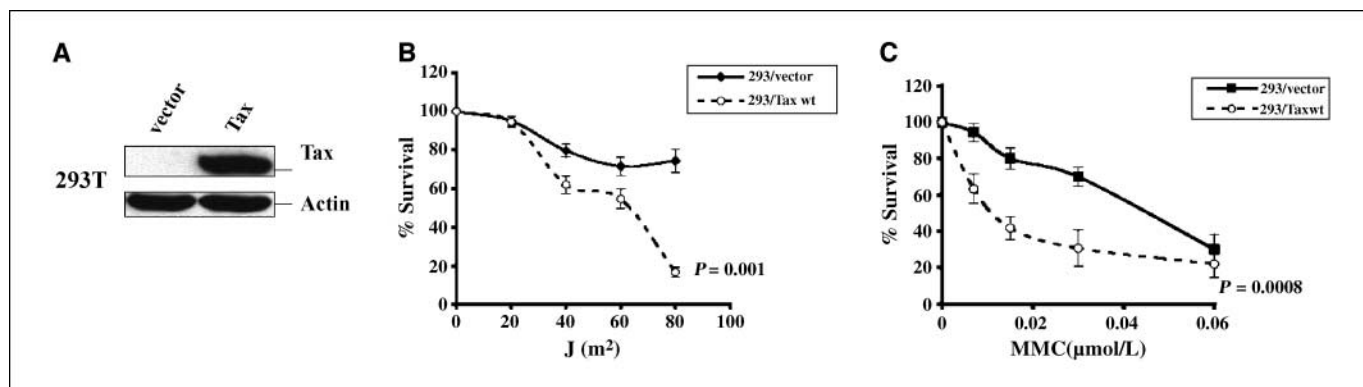


Figure 1. Tax increases cytotoxicity caused by the DNA-damaging agents UV and MMC. 293T cells were transfected with either empty vector or c-myc-Tax and subjected to UV treatment. **A**, lysates were analyzed by SDS-PAGE and immunoblotting, revealing Tax expression in the transfected cells. **B**, cells were analyzed by crystal violet assay to measure cell survival. Cells transfected with Tax were more sensitive to UV treatment than those receiving empty vector. Cytotoxicity experiments were conducted at least three independent times. **C**, the same experiment was conducted using MMC. Cells transfected with Tax were more sensitive to MMC.

in the case of the experiments using MEFs, the parental MCF7 (wt p53) became resistant with the provision of Tax expression. Tax transfection into cells with siRNA against lamin A had the same effect as the parental MCF7 cells (data not shown).

As an additional system for analysis of the Tax effect, we transfected HeLa cells, which are p53 inactivated because of long-standing human papillomavirus infection. As in the previous cell systems, Tax-expressing HeLa cells displayed increased sensitivity to MMC (Fig. 2C). We supplied the contrasting p53^{+/+} model by overexpression of wt p53 by transient transfection into HeLa. HeLa cells transfected with wt p53 were able to express detectable p53 protein in spite of HPV infection. These HeLa cells transfected with p53 became sensitive to MMC. On provision of *Tax*, these cells were rendered resistant, again consistent with the data obtained using the MCF7 and MEF model systems. Thus, in four different p53-null cell backgrounds, Tax exerts a chemosensitization effect while conferring resistance on p53 wt cells. These data are summarized in Fig. 3C.

Tax chemosensitizes some p53 mutant-expressing cells. Cancers that are p53 mutant make up a majority of all tumors. Within that group, at least two thirds constitutively express a mutant p53 protein, consisting of gain of function, loss of function, and dominant negative mutations. To test other cell lines with constitutive p53 expression, we first used a Jurkat-derived line that expresses a non-epitope-tagged Tax under control of an inducible metallothionein promoter. These JPX9 cells display constitutive p53 expression (Fig. 3A). Incubation with 10 µmol/L CdCl₂ induced Tax expression within 24 hours. Concomitant exposure to MMC induced marked chemosensitivity in JPX9 cells expressing Tax. No deleterious effect was noted with cadmium exposure alone in Jurkat cells (data not shown). Not every case of mutant-expressed p53-containing cell lines displayed chemosensitization in the presence of Tax, as breast cancer cell line T47D was unaffected (Fig. 3B). These data suggest that at least a subset of p53-mutant expressing cells is amenable to the Tax chemosensitization effect. This is significant because a majority of tumors mutant for p53 express mutant p53 protein constitutively. All data for the cell lines above are summarized in Fig. 3C.

Tax expression induces cell death by apoptosis. We next wanted to determine whether the cell death observed in damaged Tax-expressing cells was due to apoptosis. We analyzed MMC-treated JPX9 cells (Fig. 4A) by Annexin V staining and subjected the

resulting permeabilized cells to flow cytometric analysis. A histogram showing Annexin V staining shows that expression of Tax alone did not result in a dramatic induction of apoptosis in cells in the absence of DNA damage. Whereas a modest degree of apoptosis was seen in cells treated with MMC only, a significantly increased amount of apoptosis was seen in these treated cells expressing Tax. To confirm the activation and cleavage of caspase, an early indicator of apoptosis, we immunoblotted lysates prepared from similarly treated cells. Analysis of caspase-3 revealed diminution correlating with increased apoptosis. A modest degree of caspase decrease can be seen in lane 2, but a significantly greater amount of decrease is seen in lane 4, in which cells express Tax. Densitometric analysis confirmed diminished full-length caspase-3. A cleavage byproduct of caspase-3 was seen only in lane 4 in the presence of Tax and DNA damage due to a significant loss of full-length caspase. To confirm that Tax-expressing cells were actually dead and not cytostatic, we counted trypan blue-stained cells in parallel to the apoptosis analysis in JPX9 cells, with cells staining blue counted as dead. Trypan blue staining was confirmatory of the apoptosis and the cell survival data showing that Tax sensitized JPX9 cells (Supplementary Fig. S2). We additionally repeated the analysis on all the cell lines from Fig. 2, showing that the p53 mutants died in the presence of Tax and MMC, whereas p53 wt cells were resistant. This result was also seen in all the cell lines that were examined in Figs. 2 and 3, confirming the Tax chemosensitization of p53 mutant or knockdown cells and the resistance of wt p53 cells (data not shown for lamin A knockdown).

To confirm that Tax did not result in cell cycle arrest, we measured DNA flow histograms on 293T and JPX9 cells (Fig. 4B). This assay was done at 12 hours of drug exposure, well before any detectable onset of apoptosis. Both cell lines on Tax expression resulted in either no change in cell cycle flow (293T) or increased S phase (JPX9). DNA-damaging conditions resulted in marked elevation in S phase. These data indicate that (a) tax transfection does not result in cell cycle arrest in these cells and (b) p53-mutant cells expressing Tax are able to progress into S phase.

Tax targets retinoblastoma protein and downstream effectors of apoptosis. The retinoblastoma protein (pRB) is a prototypical tumor suppressor. At the cellular level, RB protein inhibits S-phase entry and cell cycle progression (14); promotes cell differentiation, inducing tissue-specific gene expression (15); and

promotes cellular survival as a response to various proapoptotic stresses (16).

The best-characterized targets for the pRB are the E2F transcription factors (17). The ability of pRB to suppress apoptosis has been attributed to its repression of E2F family and downstream of E2F target genes. Disruption of the pRB pathway induces

apoptosis through up-regulation of Apaf-1, which is an essential stimulator of caspase activity (18, 19).

Deletion of E2F1 or E2F3 protected RB-null cells from apoptosis (20, 21). Recent studies have identified E2F binding site in the promoters of several caspase genes, including caspase-3, caspase-7, caspase-8, and caspase-9 (22). In addition, E2F1 up-regulates

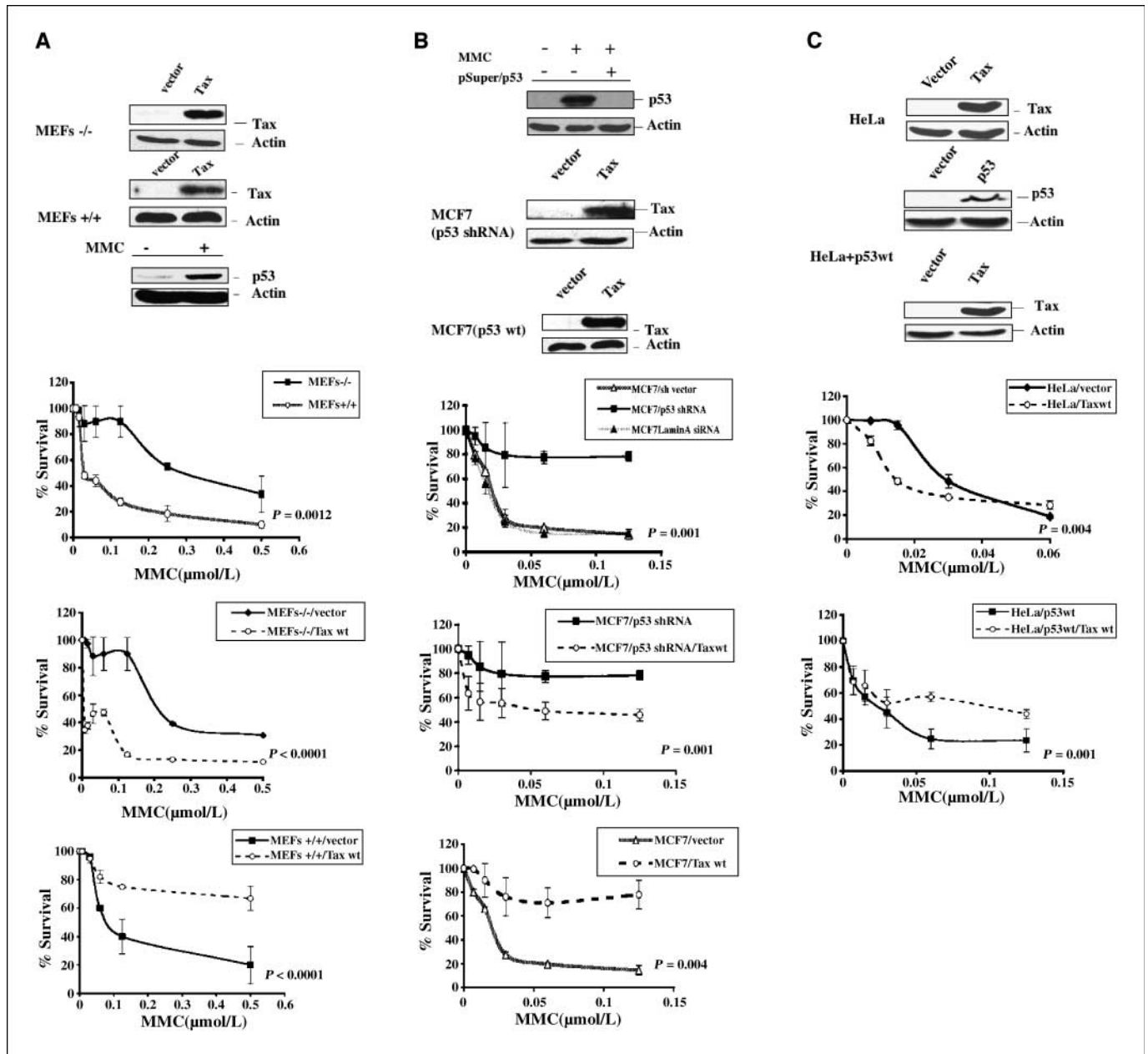


Figure 2. Tax chemosensitizes p53-null cells but confers resistance on p53 wt cells. **A**, MEFs either wt or null for p53 were transfected with either empty vector or c-myc-Tax and then treated with 1 $\mu\text{mol/L}$ MMC. Lysates from both sets of MEFs were analyzed for Tax expression by immunoblotting for c-myc. p53 wt MEFs and p53-null cells were treated with MMC at the indicated doses and analyzed by crystal violet staining to assess cytotoxicity. MEFs null for p53 were more resistant than wt MEFs. On Tax expression, p53-null MEFs became more sensitive, whereas wt p53 cells became more resistant. **B**, MCF7 cells were transfected with pSUPER-p53 to knock down expression of p53. The resulting cells were then transfected with empty vector or c-myc-Tax. Lysates from these transfected cells were prepared and analyzed for p53 and Tax expression. The MCF7 transfectants were analyzed at the indicated MMC doses and analyzed by crystal violet assay to measure cytotoxicity. The resultant p53-null MCF7 cells were more resistant than p53 wt cells. Transfection of control siRNA directed against lamin A had no effect on cell sensitivity to MMC. On Tax expression, p53-null MCF7 cells became more sensitive to MMC, whereas wt p53 MCF7 cells were more resistant to MMC. **C**, HeLa cells were transfected with Tax and subjected to crystal violet cytotoxicity assay, and lysates were immunoblotted for Tax expression. HeLa cells expressing Tax became more sensitive to MMC. HeLa cells were transfected with p53 to overcome the inhibition of endogenous p53 engendered by HPV infection. On Tax transfection, cells were analyzed by crystal violet assay for cytotoxicity, and lysates were immunoblotted to measure Tax and p53 expression. On MMC treatment, measurable p53 was detected by immunoblot. HeLa cells exogenously expressing p53 became more sensitive. On Tax expression, exogenously p53-expressing HeLa cells were more resistant. Cytotoxicity experiments were conducted at least three independent times.

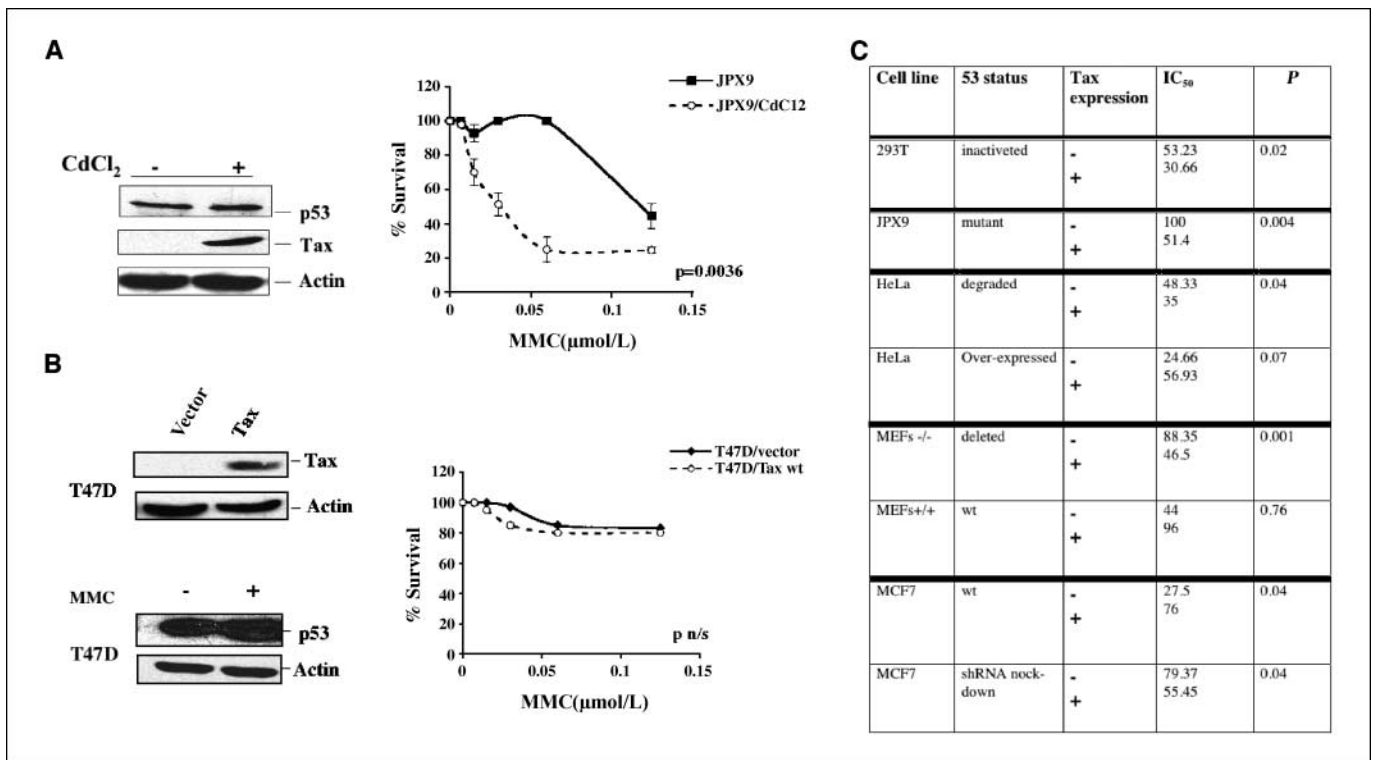


Figure 3. Tax chemosensitizes some p53 mutant-expressing cells. **A**, JPX9 cells expressing Tax under the influence of a Cd inducible promoter were treated with MMC. Lysates were analyzed by immunoblotting. Tax expression was induced on Cd exposure. The same cells were subjected to MMC treatment. Crystal violet assay to measure cytotoxicity showed that JPX9 cells became more sensitive to MMC on Tax expression. **B**, T47D were transfected with Tax and analyzed by immunoblot and cytotoxicity assay. The cells expressed p53 constitutively. T47D cells remained resistant on Tax expression. Cytotoxicity experiments were conducted at least three independent times. **C**, IC₅₀ for MMC was ascertained from the graphs in Fig. 2 and this figure and tabulated in a summary form.

the expression of proapoptotic (BH3)-only proteins, Puma, Noxa, Bim, Hrk/DP5, and Bik, through a direct transcriptional mechanism (23, 24).

To investigate the potential role of pRB in Tax-mediated apoptosis, we analyzed protein extracts prepared from cells inducibly expressing Tax. The subsequent blots were immunoassayed for pRB. As shown in Fig. 5A, in the presence of Tax in p53-null (293T) or mutant-expressed p53-containing cells (JPX), the level of pRB decreased significantly.

Earlier we observed that the Tax effect was reproducible in other species and we therefore analyzed the MEFs for pRB expression in the presence of Tax. In MEFs wt for p53, we detected stable expression of pRB protein, whereas pRB was diminished in p53-null MEFs (Fig. 5B). In all these situations, E2F1 was concomitantly increased on RB decrease.

It is well known that a variety of E2F1 target genes contribute to apoptosis. Apoptotic activity of E2F1 in p53-null cells and transgenic mice has been documented in several studies (25, 26). Therefore, to investigate these downstream targets of the pRB pathway, we immunoblotted protein extracts from Tax-expressing cells in the presence or absence of MMC with antibody detecting Puma or Noxa proteins. We analyzed both JPX-9 cells with the inducible Tax system and 293T cells transfected with either vector only or Tax. Increased expression of Puma or Noxa was detected in both cell lines on Tax expression (Fig. 5C).

To show that other species displayed a similar effect, we analyzed the MEF p53 wt and p53^{-/-} cells transfected with either control vector or Tax. In this case, blotting was only done with Puma due to availability of murine antiserum. Again, Tax induced

increased expression of Puma in p53-null cells but not in MEFs cells expressing wt p53 (Fig. 5C). In all cell types that were sensitive, we detected Bax dimers or oligomers in response to Tax, even in the absence of MMC, indicating Bax activation.

We previously showed that not all p53 mutant-expressing cells were sensitized by p53 mutant-expressing cells, such as T47D (Fig. 3B; negative control in our work). To test the effect of Tax expression on these effectors in T47D, we immunoblotted for Puma, Noxa, and Bax with extracts derived from cells transfected with either vector or Tax. We did not detect expression of Puma, Noxa, or Bax dimers or oligomers, although pRB protein level decreased and E2F1 increased significantly, respectively (Fig. 5D).

These data are consistent with the idea that Puma and Bax are down-regulated in p53 wt cells but up-regulated in p53-deleted cells and some p53 mutant-expressing cells in the presence of Tax. Changes of the protein levels of either Bcl_{XL} or Bcl2 were not detected (data not shown). These data support the idea that Tax mediates apoptosis through a common p53-independent pathway via E2F, whereas the downstream targets of E2F may be cell type specific. E2F1 has been implicated in death pathways either in a p53-dependent or a p53-independent manner, likely as a result of cell type differences as well as heterogeneous genetic and epigenetic factors (27–29).

Tax1 chemosensitization effect restricted to the NH₂ terminus. Tax1 contains several identifiable domains, including a cyclic AMP-responsive element binding protein (CREB) binding segment (2–34 amino acids), cyclin-dependent kinase (cdk) binding motif (11–25 amino acids), CPB binding domain (59–98 amino acids), and PBD (342–353 amino acids; refs. 30–32). To identify the

domain responsible for the chemosensitization effect, we used a series of deletion mutants transfected into 293T cells. The transfectants were tested for MMC sensitivity as above after verification of expression (Fig. 6A). The deletion of the NH₂-terminal 40 amino acids (corresponding to the CREB and cdk4 binding site) resulted in complete abrogation of the Tax effect. To show the specificity of Tax activity and its location in the NH₂ terminus, we also tested other deletion mutants of Tax in which 40-amino-acid regions were deleted downstream in tax1, including Δ99-141 and Δ320-353. The 99-141 mutant is notable for encompassing the NF-κB binding domain. In both cases, expression of these deletion mutants resulted in chemosensitivity (Fig. 6A). In addition, we checked the response of JPX9 cells expressing a point mutant (R222K) localized in the leucine zipper domain. Expression of the point mutant sensitized the cells to MMC to the same extent as wt Tax (Fig. 6B). Thus, the entire activity of Tax in p53-null cells is contained within the NH₂-terminal portion of the protein.

To show the importance of the NH₂ terminus of Tax in the induction of apoptosis, we analyzed RB and E2F1 expression in the setting of the different Tax deletion mutants. Whereas full-length Tax and the downstream deletions resulted in RB diminution and increased E2F1 expression, removal of the NH₂ terminus resulted in persistent RB expression and diminished E2F1 (Fig. 6C). To verify the transcriptional activity of the increased E2F1, we transfected 293 cells with the deletion mutants of Tax, along with a reporter plasmid with E2F1 sites in the promoter in frame with a luciferase reporter construct. Consistent with immunoblotting and survival data, E2F1 responsiveness only occurred when the NH₂ terminus was intact.

Discussion

In this study, we address a major issue in cancer therapeutics: how to overcome the resistance engendered by a mutant p53 gene.

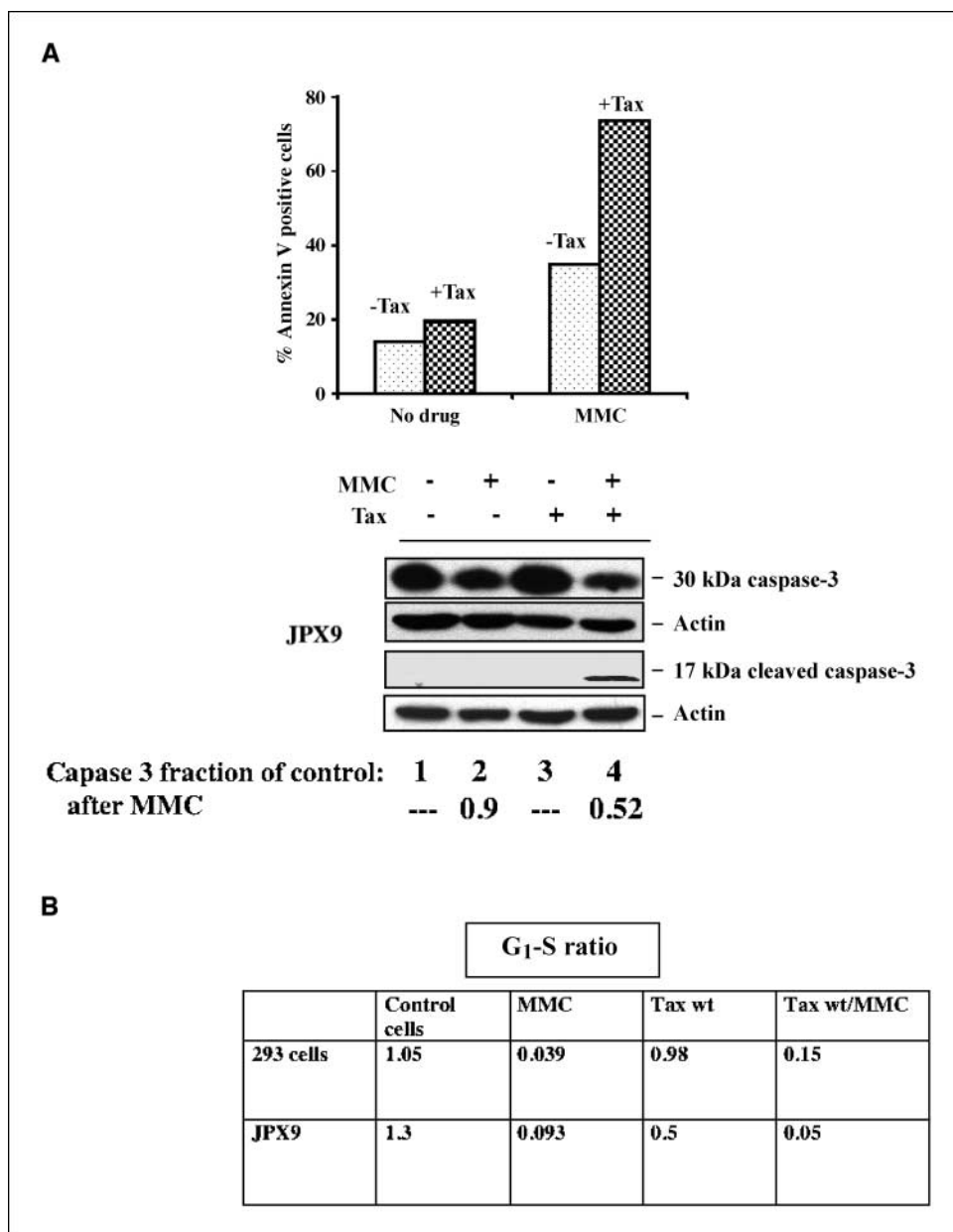


Figure 4. Tax expression induces cell death by apoptosis. **A**, JPX9 cells uninduced or induced for Tax expression were permeabilized and analyzed with Annexin V antibodies. Flow cytometric analysis showed markedly increased Annexin V staining in Tax-expressing cells on MMC treatment. Lysates prepared from the same cells were immunoblotted for caspase-3. Densitometric analysis revealed the most diminished caspase-3 on MMC treatment in the presence of Tax, and the cleavage product of caspase-3 was observed only in these cells. **B**, JPX9 cells and 293T cells transfected with Tax were treated with 1 μmol/L MMC and subjected to DNA flow analysis after 24 h of exposure. Results indicated that Tax did not induce an arrest in G₁ and that cells increased in S phase following MMC treatment.

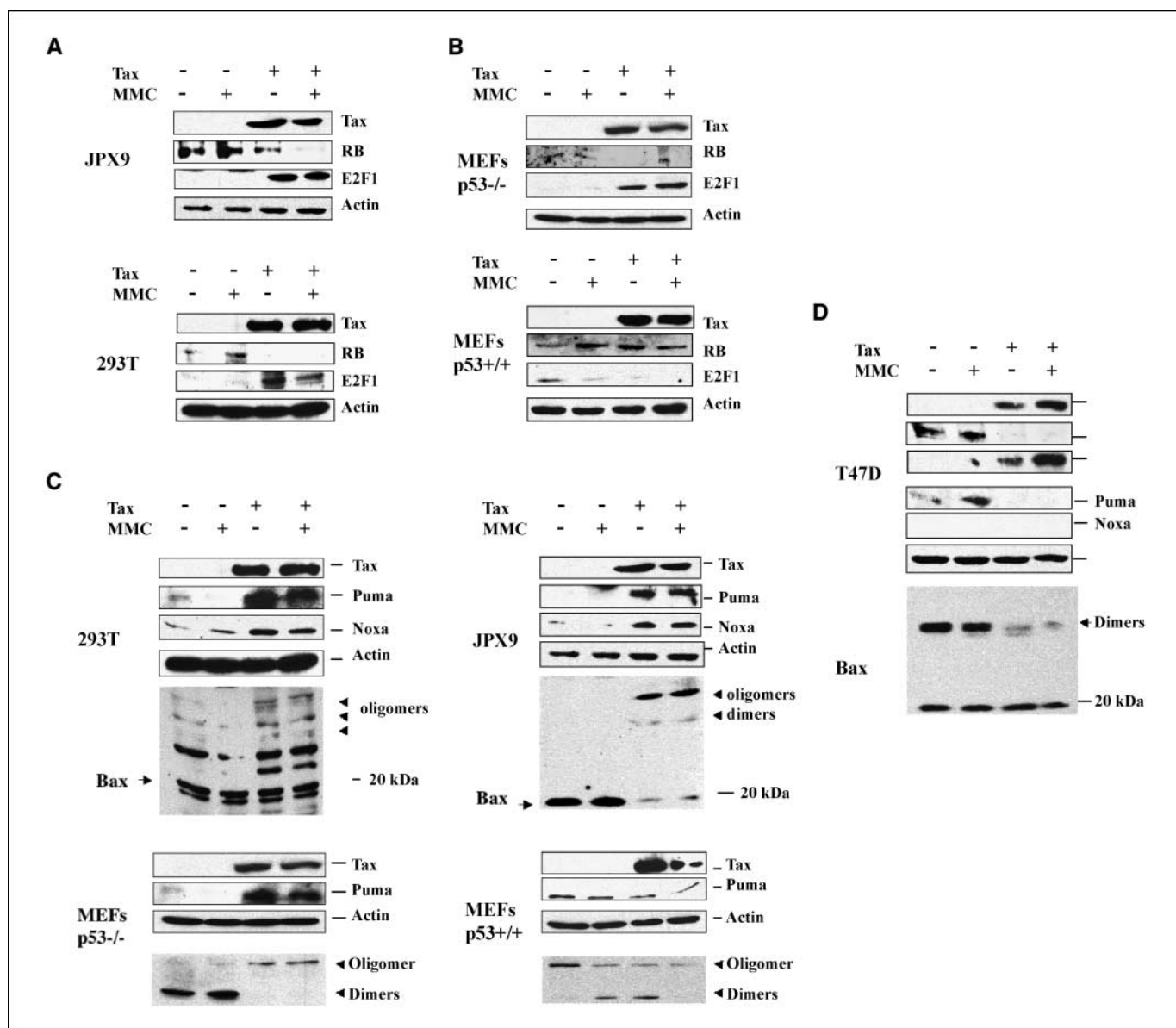


Figure 5. Tax induces decreased pRB and increased expression of E2F1 and downstream effectors of apoptosis. *A*, 293T cells were transfected with Tax, and JPX-9 cells were either uninduced or induced with CdCl₂ to turn on Tax expression. The cells were treated with MMC, and lysates prepared from them were immunoblotted for Tax, pRB, E2F1, and actin. Tax induced decreased pRB and increased E2F1. *B*, MEFs either p53 wt or p53^{-/-} were transfected with either control vector or Tax. The cells were then treated with MMC, and lysates prepared from them were immunoblotted for Tax and RB. Tax induced decreased pRB and increased E2F1 in p53^{-/-} MEF cells. *C*, JPX-9 and 293T cells prepared as in *A* were analyzed by immunoblotting to measure the proapoptotic proteins Puma, Noxa, and Bax. Tax induced expression of these proteins in nontreated and MMC-treated cells. MEFs prepared as in *B* were analyzed by immunoblotting to measure the proapoptotic proteins Puma and Bax. Tax induced expression of these proteins in sensitized cells. *D*, the same proteins as in *A* to *C* were analyzed in the Tax-resistant p53 mutant-expressing cell line T47D. Whereas protein level of RB was decreased by Tax, up-regulation of Puma and Noxa was not detected.

In the course of our work on HTLV-I Tax, we have made the observation that Tax conferred increased apoptosis in p53-null cells on UV treatment (9). Building on this finding, we asked if Tax could generally result in increased sensitivity to a range of chemotherapeutic agents commonly used in clinical oncology. Interestingly, whereas agents such as MMC and etoposide induced increased cytotoxicity in the presence of Tax, ionizing radiation and vinblastine did not. This observation applied to all p53-null cell lines we tested as well as to most mutant p53-expressing cells, suggesting that the Tax effect may be generally applicable to most p53-mutant cells. Unexpectedly, p53 wt cells achieved protection from DNA damage in the presence of Tax, suggesting a side benefit

of the concomitant administration of drug and Tax. We further show that, in sensitizing cells to DNA damage, Tax triggers a p53-independent proapoptotic pathway via increased expression of E2F1.

HTLV-I is known as the causative agent in progression to adult T-cell leukemia, and Tax has been implicated as an oncogene with transforming ability. Tax, when expressed in cells, results in genomic instability, which is thought to be the driving force of its oncogenic effect (33, 34). On the other hand, Tax is widely thought to affect the cell cycle via induction of p21 and p27 and interaction with cdk4, leading to phosphorylation of RB, RB degradation, and thus increased E2F1, although the dependence on p53 is unclear

(35, 36). Tax has been proposed to bind to RB and promote its degradation (37).

Tax has also been shown to constitutively activate c-jun kinase in the stress response, using activating transcription factor 2 rather than CREB or NF- κ B (38, 39). This would argue against the importance of the CREB site and in favor of the cdk4 site in the NH₂ terminus.

Thus, Tax has a well-described mode of action of perturbation of the cell cycle that could explain, at least in part, our findings. First, Tax is known to activate E2F1 via a transcriptional mechanism (40), although it is not thought to occur via direct interaction with the promoter (41). Some suggest a mechanism through binding of and perturbation by Tax with RB (37, 42). Second, because p53-mutant cells do not arrest normally at the G₁-S border in the wake of DNA damage, they then proceed into S phase in an E2F1-mediated fashion, where activation of the apoptotic pathway occurs. Tax has been shown to promote transition to S phase in a human T-cell line (Kit 225) and peripheral blood lymphocytes through E2F1 (43). By a similar mechanism, another virus oncoprotein, E7, overcomes p21-mediated cell cycle arrest through deregulation of E2F activity by interacting with RB (44, 45). Kao et al. (46) showed that Tax stimulated DNA synthesis in the presence of DNA damage but inhibited repair, thereby activating apoptosis. Our data show that Tax triggers a p53-independent proapoptotic pathway via disruption

of the RB pathway. These effects have proved to be a bit more variable, as Noxa and Puma are variably expressed at higher levels, although terminal Bax activation and oligomerization are common events among all the cell lines examined.

Unexpectedly, p53 wt cells achieved protection from DNA damage in the presence of Tax, suggesting a side benefit of the concomitant administration of drug and Tax. Some have postulated that Tax inhibits the activity of p53 (47, 48), allowing the bypass of the G₁-S checkpoint and an antiapoptotic state. Our data support this supposition, at least in part. Whereas it is possible that the checkpoint is no longer engaged in the p53 wt state in the presence of Tax, nonetheless some function of p53 is left intact, as apoptosis is prevented even after DNA damage. Such interaction with checkpoint control may be governed via Tax interaction with another kinase, chk2, which phosphorylates p53 and may play a role in stabilization of p53 in the setting of Tax (49).

The ultimate goal of these studies is to lay the groundwork for eventual application for enhanced cancer therapy. At least half of all cancers are mutant for p53, and the most common cause for therapy resistance can be attributed to p53 mutations. Whereas most p53 mutants in cancer expressed p53 protein, up to a third may be null mutants (5, 50). In our study, all the null cell lines were sensitized, whereas a subset of those with expressed p53 mutants were sensitized. Thus, it is likely that Tax as a therapeutic adjunct will not be effective in the setting of all p53 mutants. Nonetheless,

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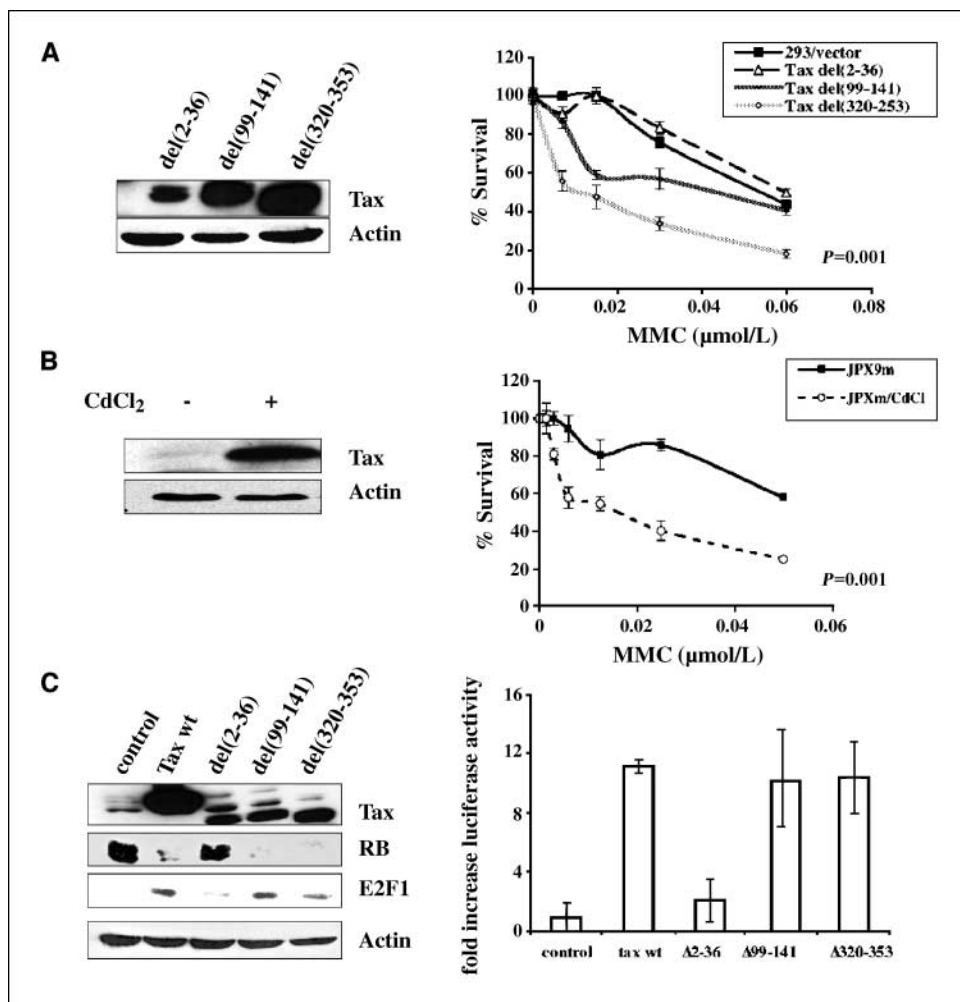


Figure 6. Tax1 chemosensitization effect restricted to the NH₂ terminus. **A**, deletion mutants of Tax were transfected into 293T cells. Lysates from the resulting cells were immunoblotted to confirm Tax expression. The same cells were subjected to a crystal violet survival assay in the presence of MMC. Only the deletion of the NH₂-terminal 36 amino acids abrogated the Tax sensitization effect. **B**, JPY9m cells expressing a Tax protein mutated at amino acid 222 were subjected to crystal violet assay in the presence of MMC. Mutation of this amino acid did not change the sensitization effect. **C**, cells from **A** were used to assess the effect of the deletion on pRB and E2F1 expression. Deletion of the NH₂-terminal amino acids resulted in persistence of pRB and lack of E2F1 induction. A luciferase assay with an E2F1 inducible promoter revealed that deletion of the NH₂ terminus abrogated induction of luciferase activity.

even if only effective in a subset of p53-mutant tumors, such an approach would still have the advantage of being used in a large number of cases. By necessity, this approach would require the screening of each case by PCR and/or immunohistochemistry to both define the type of p53 mutation and determine if protein is expressed.

Long-term infection by HTLV-I has been associated with long-term risk of T-cell leukemia (i.e., after 20–50 years), raising concerns that Tax exposure would cause potential for cancer risk after therapy. This is obviated by the fact that such therapy would be envisioned for resistant tumors and, thus, in patients with otherwise short-term survival. In addition, such exposure would be relatively limited in time. An additional difficulty concerns the delivery of Tax to the patient and tumor, especially given the problems associated with gene therapy strategies. Toward that end, we have begun to map domains associated with both the sensitization activity of Tax in p53-mutant cells and the resistant phenotype in p53 wt cells. Preliminary data suggest the possibility of using actual protein fragments of Tax. Such uses of proteins would not be especially immunogenic in patients having undergone considerable previous chemotherapy. Most intriguingly, wt p53 cells (i.e., the healthy cells of the patient) would be protected

under such a scenario, as our data imply that Tax confers resistance on such cells. Patients would thus suffer fewer side effects and achieve greater efficacy from their cancer therapy as a result of an even greater therapeutic window. Such an outcome is especially important in patients whose tumor has not been responsive to first-line therapy or who have relapsed with resistant disease. Identification of the mechanism of Tax sensitization and protection may also identify new targets that can be manipulated by other agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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