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# Identification and Characterization of the Cytoplasmic Protein TRAF4 as a p53-regulated Proapoptotic Gene*

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**The role of p53 in tumor suppression partly relies on its ability to transcriptionally regulate target genes involved in the initiation of cell cycle arrest or the activation of programmed cell death. In recent years many genes have been identified as p53-regulated genes; however, no single target gene has been shown to be required for the full apoptotic effect. We have identified TRAF4 as a p53-regulated gene in a microarray screen using a Murine 11K Affymetrix GeneChip hybridized with cRNA from the p53 temperature-sensitive cell line, Vm10. TRAF4 is a member the TRAF family of adaptor proteins that mediate cellular signaling by binding to various members of the tumor necrosis family receptor superfamily and interleukin-1/Toll-like receptor superfamily. In contrast to its other family members, TRAF4 has not been shown to bind to a member of the tumor necrosis factor receptor superfamily *in vivo*, nor has it been shown to regulate signaling pathways common to its other family members. Therefore the role of TRAF4 in a signaling pathway has not yet been established and requires further study. TRAF4 is specifically regulated by p53 in response to temperature sensitive p53, overexpression of p53 by use of an adenovirus, and stabilization of p53 in response to DNA damage. The murine TRAF4 promoter contains a functional p53 DNA-binding site ~1 kilobase upstream of the initiating methionine. TRAF4 localizes to the cytoplasm and appears to remain in the cytoplasm following DNA damage. Interestingly, the overexpression of TRAF4 induces apoptosis and suppresses colony formation. These data suggest a correlation that the orphan adaptor protein TRAF4 may play a role in p53-mediated proapoptotic signaling in the response to cellular stress.**

The tumor suppressor gene p53 is activated in response to a variety of cellular stresses including DNA damage, nucleotide depletion, and oncogene stimulation (1, 2). The most well understood role of p53 in tumor suppression is the ability to activate or repress target genes involved in cell cycle arrest, senescence, and apoptosis (2, 3). The cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ is required for p53-mediated $G_1$ arrest and 14-3-3$\sigma$ and GADD45 are p53 target genes important for the maintenance of a $G_2$ arrest (4–9). However, the role of p53 in apoptosis is less well understood. To date, numerous p53 transcriptionally regulated target genes have been identified that are involved in p53-mediated apoptosis including BAX, BID, NOXA, PUMA, PIDD, p53AIP1, PERP, FAS/APO1, KILLER/DR5, and PIGs; however, no single target gene has been shown to be required for the full effect (10–21).

The tumor necrosis factor receptor (TNFR)[1]-associated factor (TRAF) family of proteins is involved with both pro- and anti-survival signals. Originally identified as adaptor proteins for members of the tumor necrosis factor receptor (TNFR) superfamily, the TRAF proteins are involved with the activation of the stress response culminating in the activation of either c-Jun or NF-$\kappa$B and/or the initiation of apoptosis depending on the cellular circumstance (22–24). To date, all members of the TRAF family, with the exception of TRAF4 have been shown to bind to various members of the TNFR family and the Toll/IL-1 receptor family to initiate their respective signaling pathways (reviewed in Refs. 22–25).

Unlike its other family members, TRAF4 has not been shown to bind strongly to a receptor, exceptions being weak interactions with lymphotoxin-$\beta$ receptor and p75 neural growth factor receptor; however, the *in vivo* significance of these interactions remains unclear (26, 27). Originally cloned from a screen using metastatic breast cancer samples, TRAF4 was found to be located on human chromosome 17q11-q12, in the region that contains the oncogene c-erbB2 (28, 29). While TRAF4 contains both the conserved C-terminal TRAF domain and the N-terminal RING finger domain common to all TRAF family members (except TRAF1 does not contain a RING finger domain), it also appears to have two potential nuclear localization signals, distinguishing it from other family members (29). However, conflicting evidence reported the localization of TRAF4 to be predominantly in the cytoplasm or the nucleus (26, 29, 30). TRAF4 is expressed at high levels during embryogenesis and TRAF4-deficient mice have some developmental defects (31–33). Knockout of TRAF4 leads to improper development of the trachea and the mice also present with pulmonary defects (32). An additional study generated the TRAF4 knock-out mice in a different genetic background, and these mice had both spinal and pulmonary abnormalities (33). In both cases, the immune system appeared normal, a phenotype distinct from other TRAF family member-deficient mice, which appear to have varying degrees of defects in the immune system (reviewed in Ref. 24). Taken together, little is known about the biological function or regulation of TRAF4.

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[1] The abbreviations used are: TNFR, tumor necrosis factor receptor; TRAF, tumor necrosis factor receptor-associated factor; Z, benzyloxycarbonyl; DAPI, 4′,6-diamidino-2-phenylindole; NLS, nuclear localization signal; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

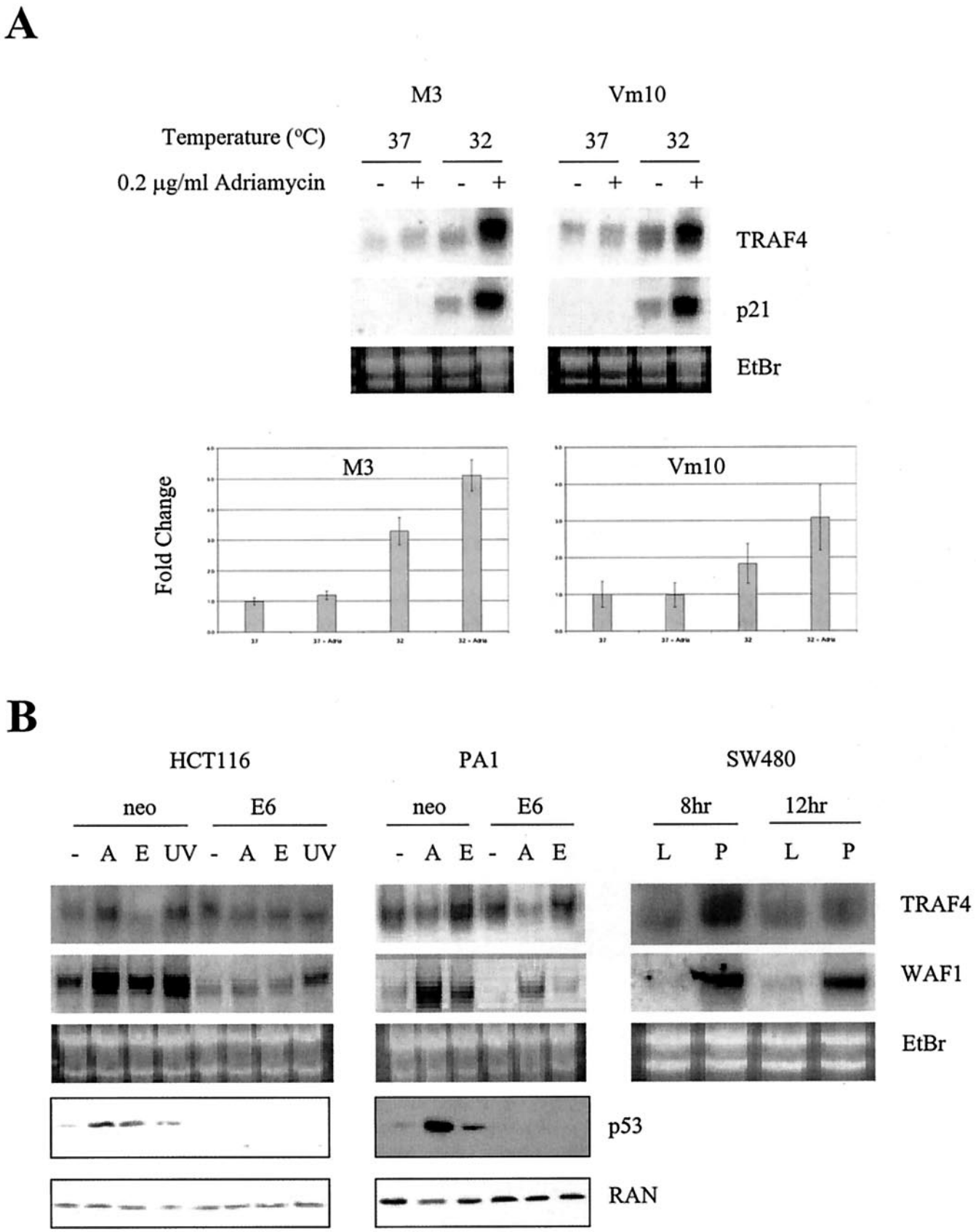


FIG. 1. **TRAF4 mRNA is up-regulated by p53.** *A*, two p53 temperature-sensitive cell lines, Vm10, and M3 were shifted from the restrictive temperature (37 °C) to the permissive temperature (32 °C) where p53 is in the wild-type conformation. When p53 is in the wild-type conformation there is a slight induction of TRAF4 mRNA. The addition of adriamycin causes an even greater induction of TRAF4 only when p53 is in the wild-type conformation, shown by Northern blot (*top*) and quantitative Taqman RT-PCR analysis (*bottom*). The inductions of TRAF4 are comparable to those of the positive control, p21 mRNA. *B*, two different wild-type p53 stable cell lines expressing either the neo cassette or the viral E6 protein, which degrades p53, were treated with various forms of DNA damage including 0.2 $\mu$g/ml adriamycin (*A*), 0.2 $\mu$M etoposide (*E*), or 50 joules/m$^2$ UVC (*UV*). TRAF4 mRNA is specifically induced by DNA damage in the neo cell lines but not in the cell lines that express E6.

In the present studies, we have identified TRAF4 as a p53-responsive gene. TRAF4 mRNA is induced in response to temperature-sensitive p53 at the permissive temperature, overexpression of wild-type p53, and to a lesser extent by the stabilization of p53 in response to exposure to DNA-damaging agents. The murine TRAF4 genomic locus contains a functional p53 DNA-binding element located ~1 kilobase (kb) upstream of the translation start site. Localization by immunofluorescence shows that TRAF4 appears to reside in the cytoplasm, even after treatment of cells with chemotherapeutic agents. Overexpression of TRAF4 induces apoptosis and suppresses colony formation in multiple tumor cell lines suggesting a role for TRAF4 in p53-mediated apoptosis.

Taken together, our results suggest a correlation that TRAF4 is a p53-regulated gene encoding a cytoplasmic protein that may mediate a role for p53 in tumor growth suppression. TRAF4 is the first adaptor protein shown to be directly regulated by p53 and thus may have a role in determining cell fate in response to stabilization of p53.

## MATERIALS AND METHODS

*Cell Lines and Culture Conditions*—We maintained the Vm10, M3, SW480, SAOS2, CALU-6, and H460 cell lines in culture as previously described (11). The HCT116 neo and E6 clones and the PA1 neo and E6 clones were maintained in culture as previously described (34). DNA damage treatments were performed as previously described (19).

*Adenovirus Preparation and Infection*—The replication-deficient adenovirus recombinants expressing either wild-type p53 (P) or β-galactosidase (L) were prepared as previously described (4). SW480, SAOS2, and CALU-6 cell lines were infected at a multiplicity of infection (MOI) of 50.

*Northern Analysis*—Isolation of total RNA and Northern blotting was carried out as described (4). The mouse and human plasmids carrying the p21 cDNA for each probe was used as described (11). Mouse and human TRAF4 were cloned by PCR using cDNA libraries, inserted into the pcDNA3 vector and sequenced. We used a *Hin*dIII-*Bam*HI fragment of about 1400 base pairs (bp) from the pcDNA3 plasmid carrying the mouse TRAF4 cDNA as a probe for Northern blots of mouse TRAF4 RNA. We used a *Bam*HI-*Eco*RI fragment of about 1400 bp from the pcDNA3 plasmid carrying the human TRAF4 cDNA as a probe for Northern blots of human TRAF4 RNA.

*Western Analysis*—Western blotting was carried out as previously described (35) using rabbit anti-mouse p53 (CM5, Novocastra Laboratories), mouse anti-human p53 (Ab-2, Oncogene Research Products) and mouse anti-RAN (BD Transduction Laboratories). The HCT116 neo and E6 cells were analyzed for p53 stabilization 12-h post-treatment and the PA1 neo and E6 cells lines were analyzed for p53 stabilization 18-h post-treatment.

*Taqman Real-time Quantitative RT-PCR*—Taqman RT-PCR was carried out as described (36). The mouse and human GAPDH primer and probe set were obtained from PE Applied Biosystems. Both mouse and human TRAF4 primers and probe crossed over an intron/exon boundary to prevent amplification of genomic DNA and were designed using Primer Express Version 1.0 (PE Applied Biosystems). hTRAF4 primers: Forward, 5′-GCGCACTCAGCCCTGC-3′, Reverse, 5′-GCACTGGTACTGGTGGCTCTG-3′. hTRAF4 probe: 6FAM-CCTACTGCACTAAGGAGTTCGTCTTTGA-TAMRA. mTRAF4 primers: Forward, 5′-GGCCTACGAGAGTCACGAGG-3′, Reverse, 5′-GGCCCCACACTTGTTCTCAC-3′. mTRAF4 probe: 6FAM-CGTGTGCCCCCAAGAGAGTGTGTTC-TAMRA. The probe and primers designed for mouse p21 have been previously described (36).

*Mice and Treatments*—Irradiation of healthy p53 wild-type and p53 knock-out mice was performed as previously described (36). Tissues were harvested 6-h postirradiation and snap frozen. RNA was purified from tissues as described (36).

*EMSA$^{NI}$*—EMSA$^{NI}$ was carried out as described (11) using 40 fmol/reaction of WAF1 oligonucleotide and 80 fmol/reaction of TRAF4 oligonucleotide. The double-stranded probe for TRAF4 contained the following sequence: 5′-biotin-GAA TAG GGC AAG CCA GGG CTT GTT TGG ACT-3′. The cold competitor oligonucleotide did not contain a 5′-biotin modification.

*Plasmid Expression Constructs*—Full-length TRAF4 expression plasmids were constructed by cloning PCR products into the pcDNA3 vector or pCEP4 vector (Invitrogen). Deletion mutants of either the RING finger domain (DM-RING) or TRAF domain (DM-TRAF) were constructed similarly and placed into both the pcDNA3 and pCEP4 vectors. The C-terminal V5-tagged TRAF4 (T4-V5) construct was generated by PCR reaction using the TRAF4-pcDNA3 plasmid as template and then ligated into the pcDNA3.1/V5-His A vector (Invitrogen). The T4-V5 construct does not contain the stop codon contained within the full-length TRAF4 cDNA sequence. All constructs were verified by sequencing. Vector construction and sequences of the primers are available upon request.

*Plasmid Reporter Constructs and Luciferase Assays*—Both full-length TRAF4 (T4FL-Luc) containing the p53 DNA-binding element and the promoter and deletion mutant TRAF4 (T4DM-Luc) containing the promoter region without the p53 DNA-binding element were generated by a PCR reaction using Vm10 genomic DNA as template. The PCR products were digested with *Kpn* and *Xho*I and ligated into the pGL2-basic luciferase vector (Promega). PCR-generated clones were confirmed by sequencing. The p21 promoter luciferase construct (WWP-Luc) was previously described (4). The expression vectors for wild-type p53 (pCEP4-p53) and for mutant p53 (pCEP4-A273H) were provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD) (4, 37). Luciferase assays in CALU-6 cells were carried out as described (11). Luciferase assays in SW480 cells were carried out by plating ~$2.5 \times 10^5$ cells per well in 12-well plates, transfecting cells with indicated plasmids using a LipofectAMINE 2000/DNA conjugate according to the manufacturer's instructions (Invitrogen) and assaying for luciferase activity ~24 h later as previously described (11). U2OS cells, which contain wild-type p53, were seeded ~$1 \times 10^5$ cells per well in 12-well plates, transfected with the T4FL-Luc construct in a LipofectAMINE 2000/DNA conjugate (Invitrogen) and treated with 0.4 μg/ml adriamycin for ~22 h and assayed for luciferase activity as described (11). All assays were performed in triplicate.

*Apoptosis Assays*—Cells were co-transfected with the indicated expression plasmids and GFP-Spectrin as 10% of total transfected DNA, harvested at indicated time points, stained with propidium iodide and analyzed by flow cytometric gating for both GFP and propidium iodide. Cells with less than 2N DNA content are scored as apoptotic cells. For caspase inhibitor experiments, cells were pretreated with $Me_2SO$ or 20 μM z-VAD for ~1.5 h and then co-transfected with the indicated plasmid including GFP-Spectrin at 10% of the total DNA. Cells were treated with either $Me_2SO$ or z-VAD containing media every 10–14 h for the duration of the experiments. Cells were then harvested at the indicated time points, stained with prodium iodide, and analyzed by flow cytometry as described above.

Active caspase-3 was measured by fixing transfected cells using the Cytofix/Cytoperm solutions (BD Pharmingen), incubating cells with 0.25 μg/sample anti-active caspsae-3 monoclonal antibody (BD Pharmingen), washed in Cytoperm, and then incubation with 0.25 μg per sample anti-rabbit IgG-PE (Caltag) and analyzed by flow cytometry.

*Colony Assays*—Approximately $3 \times 10^5$ U2OS cells, $5 \times 10^5$ SW480 cells, $5 \times 10^5$ CALU-6 cells and $4 \times 10^5$ HCT116 cells were plated for 18–24 h prior to transfection in 6-well plates. Cells were transfected using a LipofectAMINE 2000/DNA conjugate suspended in Opti-Mem medium (Invitrogen). Approximately 24 h of post-transfection, cells were trypsinized and one-half of each well was placed into a T-25 in media under selection. U2OS and CALU-6 cells were selected with 0.25 mg/ml Hygromycin B. SW480 cells were selected with 0.4 mg/ml Hygromycin B. HCT116 cells were selected with 0.1 mg/ml Hygromycin B. Medium was changed every 2–3 days and cells were stained with Coomassie Blue 12–14 days after the beginning of selection. Each colony assay was repeated on at least two independent occasions in each cell line. Visible colonies were counted and percent colony suppression was calculated by dividing the number of colonies for each TRAF4 construct by pCEP4 and multiplying that number by 100. The percent values shown are an average of two independent experiments.

*Immunofluorescence*—U2OS cells were seeded on Nalge Nunc glass chamber slides 18–24 h prior to transfection. Cells were transfected

Overexpression of p53 by use of an adenovirus (*P*) in the mutant p53-expressing cell line SW480 led to induction of TRAF4 mRNA as compared with cells infected with a control virus expressing β-galactosidase (*L*). A Northern blot for p21 is used as a positive control for all experiments. A Western blot shows stabilization of p53 only in the neo stable cell lines treated with DNA-damaging agents and not in the E6 (p53 degrading) stable cell lines. RAN is used as a loading control for Western blots. Ethidium bromide staining of the 28 S and 18 S ribosomes is used as a loading control for RNA. All RNA was harvested at a 10-h post-treatment unless otherwise noted.

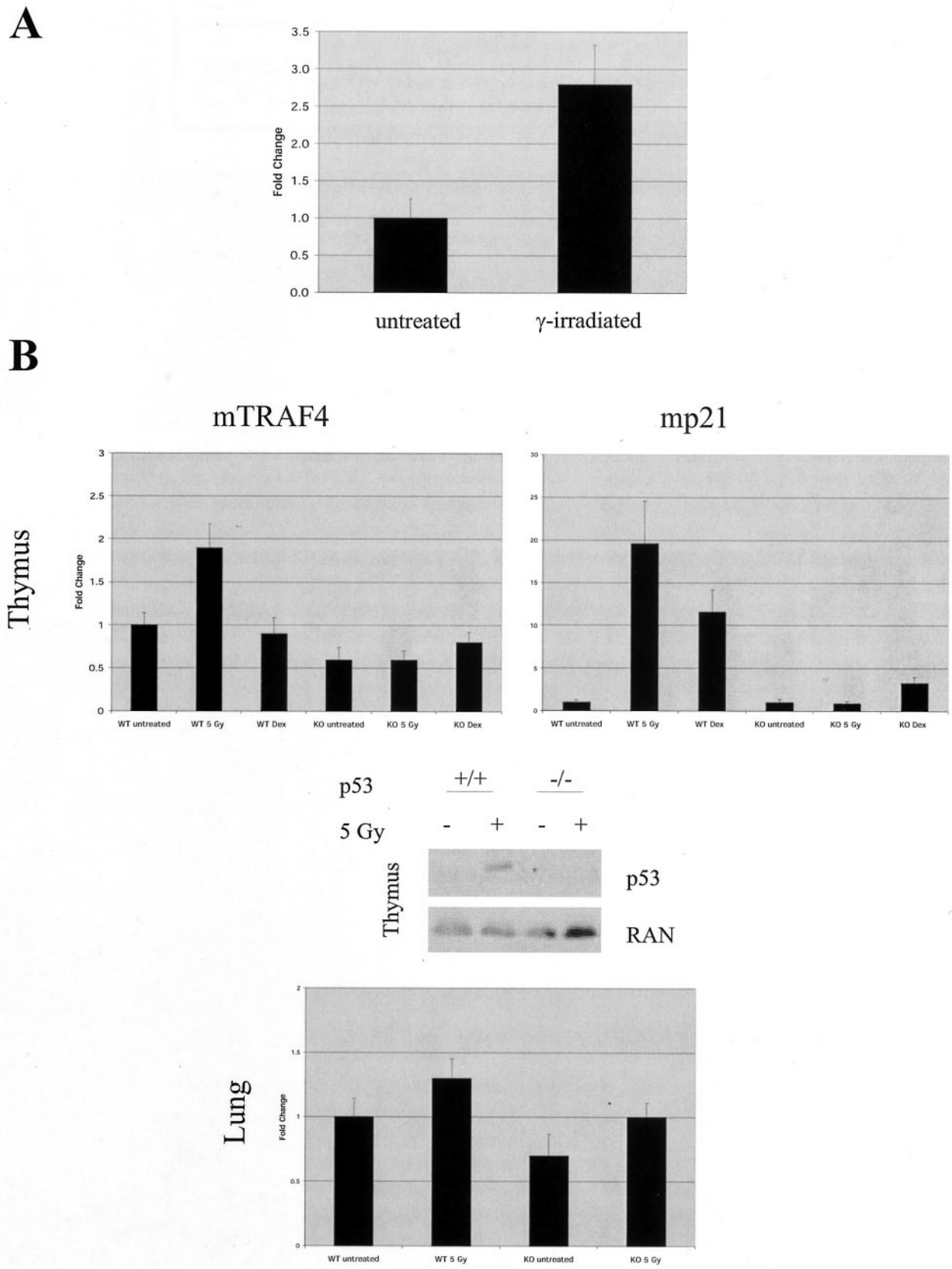


FIG. 2. **TRAF4 mRNA is up-regulated by p53 in response to γ-irradiation *in vitro* and *in vivo*.** *A*, irradiation of the wild-type p53 expressing lung cancer cell line H460 showed an approximate 3-fold induction of TRAF4 mRNA as assessed by quantitative Taqman RT-PCR analysis. RNA was harvested at 10-h post- 20Gy irradiation. *B*, the thymus of γ-irradiated (5 Gy) mice showed an induction of TRAF4 mRNA in p53 wild-type (*WT*) mice as compared with p53 knock-out (*KO*) mice analyzed by Taqman RT-PCR. Quantitative Taqman RT-PCR using p21 as the probe is used as a positive control for the p53-dependent induction in response to irradiation. Dexamethasone (*Dex*) is used as a control treatment. A Western blot shows the stabilization of p53 only in the wild-type irradiated thymus. RAN is used as a loading control for the Western blot. Irradiated lung of p53 wild-type mice did not reveal a p53-dependent induction of TRAF4 mRNA in this tissue.

with the indicated expression plasmids and fixed with 4% paraformaldehyde in phosphate-buffered saline ~20–24-h post-transfection. Cells were permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Cells were washed three times with phosphate-buffered saline, blocked in goat serum for 20 min at room temperature and then incubated with anti-V5 antibody (Invitrogen) at a 1:100 dilution in goat serum for 2–4 h at room temperature or at a 1:200 dilution overnight at 4 °C. Cells were washed with phosphate-buffered saline for 30 min and then incubated in goat anti-mouse FITC (Jackson Laboratories) at a 1:100 dilution in goat serum for 1 h at room temperature. After washing with phosphate-buffered saline for 30 min, slides were mounted with DAPI-containing mounting media (Vector Laboratories) and analyzed by fluorescence microscopy. Samples were treated with the indicated chemotherapeutic agents for 8 h ~16–20-h post-transfection. Secondary antibody-only controls were performed and did not show detectable fluorescence.

## RESULTS

*TRAF4 mRNA Is Specifically Up-regulated in Response to p53*—We used an oligonucleotide approach to screen for p53-activated transcriptional targets using cRNA derived from the p53-expressing temperature-sensitive cell line Vm10 cultured at different temperatures (11, 38, 39). On the murine 11K Affymetrix GeneChip, levels of TRAF4 mRNA were 32.7-fold higher in the Vm10 cRNA from cells incubated at 32 °C (permissive temperature; wild-type p53 conformation) compared with cells incubated at 39 °C (restrictive temperature; mutant p53 conformation) (data not shown).

Using two p53 temperature-sensitive murine cell lines, Vm10, and M3, we confirmed the up-regulation of TRAF4 mRNA in response to wild-type p53 at the permissive temperature by Northern blotting and quantitative Taqman RT-PCR analysis (Fig. 1*A*). An even greater induction of TRAF4 mRNA was observed following treatment with Adriamycin only when p53 was in the wild-type conformation and not when p53 was in the mutant conformation (Fig. 1*A*). Moreover, TRAF4 mRNA was specifically induced by wild-type p53 in response to DNA damage in both HCT116 and PA1 human cancer cells and not in the E6-p53-degrading clones previously derived from these cell lines (34) (Fig. 1*B*). TRAF4 mRNA does not appear to be obviously up-regulated by etoposide in the HCT116-neo or by adriamycin in the PAI-neo, however this could be due to different responses of each cell line to different chemotherapeutic treatments. More importantly, TRAF4 mRNA is specifically induced by p53 in response to various forms of DNA damage in more than one cell line, suggesting a correlation between stabilization of p53 by DNA-damaging agents and induction of TRAF4 mRNA (Fig. 1, *A* and *B*). Overexpression of p53 by use of an adenovirus also induced the TRAF4 transcript in SW480 cells (Fig. 1*B*). Of note, there appears to be two transcripts of TRAF4 in the human cell lines that are very similar in size. Both transcripts appear to be positively regulated by p53. Northern blotting for the p21 transcript serves as a positive control for the p53-dependent induction of a known p53 target gene expressed in these cell lines. In addition, a Western blot for p53 protein stabilization in response to treatment with DNA-damaging agents in the HCT116 and PA1 stable cell lines shows p53 stabilization only in the neo stable clones and not in the E6, p53-degrading, stable clones (Fig. 1*B*).

Finally, using a quantitative Taqman RT-PCR assay, TRAF4 mRNA was up-regulated by γ-irradiation in the wild-type p53 expressing cell line H460 (Fig. 2*A*). TRAF4 mRNA was also induced in the thymus of irradiated p53 wild-type mice but not in the irradiated p53 knock-out mice (Fig. 2*B*). As a positive control, quantitative Taqman RT-PCR analysis using p21 as the probe shows the p53-dependent up-regulation of p21 in the irradiated thymus (Fig. 2*B*). In addition, a Western blot against p53 protein shows the stabilization of p53 in wild-type irradiated thymus and not in the knock-out irradiated thymus (Fig. 2*B*). TRAF4 mRNA was not specifically induced by p53 in other tissues tested including the small intestine, spleen or lungs of irradiated p53 wild-type mice as compared with the p53 knock-out mice. The Taqman RT-PCR of irradiated lung shown in Fig. 2*B* shows a similar, albeit small, level of induction of TRAF4 mRNA in irradiated lung in both wild-type p53 and p53-null mice when compared with the non-irradiated lung, respectively, suggesting TRAF4 may not specifically be regulated by p53 in this tissue (Fig. 2*B*). Taken together, these results suggest that the presence of p53 in irradiated thymus correlates with a small induction of TRAF4 and there could potentially be some tissue specificity to the radiation response.

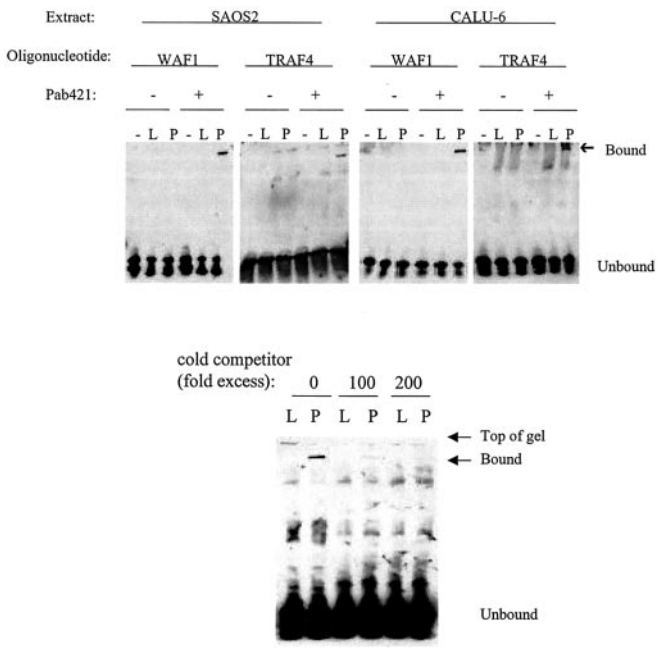


FIG. 3. **The TRAF4 promoter contains a putative p53 DNA-binding element.** Non-isotopic EMSA$^{NI}$ shows that a putative p53 DNA-binding element in the TRAF4 promoter only forms a protein-DNA complex in nuclear extracts that had been infected with an adenovirus expressing p53 (*P*) and not in extracts that had been infected with an adenovirus expression β-galactosidase (*L*). (−) denotes no extract added to that reaction. The protein-DNA complex is only formed with the addition of the p53 C-terminal modifying antibody Pab421. The p53 DNA-binding element in the p21 promoter is used as a positive control for these assays. The visible p53-DNA complex can be competed out using excess cold-competitor in CALU-6 nuclear extracts expressing either β-galactosidase (*L*) or p53 (*P*) and in the presence of Pab421.

*The TRAF4 Promoter is p53-responsive*—In order to activate transcription, p53 binds to a consensus DNA sequence consisting of two conserved decamers separated by 0–13 base pairs (40). Analysis of the regulatory region of the murine TRAF4 locus revealed a potential p53 DNA-binding element ~1 kb upstream of the initiator methionine (sequence of p53 DNA-binding element shown schematically in Fig. 4). Non-isotopic Electrophoretic Mobility Shift Assay (EMSA$^{NI}$) showed that p53 specifically formed a protein-DNA complex in nuclear extracts where p53 was overexpressed by use of an adenovirus (Fig. 3). The protein-DNA complex could only be seen in the presence of the p53 C-terminal modifying antibody Pab421. The p53 DNA-binding element in the p21$^{WAF1/Cip1}$ promoter was used as a positive control in these assays (Fig. 3). In addition, the binding of p53 to the biotin labeled TRAF4 oligonucleotide could be competed out using excess cold (*i.e.* unlabeled) competitor oligonucleotide (Fig. 3).

Wild-type p53 also efficiently transactivated a luciferase reporter construct, T4FL-Luc, which contained the murine TRAF4 promoter region including the p53 DNA-binding ele-

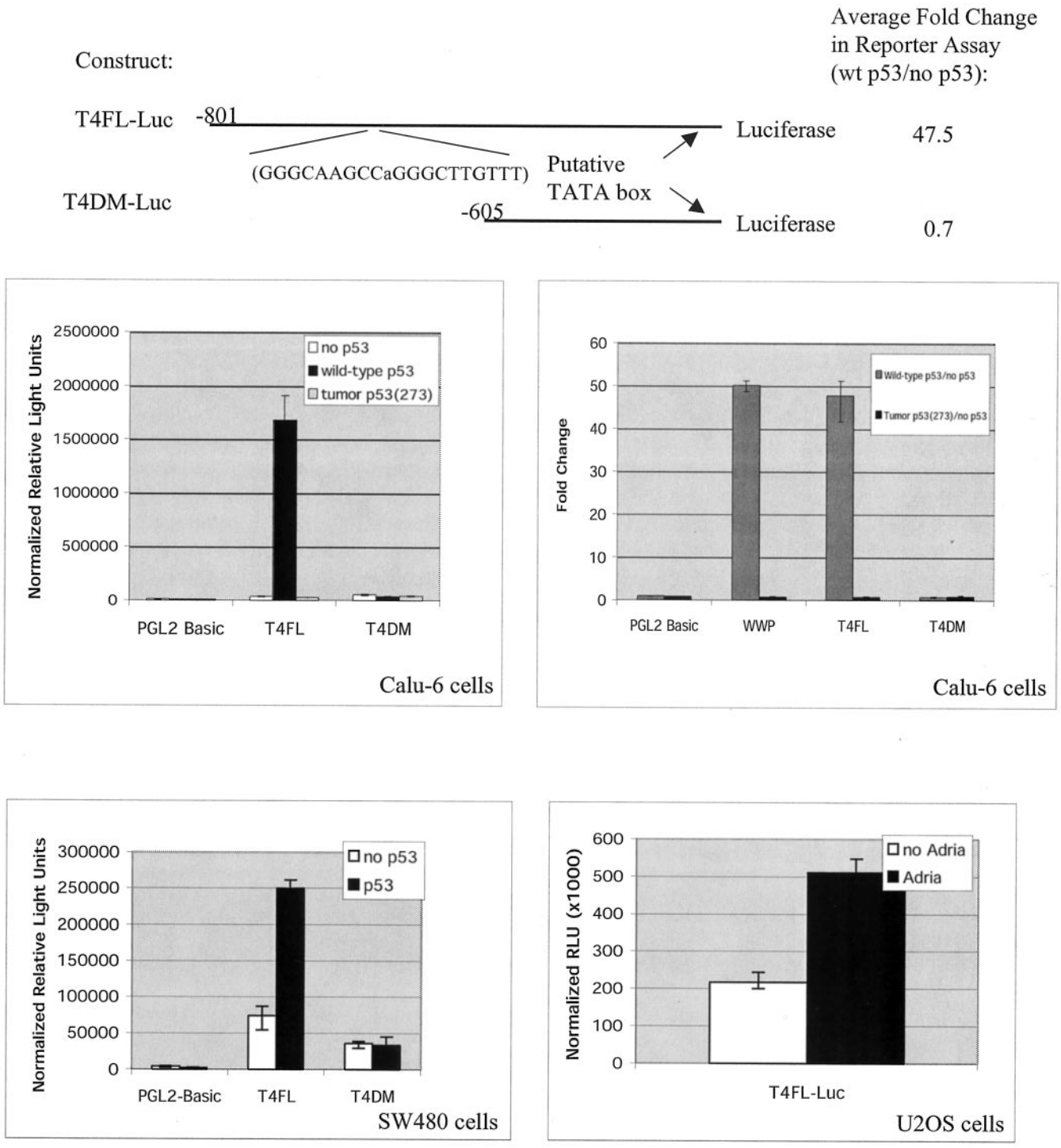


FIG. 4. **The TRAF4 promoter can be transactivated by p53.** The full-length murine TRAF4 promoter, containing the p53 DNA-binding element, placed upstream of a luciferase reporter (T4FL-Luc) can be specifically transactivated by co-transfection with a wild-type p53 expression plasmid. Co-transfection of T4FL-Luc with empty vector or a plasmid expressing a tumor-derived p53 mutant (p53 ($Arg^{273} \rightarrow His$)) is not able to transactivate the TRAF4 promoter. A luciferase reporter that does not contain the p53 DNA-binding element in the TRAF4 promoter, T4DM-Luc, cannot be transactivated by co-transfection with wild-type p53 in CALU-6 or SW480 cells. The p21 promoter luciferase reporter construct, WWP-Luc, is used as a positive control in these assays. The T4FL-Luc construct can be transactivated by the stabilization of endogenous p53 by treatment with 0.4 $\mu$g/ml adriamycin (Adria) in U2OS cells. All assays were normalized to $\beta$-galctosidase expression (see "Materials and Methods").

ment (Fig. 4). Indeed the transactivation of the TRAF4 promoter region was comparable to that of the p21 promoter luciferase construct, WWP-Luc (~47-fold and 50-fold, respectively) (Fig. 4). The transactivation of the full-length TRAF4 construct could only be seen with co-transfection of wild-type p53 and not with empty vector or a tumor-derived mutant p53 ($Arg^{273} \rightarrow His$), which contains a mutation in the DNA binding domain rendering a transactivation-deficient p53 protein. Moreover, deletion of the p53 DNA-binding element in the TRAF4 luciferase reporter construct that still retained the promoter region, T4DM-Luc, was not transactivated by wild-type p53 in both CALU-6 and SW480 cell lines (Fig. 4). Furthermore, the T4FL-Luc construct could be transactivated by stabilization of endogenous p53 by the chemotherapeutic agent, adriamycin, in the wild-type p53-expressing cell line U2OS (Fig. 4). Taken together, these results suggest that the

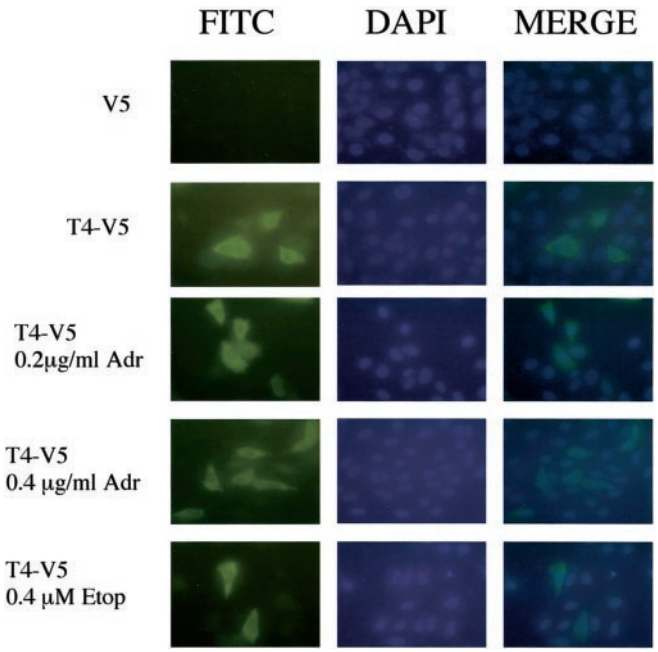


FIG. 5. **TRAF4 localizes to the cytoplasm.** A C-terminal V5-tagged TRAF4 expression vector localizes to the cytoplasm of U2OS cells as analyzed by immunofluorescence. DAPI staining is used to identify the nucleus and the merged pictures show no overlap between the fluorescence from TRAF4-V5 and the fluorescence from the DAPI. Treatment of cells with the chemotherapeutic agents adriamycin (*Adr*) and etoposide (*Etop*) at the indicated concentrations did not alter the cytoplasmic localization of TRAF4.

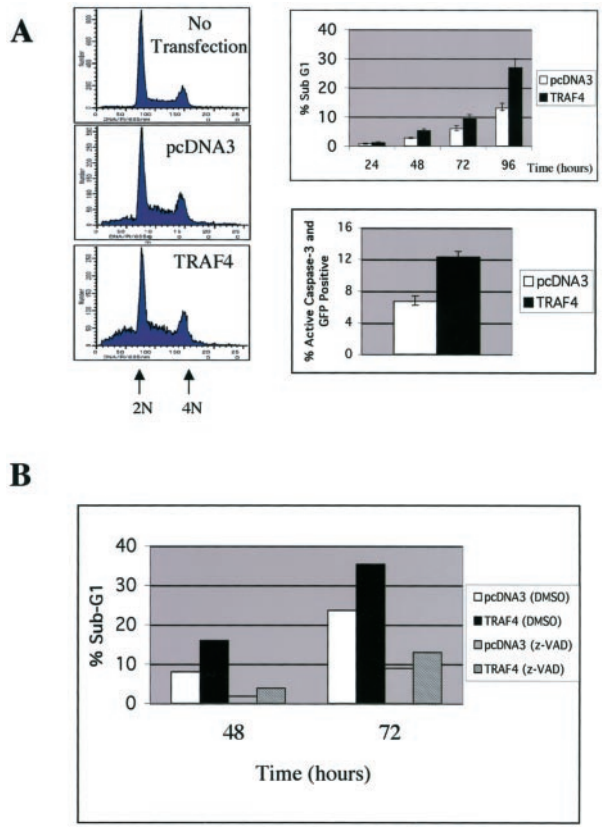


FIG. 6. **Overexpression of TRAF4 induces apoptosis.** *A*, propidium iodide staining and cell cycle analysis of U2OS cells that have not been transfected, transfected with empty vector (pcDNA3), or transfected with TRAF4. *Arrows* at bottom of profiles denote DNA content. TRAF4 overexpression induces apoptosis as measured by an increase in the sub-$G_1$ population. Analysis of the sub-$G_1$ population after transfection of either pcDNA3 or TRAF4 shows an increase in apoptosis over time in cells expressing TRAF4 as compared with empty vector. An active caspase-3 assay was used to show that overexpression of TRAF4 specifically induces apoptosis as compared with empty vector. *B*, TRAF4-induced cell death can be inhibited by the pan-caspase inhibitor z-VAD. Cells were treated with either $Me_2SO$ or 20 $\mu$M z-VAD, transfected with either empty vector or TRAF4 and then analyzed by propidium iodide staining and flow cytometry at the indicated time points.

p53 DNA-binding element ~1 kb upstream of the translation start site can be bound by p53 and mediate p53-dependent transactivation of the TRAF4 gene.

*TRAF4 Localizes to the Cytoplasm*—Conflicting reports in the literature place TRAF4 in either the cytoplasm or the nucleus (24, 26, 29, 30). In order to evaluate the localization of TRAF4 we placed TRAF4 in an expression vector with a V5 tag at the C terminus. The overexpression of the tagged TRAF4 (T4-V5) and the analysis of localization by immunofluorescence (IF) showed that T4-V5 localizes to the cytoplasm in U2OS cells (Fig. 5). We also investigated the localization of T4-V5 in COS-7 cells and observed essentially identical staining as in the U2OS cells (data not shown). This is in agreement with the recent report by Glauner *et al.* (30), who also found that full-length TRAF4 localizes to the cytoplasm. TRAF4 contains two putative nuclear localization sequences (NLS) and though it appears that TRAF4 localizes to the cytoplasm, we also mutated those two NLS sites and performed an immunofluorescence analysis. As expected, the localization of the two NLS mutants was identical to the wild type (data not shown).

Because TRAF4 is up-regulated in response to DNA damage in a p53-dependent manner, we sought to determine whether treatment of cells with DNA-damaging agents might alter the localization of TRAF4. However, the combination of overexpression of TRAF-V5 and treatment with the chemotherapeutic agents adriamycin and etoposide, also did not alter the localization of TRAF4 from the cytoplasm (Fig. 5).

*TRAF4 Overexpression Induces Apoptosis and Suppresses Colony Formation*—In an attempt to understand why p53 upregulates TRAF4, we overexpressed the protein in U2OS cells and analyzed the cellular DNA content by propidium iodide staining. Overexpression of TRAF4 did not cause a cell cycle arrest, but it increased the sub-$G_1$ population (Fig. 6*A*). Indeed, TRAF4-induced apoptosis occurred at a slow rate (Fig. 6*A*) suggesting that TRAF4 may not directly induce apoptosis but may be a late mediator in a proapoptotic signaling pathway. In order to show that the sub-$G_1$ population was caused by apoptosis, we overexpressed TRAF4 and analyzed for cell death more directly by measuring the levels of active caspase-3 protein. We found that overexpression of TRAF4 for 48 h increases the cellular content of active-caspase-3 ~2-fold as compared with vector alone transfectants (Fig. 6*A*). Moreover, TRAF4-induced cell death could be inhibited by the addition of the pan-caspase inhibitor z-VAD (Fig. 6*B*).

To determine the effect of TRAF4 on the growth of human tumor cell lines, we placed TRAF4 in the episomal plasmid pCEP4 containing the selection marker Hygromycin B. We transfected this plasmid into four different human tumor cell lines, U2OS, CALU-6, SW480, and HCT116, and placed the cells under selection for 12–14 days. Regardless of the p53 status of the cell line, TRAF4 suppressed colony formation in all cell lines tested (Fig. 7 and Table I). The results in SW480 were not as dramatic as the other cell lines; however, TRAF4 appeared to suppress colony formation as compared with vector alone. An expression plasmid carrying wild-type p53 in the pCEP4 vector was used as a positive control (Fig. 7).

In order to determine which domain of TRAF4 may be important for TRAF4 induced apoptosis and colony suppression we generated deletion mutants. TRAF4 shares sequence homology with its other family members in the N-terminal RING

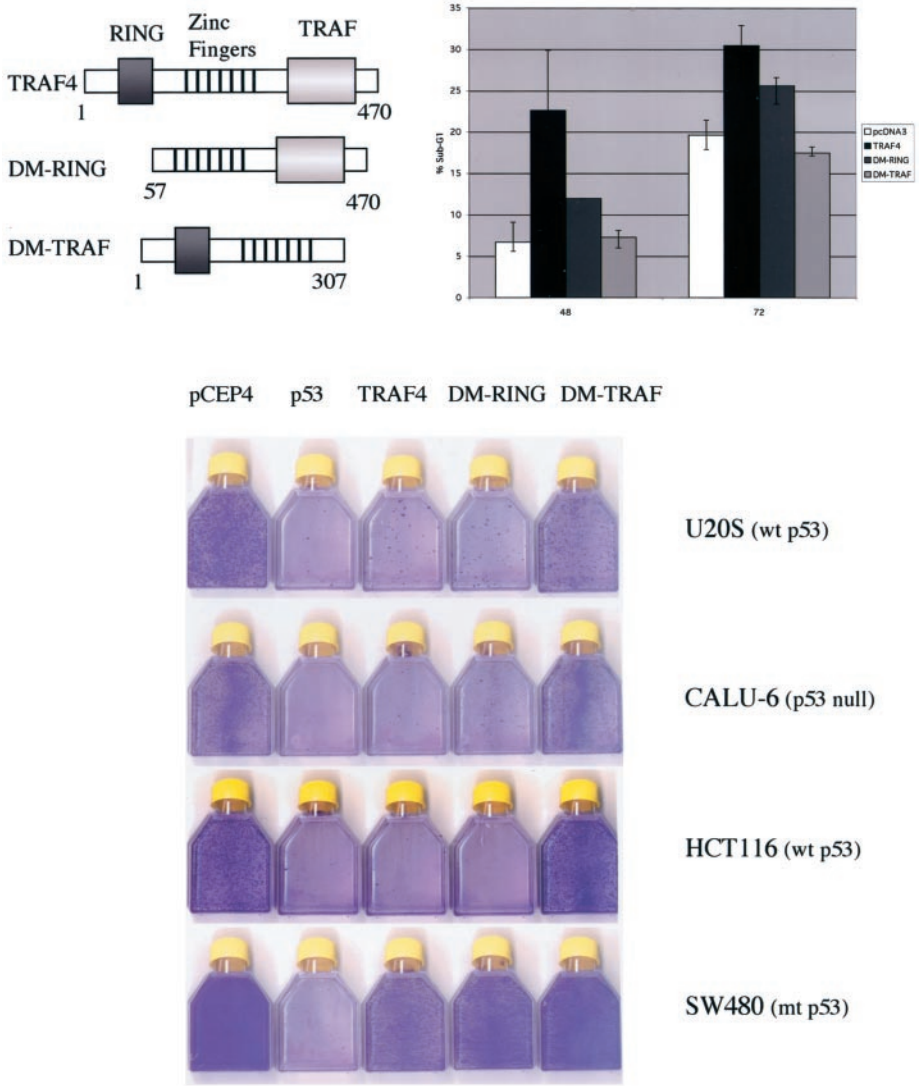


FIG. 7. **TRAF4 suppresses colony formation in multiple tumor cell lines and requires the TRAF domain for colony suppression.** Shown schematically are the full-length and two deletion mutants of TRAF4 used in propidium iodide staining and colony assays. TRAF4 is a 470-amino acid protein. At the N terminus is a RING finger domain followed by seven zinc fingers. The TRAF domain is at the C terminus of the protein. DM-RING denotes deletion of the first 57 amino acid residues that contain the RING domain. DM-TRAF denotes deletion of the last 163 amino acid residues that contain the TRAF domain. Full-length TRAF4 and the deletion mutants were transfected into U2OS cells and analyzed for cell death by propidium iodide staining and flow cytometry. U2OS, CALU-6, SW480, and HCT116 were transfected with the indicated plasmids and allowed to grow under Hygromycin B selection for 12–14 days. Colonies were visualized by Coomassie Blue staining. pCEP4 is the empty vector control and p53 is used as a positive control for these assays. Full-length TRAF4 suppresses colony formation. Deletion of the RING domain does not affect the colony suppression; however, deletion of the TRAF domain ablates the ability of TRAF4 to suppress colony formation.

TABLE I
*Number of colonies in colony assays and percent colony suppression*

| | | Colony numbers | | | | Colony suppression | | |
|---|---|---|---|---|---|---|---|---|
| | | pCEP4 | TRAF4 | DM-RING | DM-TRAF | TRAF4 | DM-RING | DM-TRAF |
| | | | | | | | % | |
| U2OS | Expt. 1 | 410 | 34 | 36 | 378 | 8 | 11 | 86 |
| | Expt. 2 | 522 | 42 | 72 | 410 | | | |
| CALU-6 | Expt. 1 | 482 | 10 | 80 | 444 | 6 | 18 | 82 |
| | Expt. 2 | 350 | 34 | 66 | 248 | | | |
| HCT116 | Expt. 1 | 540 | 60 | 62 | 456 | 10 | 9 | 79 |
| | Expt. 2 | 584 | 52 | 34 | 428 | | | |
| SW480 | Expt. 1 | 954 | 540 | 480 | 864 | 72 | 66 | 109 |
| | Expt. 2 | 668 | 574 | 538 | 852 | | | |

finger domain as well as the C-terminal TRAF domain. Therefore both N-terminal and C-terminal deletion mutants were generated. Propidium iodide staining of transfected U2OS cells revealed an increase in the sub-$G_1$ population in cells overexpressing either full-length TRAF4 or the DM-RING mutant (Fig. 7). However, cells overexpressing the DM-TRAF construct did not appear to be undergoing apoptosis. Indeed, in long-term colony assays, the N-terminal RING finger deletion mutant appeared to suppress colony formation as well as the full-length TRAF4 (Fig. 7 and Table I). However, the C-terminal TRAF domain mutant did not suppress colony formation (Fig. 7 and Table I). These results suggest that TRAF4 may require its TRAF domain to suppress colony formation.

## DISCUSSION

Attempts to identify novel p53 target genes involved in cell cycle arrest and apoptosis has been ongoing over the last few years. While the p53-mediated $G_1$ cell cycle arrest is primarily mediated by $p21^{WAF1/CIP1}$, no single target gene has been shown to be required for the full p53-mediated apoptotic response. In addition to the ability of p53 to regulate multiple genes involved in cell cycle arrest and apoptosis, the mechanism determining cell fate remains unclear (41). Recent studies have shown that contributions of the other family members, p63 and p73, as well as post-translational modifications of p53 may impact on p53-mediated apoptosis (16, 41–44).

Here we identify TRAF4 as a p53 regulated gene. TRAF4 mRNA is regulated in response to temperature-sensitive p53 at the permissive temperature, overexpression of p53, and exposure to DNA-damaging agents correlating to a p53-dependent manner. TRAF4 mRNA was specifically induced in the thymus of irradiated p53 wild-type mice and not in irradiated p53 knock-out mice. The TRAF4 promoter contains a functional p53 DNA-binding element that can form a protein-DNA complex as shown by EMSA[NI] and the promoter can be highly transactivated by wild-type p53 and not by a tumor-derived mutant.

Recent reports have localized TRAF4 to either the nucleus or cytoplasm using different antibodies or expression constructs (26, 29, 30). Using a V5 tagged full-length TRAF4 construct and immunofluorescence we observed cytoplasmic localization. Unlike other TRAF family members, TRAF4 contains two putative nuclear localization sequences, suggesting that TRAF4 may reside in the nucleus. Because TRAF4 is up-regulated in response to the chemotherapeutic agents adriamycin and etoposide (Fig. 1) we sought to determine whether TRAF4 may localize to the nucleus after DNA damage. However, it appears that TRAF4 remains cytoplasmic even after the addition of adriamycin or etoposide (Fig. 5). This does not rule out the possibility that under different circumstances TRAF4 may localize to the nucleus, but we did not detect nuclear localization in our assays.

Overexpression of TRAF4 induces apoptosis and suppresses colony formation. Interestingly, TRAF4-induced apoptosis occurs at a slower rate than many other proteins that directly activate death pathways. This could suggest that TRAF4 may not directly activate death but may regulate another pathway to allow the cell to undergo apoptosis. Attempts to determine whether TRAF4 positively or negatively regulates multiple signaling pathways including NF-κB, JNK, MAPK, or Rho-GTPase by using commercially available reporter assays (Stratagene) failed to uncover a relationship or an effect on these particular stress pathways (data not shown). However, the combination of the cytoplasmic localization, the slow rate of apoptosis, and the requirement of the TRAF domain, which has been shown to mediate protein-protein interactions in other TRAF family members, for colony suppression suggests that TRAF4 may interact with other cytoplasmic proteins to regulate cell death and growth suppression in the p53 response.

A recent study identified TRAF4 as a binding partner for $p47^{phox}$ in endothelial cells (45). Co-overexpression of both TRAF4 and $p47^{phox}$ led to JNK activation and a measurable increase in oxidant production (45). While the overexpression of TRAF4 alone had little to no effect on JNK activity, the TRAF4 homolog in *Drosophila* (DTRAF1) has been shown to interact with the putative MAP4K protein, Misshapen (45, 46). These data suggest TRAF4 may be involved with stress-related signaling; however, to date, TRAF4 has not been placed in a clear signaling pathway and future studies will aim to determine the mechanism behind TRAF4-induced apoptosis.

While much is known with respect to the other TRAF family members especially TRAF2 and TRAF6, little is known about the function of TRAF4. TRAF4 is expressed at basal levels in most adult tissues and specifically highly expressed in the developing embryo in the central and peripheral nervous systems (31, 33). In one study many TRAF4 knock-out mice exhibit spina bifida probably due to impaired neural tube closure as well as the tracheal formation problems found in another TRAF4 knock-out mouse model (32, 33). This is in contrast to other TRAF family member knock-out mice, which have varying defects in their immune system and NF-κB and JNK signaling pathways (reviewed in Refs. 22, 24, 25). In addition, TRAF4 has not been shown to associate with many of the TNFR superfamily members known to bind to other TRAF family members, nor has TRAF4 been shown to bind to other adaptor proteins. Taken together, these data suggest that while TRAF4 shares high sequence homology to the other family members, it does not share many of their common characteristics.

Much evidence suggests that the p53-dependent apoptotic response involves multiple downstream target genes. It is possible that TRAF4 may not uniquely substitute for p53 in death signaling. To support this notion our preliminary TRAF4 RNAi data revealed no phenotype toward either DNA damage or p53-dependent cell death. However, this is not necessarily surprising nor does it indicate a lack of importance of TRAF4. To the contrary, we believe the high magnitude of TRAF4 induction as well as its structural links to death signaling and the apoptotic phenotype upon overexpression strongly implicates the gene in the p53-apoptotic response pathway. In the future it will be important to target blockade of TRAF4 plus other apoptotic targets of p53, both in cell lines and in mice.

TRAF4 was identified as a p53 regulated gene on an Affymetrix GeneChip array. While other family members were represented on the GeneChip, TRAF4 was the only member to be up-regulated by p53 (data not shown). The correlation of TRAF4 regulation by p53 distinguishes this family member, yet again, from the other TRAFs. TRAF4 is able to induce apoptosis that can be inhibited by a pan caspase inhibitor. Although the mechanism behind TRAF4-induced apoptosis remains unclear at this time, our data suggest that p53 specifically up-regulates this gene in order to facilitate the cellular process of apoptosis and growth suppression.

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