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TrkA Induces Apoptosis of Neuroblastoma Cells and Does So via a p53-dependent Mechanism*[S]

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Neuroblastoma (NB) is the most frequent solid extracranial tumor in children. Its clinical prognosis correlates with the expression of members of the Trk neurotrophin receptor family, which includes TrkA and TrkB. TrkA expression is associated with favorable prognosis, whereas TrkB expression is associated with poor prognosis. Here we show that TrkA expression induces the apoptosis of NB cells and does so by modulating the levels or activities of a number of proteins involved in regulating cell survival and apoptosis, including p53, Bcl-2, and caspase-3. TrkA increased the expression of p53 target proteins and failed to induce apoptosis in cells where p53 was inactivated by mutation or via expression of dominant inhibitory p53 or E1B55K, indicating that TrkA mediates apoptosis, at least in part, through p53. Treatment with a caspase inhibitor or overexpression of Bcl-X_L also prevented TrkA from inducing apoptosis. In contrast, elevated expression of TrkA in non-transformed sympathetic neurons resulted in the suppression of p53 levels and enhanced survival. These results identify apoptosis as a novel biological response of TrkA in NB cells and imply that TrkA is a good prognosis marker for NB due in part to its ability to mediate apoptosis when expressed at sufficient levels.

Neuroblastoma (NB)[1] is the most common solid extracranial solid tumor of childhood and likely arises from sympathoadrenal precursor cells. The median age at diagnosis is ~18 months, and spontaneous tumor regression is frequently observed in patients diagnosed at 1 year of age or younger. In contrast, children older than 1 year diagnosed with NB often experience aggressive tumors that are disseminated, resistant to chemotherapy, and metastasized to bone, and often fatal. An important correlative characteristic of NB is the expression of two of the neurotrophin receptors, the TrkA/nerve growth factor (NGF) receptor and the TrkB/brain-derived neurotrophic factor receptor (1–4). The expression of TrkB, a poor prognosis marker, mediates survival, proliferation, and chemotherapeutic drug resistance (5–10), whereas expression of TrkA, a favorable prognosis marker, induces cell growth arrest and differentiation of cultured NB cells (11–13). This is consistent with TrkA being expressed in tumors that spontaneously regress. Most NB cell lines, which are derived from malignant tumors, lack or have very low levels of TrkA protein expression. Cell lines with low TrkA expression respond to NGF by differentiating into neuronal-like cells (11). Similarly, expression of TrkA by transfection converts NGF non-responsive NB cells into NGF-responsive cells, both in culture and *in vivo*, with the typical responses being the cessation of cell growth and differentiation into neural and Schwann cells (12). Thus, TrkA converts malignant NB cells into quiescent differentiated cells.

An alternative or additional explanation for why TrkA is a good prognosis marker for NB that might contribute to spontaneous tumor regression is that it may induce apoptosis. Paradoxically, TrkA is a potent pro-survival protein for sympathetic and sensory neurons (14), whereas it has been shown to induce, when overexpressed, the apoptosis of medulloblastoma cells (15). In the following study, we asked whether expression of full-length TrkA (human TrkAI) would induce the apoptosis of NB cells. We find that TrkA, expressed at levels similar to that found in neurons, caused the apoptosis of NB cells with *MYCN* amplification while stimulating the survival of non-transformed sympathetic neurons. Apoptosis by TrkA was accompanied by alterations in the levels and/or the activities of a number of proteins involved in apoptotic signaling, including p53, Bcl-2, and caspase-3. We show that wild-type p53 expression and activity, and not p38 MAPK or p42/44 MAPK activities, were required to mediate TrkA-induced cell death. Our findings suggest that high expression of TrkA that occurs in good prognosis NB tumors may result in apoptosis and that this event is mediated by p53, which is wild-type in most NB tumors.

MATERIALS AND METHODS

Cell Culture—MYCN amplified human neuroblastoma Lan-1–15N (15N) cells (16), NGP (17) cells, and p53 mutated SK-N-BE(2) NB cells (18) were cultured as described (5). Primary sympathetic neurons were isolated and cultured as described previously (19).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[1] The abbreviations used are: NB, neuroblastoma; NGF, nerve growth factor; MAPK, mitogen-activate protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun NH_2-terminal kinase; Z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethyl ketone; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; KD, kinase-dead; Ptyr, phosphotyrosine; m.o.i., multiplicity of infection; PARP, poly(ADP-ribose) polymerase; tTA, tetracycline-responsive transcriptional activator.

Neurotrophins, Antibodies, and Inhibitors—Reagents were purchased from the following companies: NGF, Cedarlane, Hornby, Ontario, Canada; brain-derived neurotrophic factor, PeproTech, Rocky Hill, NY; anti-β-galactosidase, anti-phospho-ERK, anti-phospho-p38 MAPK, and U0126, Promega Inc., Madison, WI; polyclonal anti-active JNK, BioSource, Camarillo, CA; anti-phospho Akt phosphorylated serine 473, Cell Signaling, Beverly, MA; polyclonal anti-ERK-1, anti-JNK-1, anti-Bcl-2, anti-Bcl-X_L, anti-p53 (FL393) and anti-p53 (DO-1), Santa Cruz Biotechnology, CA; anti-pan Akt, anti-cleaved caspase-3, and anti-cleaved-PARP, Cell Signaling; anti-$p21^{WAF}$, phosphotyrosine 4G10 monoclonal antibody and anti-Bax, Upstate Biotechnology, Lake Placid, NY; anti-FLAG M2 and anti-tubulin, and sorbitol, Sigma, Inc.; horseradish peroxidase-coupled anti-mouse, Bio-Rad; horseradish peroxidase-coupled anti-rat, Chemicon International, Temecula, CA; anti-mouse-fluorescein isothiocyanate and anti-rabbit-Cy3, Jackson ImmunoResearch Laboratories; and Z-VAD-fmk, Calbiochem. Anti-pan Trk 203 (20) and anti-rat TrkA (21) were previously described.

Adenoviruses and Infections—Wild-type or kinase inactive K538A human *TrkAI* cDNA (22) tagged at their extracellular domains with a FLAG epitope driven by a tTA-sensitive cytomegalovirus promoter were inserted into the pAdTR5F adenovirus vector and transfected into HEK 293 cells, and viral particles were amplified and purified as previously described (23, 24). Adenoviruses encoding wild-type or kinase-inactive *TrkB*, *GFP*, or *LacZ* were described in Atwal *et al.* (19), whereas adenoviruses encoding *Bcl-X_L* or wild-type *p53* were described in Aloyz *et al.* (39). E1B55K and $p53^{C135S}$ Ad5 were a kind gift from Dr. Philip Branton (25). Heat inactivation was performed by heating the virus at 100 °C for 5 min. For infections, NB cells were counted and plated in flasks coated with either 4% rat tail collagen (for biochemistry) or poly-D-lysine (for survival assays and immunohistochemistry) for 18–24 h. Cells were infected with adenovirus at the indicated multiplicity of infection (m.o.i.) and times. NGF (50 ng/ml) and brain-derived neurotrophic factor (100 ng/ml), and U0126 MEK and Z-VAD-fmk were added at the time of infection unless otherwise indicated.

Biochemistry—Trk immunoprecipitation: NB cells infected for 36 h were treated with neurotrophin for 5–10 min at 37 °C and lysed, and Trk proteins were immunoprecipitated and assessed for tyrosine phosphorylation in Western blots then stripped and reprobed for total protein as described before (5). Trk immunoprecipitation in neurons was performed as described previously (26). For Western blots, cell lysates were electrophoresed on a 7% to 15% gradient or 10% SDS-PAGE, and proteins were visualized using a chemiluminescence ECL™ kit (Amersham Biosciences) according to the manufacturer's instructions.

Densitometry Analysis—The densitometry analysis was performed on scanned films with Total Lab 1.0 software (Amersham Biosciences). The intensity values for p53 bands were normalized to the intensity values of the loading control.

Immunostaining Assays—Cells were plated on chamber slides (Nalgene, Naperville, IL), infected in the presence of neurotrophins for the indicated times, washed with Hanks' balanced salt solution (BioWhittaker), fixed in 4% paraformaldehyde (Fisher Scientific) for 15 min, and blocked in 3% normal goat serum (Jackson ImmunoResearch Laboratories)/Hanks' balanced salt solution for 1 h at room temperature. Cells were incubated with primary antibody (diluted in blocking solution) overnight at 4 °C, washed, and incubated with secondary antibodies coupled to fluorochrome for 1 h at room temperature. Nuclei were stained with Hoechst, and pictures were taken using an upright Zeiss microscope.

Survival Assays—MTT, Trypan blue exclusion, and TUNEL assays were performed as previously described (27).

Statistical Analysis—Statistical analyses were performed using the SAS software version 8.02, SAS Institute Inc., Cary, NC. Linear regression, *t* test, Tukey, Bonferroni, and Dunnett tests were performed using the procedure GLM, binary data were analyzed using logistic regression, and maximum likelihood ratio was performed with the procedure LOGISTIC.

RESULTS

TrkA was expressed by recombinant adenovirus in two human NB cell lines derived from malignant tumors, 15N and NGP (16, 17). Both of these cell lines exhibit *MYCN* amplification and do not express detectable endogenous TrkA or TrkB (16, 28). Recombinant adenoviruses expressing wild-type human TrkA, or a kinase-defective (KD) human TrkA encoding a mutation at Lys-538 (22) were generated. The TrkA proteins were expressed from a tTA-regulated cytomegalovirus promoter (23). Adenovirus vectors that express myc epitope-tagged wild-type or kinase-inactive TrkB (19) were also used to infect NB cells. Cells infected with the adenoviruses expressed kinase-active TrkA or TrkB, as determined by probing anti-pan Trk immunoprecipitates of lysates from infected cells with anti-phosphotyrosine (Ptyr). In cells infected with the adenoviruses and treated with the TrkA or TrkB ligands NGF or brain-derived neurotrophic factor, respectively, TrkA and TrkB were expressed and active as kinases (Fig. 1, *A* and *B*). KDTrkA and KDTrkB were also expressed at levels similar to their wild-type counterparts at the virus titers used (Fig. 1*A*). The infection rate was determined to be 50% or more by immunohistochemistry at 27 h post-infection. At this time post-infection, TrkA expressed in NB cells is lower than the endogenous levels of TrkA in cultured sympathetic neurons (Fig. 1*C*), indicating that TrkA was not greatly overexpressed. Because TrkA expression was observed in the absence of co-infection with a tTA virus to further transactivate the tTA-responsive promoter element driving TrkA expression (Fig. 1*B*), we did not use tTA co-infection in further experiments.

We next determined whether TrkA expression would alter the survival or growth of the NB cell lines. TrkA, KDTrkA, TrkB, KDTrkB, and LacZ were ectopically expressed using adenoviruses in 15N and NGP cell lines and survival determined by Trypan blue exclusion (which measures both necrosis and apoptosis) and MTT assay (for mitochondrial function) at 72 h post-infection. The survival of TrkA-infected cells was reduced by 50% as compared with uninfected cells as assessed by Trypan blue exclusion (Fig. 1*D*, $p < 0.05$, Dunnett). In contrast, the expression of TrkB, KDTrkA, KDTrkB or LacZ had no significant effect on cell survival. The cell death-inducing effect of TrkA was dose-responsive in both 15N and NGP cells, with maximal cell death of 70–85% observed at 50–200 m.o.i. as determined by MTT assay (Fig. 1, *E* and *F*). The LacZ adenovirus had no effect on cell viability at 200 m.o.i. To determine whether this effect of TrkA expression was specific to TrkA and not to a contaminant in the virus preparation, the TrkA adenovirus preparation was heat-inactivated prior to infection of NGP cells. This treatment abolished TrkA-induced cell death (Fig. 1*G*). To ascertain whether TrkA caused apoptotic cell death, TUNEL assays were performed at different time points following TrkA or LacZ infection of NGP cells. Approximately 20% of the cells were TUNEL-positive by 48 h of TrkA expression, whereas virtually no TUNEL-positive cells were observed in cells infected with the LacZ adenovirus (Fig. 1*H*). This was statistically significant with $p < 0.001$ (logistic regression). Thus, transient ectopic TrkA, and not TrkB or *KD* TrkA expression, induces the apoptotic cell death of NB cells.

A concern of these experiments is that expression of TrkA via recombinant adenovirus will induce apoptosis of all cell types. We therefore determined whether TrkA adenovirus expression would induce the death of sympathetic neurons, which is derived from the same precursor cells as NB is thought to arise from (29). Sympathetic neurons isolated from the superior cervical ganglia of newborn rats were grown for 3 days in NGF and infected with TrkA or GFP adenoviruses. After 48 h, cells were washed free of NGF, and incubated for a further 48 h in 0–20 ng/ml NGF, and MTT assays were performed. TrkA, when expressed at ~20-fold higher levels than endogenous TrkA (Fig. 1*I*), did not suppress the survival of the neurons. Rather, the adenovirus-expressed TrkA enhanced the survival of neurons grown in the absence or in low amounts of NGF (0–1 ng/ml NGF) that do not normally support survival (Fig. 1*J*), likely reflecting the auto-activation of TrkA observed at high expression levels (20). Thus, elevated levels of TrkA mediate the apoptosis of NB cells and enhance the survival of non-transformed neurons.

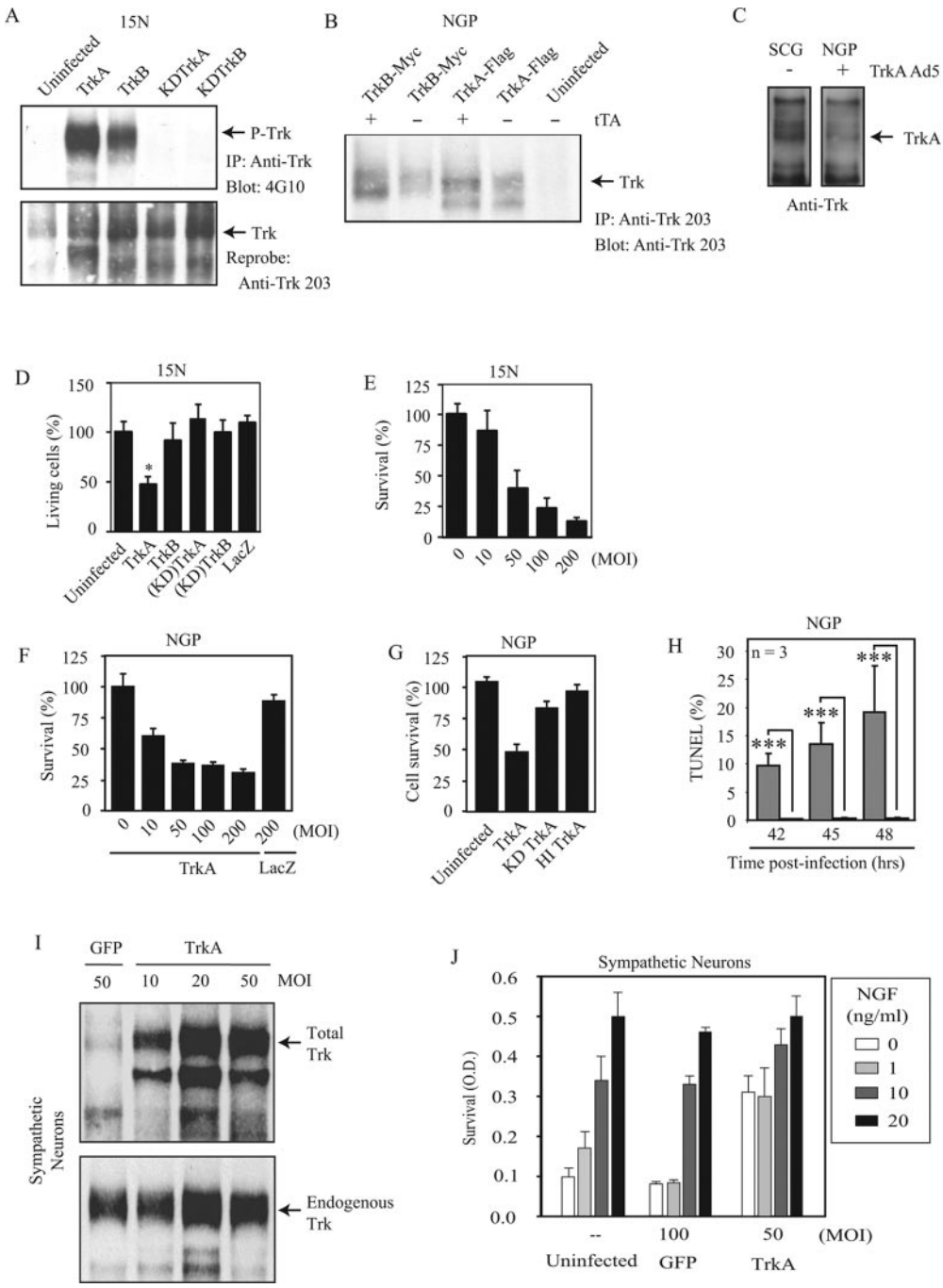

FIG. 1. **TrkA expression induces the apoptosis of NB cells and survival of sympathetic neurons.** *A* and *B*, TrkA adenovirus induces active TrkA expression in neuroblastoma cells. Trk was immunoprecipitated with anti-pan Trk from lysates of 15N (*A*) or NGP (*B*) cells infected with adenoviruses encoding the indicated proteins as described under "Materials and Methods" and stimulated with corresponding neurotrophin. Trk proteins were probed in Western blots with anti-Ptyr (*A*) or anti-pan Trk (*B*), and reprobed with anti-pan Trk (*A*). *C*, expression of TrkA protein in NGP cells infected with TrkA is less than the expression of endogenous TrkA in sympathetic neurons. Sympathetic neurons or NGP cells infected with 50 m.o.i. of TrkA adenovirus (for 27 h) were lysed, and equivalent protein was probed in Western blots with anti-pan Trk. *D–H*, TrkA expression induces cell death of NB cells. *D*, total number of living 15N cells infected with the indicated viruses for 72 h in the presence of NGF was assessed by Trypan blue exclusion assay. Cell number is expressed as a percentage of uninfected cells with its standard error ($n = 3$; *, $p < 0.05$ Dunnett). *E*, representative MTT survival assay of 15N cells infected with TrkA for 72 h at the indicated multiplicity of infection (*MOI*). *F* and *G*, representative MTT survival assay of NGP cells infected with the indicated adenoviruses (*KD*, kinase dead; *HI*, heat-inactivated) and m.o.i. for 72 h in the presence of NGF. *H*, TUNEL assay on NGP cells infected with TrkA (*gray*) or LacZ (*black*) adenoviruses for the indicated times and in the presence of NGF. Shown is the mean proportion of TUNEL-positive cells and S.E. ($n = 3$ experiments, difference between TrkA and LacZ: ***, $p < 0.001$, logistic regression). *I*, detection of recombinant TrkA in sympathetic neurons infected with TrkA or GFP adenovirus for 48 h at the indicated m.o.i. Trk was immunoprecipitated with anti-pan Trk, and blots were probed in the *top panel* with anti-pan Trk, stripped, and reprobed in the *bottom panel* with anti-rat TrkA. *J*, adenovirus-mediated expression of TrkA promotes survival of sympathetic neurons in suboptimal levels of NGF. Representative MTT survival assay of neurons infected with TrkA or GFP (50 or 100 m.o.i.) in the presence of 0 (*white*), 1 (*pale gray*), 10 (*dark gray*), or 20 (*black*) ng/ml NGF for 48 h.

We then asked whether TrkA would induce caspase-dependent apoptosis by examining caspase-3 cleavage as an indicator of caspase-3 activity, and by the cleavage of PARP, a downstream target of caspases. NGP cells treated with NGF were infected with TrkA adenovirus, and cleaved caspase-3 detected by Western blotting with an antibody specific to this protein.

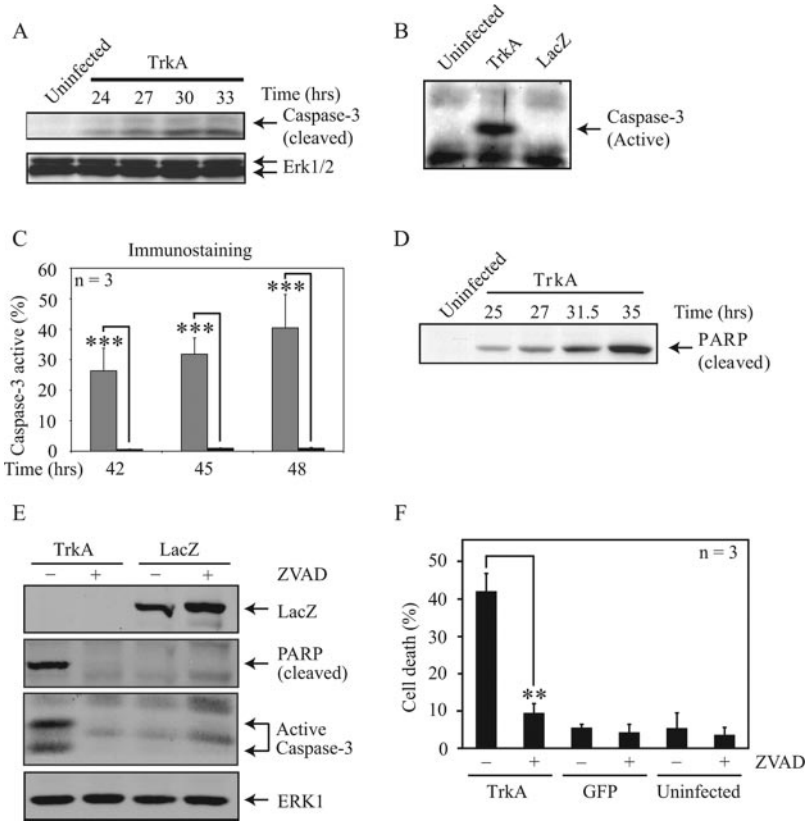

FIG. 2. **TrkA causes caspase-dependent cell death.** *A* and *B*, Western blots for cleaved caspase-3 and Erk1/2 (loading control) of NGP cells expressing (*A*) TrkA for the indicated times or (*B*) LacZ for 36 h. *C*, immunohistochemistry for cleaved caspase-3 on NGP cells infected with TrkA (*gray*) or LacZ (*black*) for the indicated times (average proportion of cleaved caspase-3-positive cells from three independent experiments with associated S.E., *** = $p < 0.001$, logistic regression). *D*, Western blot for cleaved PARP of lysates of NGP cells infected with TrkA for the indicated times. *E* and *F*, blockade of caspase activation by Z-VAD protects against TrkA-induced cell death. *E*, Western blot with antibodies to cleaved caspase-3 or PARP, Erk1, or LacZ on lysates of NGP cells treated with the caspase inhibitor Z-VAD for 36 h. *F*, trypan blue exclusion assay of NGP cells infected with TrkA or GFP adenovirus in the presence or absence of caspase inhibitor (*ZVAD*). Shown is the average of three independent experiments with S.E. (**, $p < 0.01$; Bonferroni and Tukey tests).

Caspase-3 was cleaved by 24 h and increased to maximal levels at 30 h (Fig. 2, *A* and *B*). This effect was specific to TrkA, because LacZ expression resulted in no caspase-3 activation at the 36-h time point (Fig. 2*B*). This result was confirmed by immunostaining for the cleaved form of caspase-3 in TrkA-infected NGP cells (Fig. 2*C*, $p < 0.001$, logistic regression). Similarly, the cleaved form of PARP was observed by 24 h and increased up to 35 h post-TrkA infection (Fig. 2*D*). To determine whether cell death induced by TrkA was dependent upon caspase activation, TrkA-infected cells were treated with the broad-spectrum caspase inhibitor Z-VAD-fmk (100 μM). Z-VAD-fmk prevented the appearance of cleaved caspase-3 and PARP induced by TrkA (Fig. 2*E*, *lanes 1* and *2*) and almost completely suppressed cell death induced by TrkA expression (Fig. 2*F*).

Overexpression of anti-apoptotic Bcl-2 family member proteins such as Bcl-X_L has been shown to be protective against anti-apoptotic stimuli (30). If TrkA induces caspase-3 activation via the mitochondria pathway, we hypothesized that overexpression of Bcl-X_L should suppress TrkA-induced cell death. NGP cells were infected with either Bcl-X_L or control GFP-expressing adenovirus, together with TrkA. The overexpression of Bcl-X_L was confirmed by Western blotting (Fig. 3*A*, *first panel*). Bcl-X_L expression prevented TrkA-induced caspase-3 activation and PARP cleavage (Fig. 3*A*) and significantly ($p < 0.01$, logistic regression) reduced TrkA-mediated cell death by ~50% as compared with cells co-infected with TrkA and GFP (Fig. 3*B*). These results suggest that TrkA induces apoptosis in part by modulating the levels of Bcl-2 family members. In support of this hypothesis, expression of TrkA, but not LacZ via recombinant adenovirus in NGP cells resulted in a dramatic suppression of Bcl-2 protein levels as observed 36 h post-infection (Fig. 3*C*). TrkA expression did not alter the levels of Bax (Fig. 3*D*) or Bcl-X_L (Fig. 3*A*, *TrkA versus LacZ lanes*). Thus, one mechanism whereby TrkA may induce apoptosis is by the suppression of the levels of the anti-apoptotic protein Bcl-2.

We next asked whether there were differences in the activation of intracellular signaling proteins in NB cells expressing TrkA, which induces apoptosis, and TrkB, which does not. NB cells were infected with TrkA or TrkB, and the activation of three known signal transduction cascades, MEK/Erk, phosphatidylinositol 3-kinase/Akt, and PLC-γ1 (14), was assessed after 24 h in NGF or brain-derived neurotrophic factor by probing Western blots of cell lysates with antibodies to phosphorylated and activated Akt, Erk1/2, or PLC-γ1. Although TrkA and TrkB activation resulted in the phosphorylation of both Akt and PLC-γ1 to similar extents (Fig. 4, *A* and *B*), TrkA expression resulted in much more robust Erk1/2 phosphorylation (Fig. 4*C*). No differences in Erk1/2 phosphorylation were observed in TrkA- or TrkB-expressing cells treated with NGF or brain-derived neurotrophic factor for 5 min (data not shown). These results suggest that TrkA may induce apoptosis by a MAPK-dependent mechanism. To test this hypothesis, TrkA-expressing NGP cells were treated with the selective MEK inhibitor U0126, and cell survival was assessed. U0126 treatment (20 μM) for 36 h suppressed the phosphorylation of Erk2, but not of Akt, indicating that U0126 acts selectively in these experiments (Fig. 4*D*). U0126 treatment, however, did not inhibit cell death induced by TrkA (48 h post-infection) in NGP cells as determined by MTT (Fig. 4*E*) or Trypan blue exclusion assay (Fig. 4*F*). MAPK activity is therefore not required for TrkA-induced cell death.

The MKK4/7-JNK pathway is an important pro-apoptotic pathway in sympathetic neurons (14). We therefore asked whether TrkA would induce the activation of JNK in NB cells. NGP cells were infected with TrkA or LacZ adenoviruses for 36 h and treated with 50 ng/ml NGF for either 15 min or 2 h prior to lysis. Western blot analysis using a phosphorylation and activation-specific antibody for JNK revealed that TrkA did not induce the phosphorylation of JNK1/2 (Fig. 4*G*), indicating that these JNK isoforms are likely not involved in TrkA-mediated NB cell death. We also assessed p38 MAPK activity,

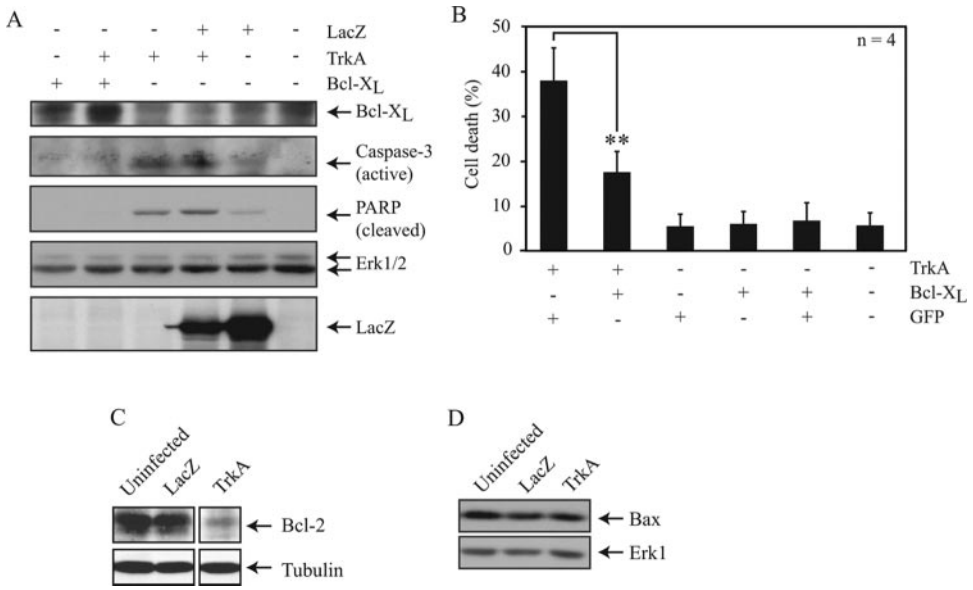

FIG. 3. **Overexpression of Bcl-X_L protects against TrkA-induced cell death, and TrkA reduces Bcl-2 levels.** *A*, Western blot for Bcl-X_L, cleaved caspase-3, PARP, Erk1/2, and LacZ of lysates from NGP cells infected for 36 h with the indicated adenoviruses in the presence of NGF. *B*, trypan blue exclusion survival assay of NGP cells infected with 50 m.o.i. of the indicated viruses. Shown is the average of four independent experiments with S.E. (**, $p < 0.01$; Bonferroni and Tukey tests). *C* and *D*, Western blots for Bcl-2 and tubulin (*C*), and Bax and Erk1 (*D*), of lysates from NGP cells infected with TrkA or LacZ adenoviruses for 36 h in the presence of NGF.

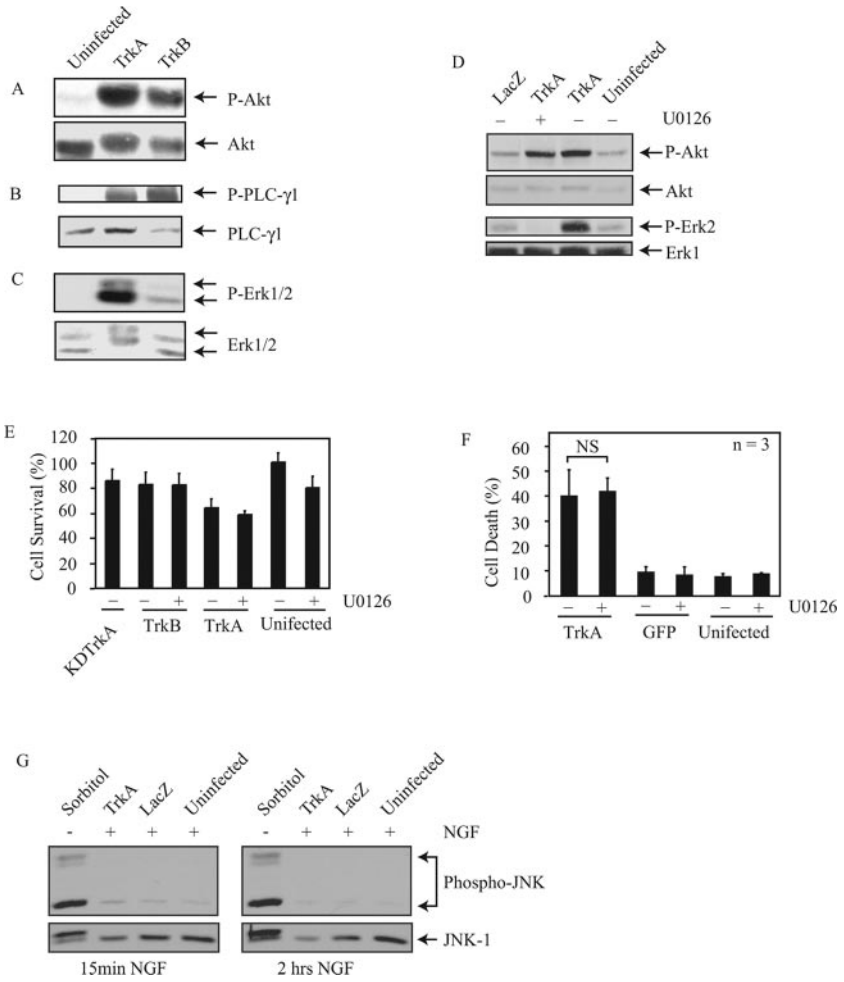

FIG. 4. **TrkA but not TrkB expression causes sustained activation of Erk1/2, although Erk1/2 activity is dispensable for TrkA-induced cell death.** *A–C*, lysates of 15N cells infected with TrkA or TrkB adenoviruses for 24 h and treated with neurotrophins for an additional 24 h were analyzed by Western blots with antibodies for the activated and phosphorylated forms of Akt, Erk1/2, and PLC-γ1, and reprobed with anti-Akt, Erk1/2, or PLC-γ1. *D–F*, inhibition of MEK/Erk pathway is not protective against TrkA-induced cell death. *D*, Western blot of lysates from NGP cells infected with the indicated adenoviruses in the presence or absence of the MEK inhibitor (U0126), with anti-phospho-Akt and anti-phospho-Erk1/2, and reprobed with anti-Akt and Erk1. *E*, representative MTT survival assays of NGP cells infected with the indicated adenoviruses for 72 h in the presence or absence of U0126. *F*, cell death of NGP cells infected with TrkA or GFP adenoviruses for 48 h in the presence of NGF and in the presence or absence of U0126 was measured by Trypan blue exclusion assay ($n = 3$ experiments; *error bar* = S.E.; *NS* = not significant). *G*, lysates of NGP cells infected with TrkA or LacZ adenovirus, treated with NGF for 15 min or 2 h, or treated with 0.5 M sorbitol for 15 min (positive control), were probed in Western blots with anti-phospho-JNK, and reprobed with anti-JNK.

which is required for efficient TrkA-induced apoptosis of PC12 cells (31). No difference in p38 MAPK activity (as determined by a phosphorylation and activation-specific antibody) was observed in NGF-treated NGP cells infected with TrkA or LacZ adenovirus (data not shown). Thus, TrkA appears to induce a p38 MAPK, Erk1/2, and JNK-independent cell death.

An important pro-apoptotic effector acting upstream of mitochondria is the p53 tumor suppressor protein. Most primary

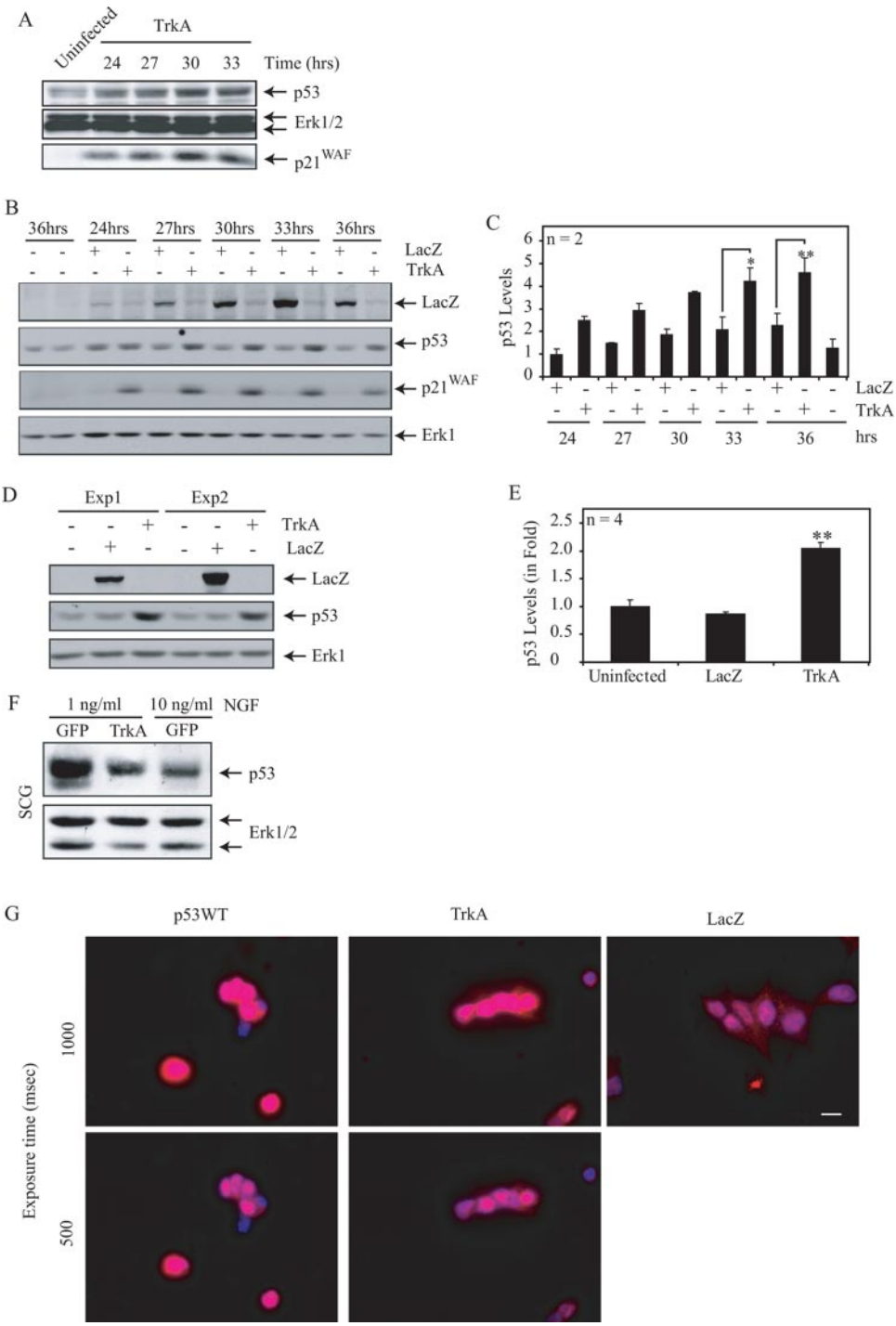

FIG. 5. **TrkA increases p53 protein levels.** *A–C*, NGP cells were infected with the indicated adenoviruses for the indicated times in the presence of NGF. *A*, cell lysates were probed in Western blots with anti-p53, $p21^{WAF}$, or Erk1/2 (as a loading control). *B*, lysates were probed in Western blots with anti-LacZ, p53, $p21^{WAF}$, or Erk1. *C*, densitometry analysis showing the mean and standard error of p53 protein levels adjusted to Erk1 protein levels (arbitrary units, $n = 2$ experiments; *, $p < 0.05$; **, $p < 0.01$; t test). *D*, two independent experiments in which NGP cells were treated as in *B*, but for 30 h, lysed, and run on the same SDS-PAGE and probed in Western blots with anti-p53, LacZ, and Erk1. *E*, densitometry analysis for p53 levels as in *C*, for the time point 30 h post-infection (mean of four independent experiments and its associated standard error; **, $p < 0.01$; Tukey). *F*, neurons grown in 1 ng/ml (suboptimal for survival) or 10 ng/ml (optimal for survival) of NGF were infected with TrkA or GFP adenoviruses for 48 h, and lysates were probed in Western blots with anti-p53 or Erk1/2 as a loading control. *G*, immunohistochemistry for p53 (*red*) and nuclei (*blue*) of NGP cells infected for 30 h with TrkA, LacZ, or wild-type p53 adenoviruses in the presence of NGF. Pictures were taken at the indicated exposure times (*scale bar* = 10 μm).

NBs from patients express wild-type p53 (32–34), and p53 is frequently sequestered in the cytoplasm of NB cells away from its transcriptional targets (35, 36). To address whether TrkA modulates p53 expression levels and/or localization, we measured the protein levels of p53 following TrkA expression by Western blotting or immunofluorescence with anti-p53. TrkA, but not LacZ control virus, induced an increase in p53 levels that was first observed at 24 h post-infection (Fig. 5, *A* and *B*). Densitometry and statistical analyses of all time points showed a significant increase in p53 levels ($p < 0.01$, linear regression adjusted for time or by t test for each time point; *, $p < 0.05$; **, $p < 0.01$, Fig. 5*C*). In addition, that increase was determined to

be of 2-fold 30 h post-infection compared with LacZ-infected cells (Fig. 5, *D* and *E*, $p < 0.01$, Tukey). The levels of $p21^{WAF-1}$, a protein product of a gene whose transcription is induced by p53, were also increased by TrkA expression (Fig. 5, *A* and *B*). In contrast, p53 levels were suppressed by TrkA adenovirus expression in sympathetic neurons grown in suboptimal (1 ng/ml) levels of NGF that do not support survival (Fig. 5*F*). The increases in p53 levels in TrkA-expressing NB cells were confirmed by immunostaining NGP cells with anti-p53 at 30 h post-infection. p53 levels were higher in TrkA-infected than in LacZ-infected cells and showed higher levels of p53 in the nucleus as compared with LacZ-infected cells (Fig. 5*G*). Thus, p53 may be a critical mediator of TrkA-induced NB cell death.

To address whether p53 activity is required for TrkA-mediated NB cell death, two types of experiments were performed. First, we asked whether TrkA would induce the death of *MYCN* amplified SK-N-BE(2) neuroblastoma cells that express only a non-functional p53 mutant (C135F) (37). SK-N-BE(2) or NGP (expressing wild-type p53 (38)) cells were infected with TrkA, wild-type p53, or LacZ adenoviruses for 48–72 h in the presence of NGF, and cell death was assessed. TrkA was expressed at 36 h post-infection and active as a kinase in the TrkA-infected SK-N-BE(2) cells, as determined by Western blotting of anti-Trk immunoprecipitates with anti-Ptyr (Fig. 6*A*, *first two panels*) and by probing with anti-phosphorylation and activation specific Erk1/2 (Fig. 6*A*, *third panel*). Although overexpression of wild-type p53 was sufficient to induce $p21^{WAF-1}$ expression in both cell lines, TrkA was unable to elevate $p21^{WAF-1}$ protein levels in SK-N-BE(2) cells (data not shown). p53 expression induced 43.6 ± 6.9% and 53.0 ± 4.0% (average proportion ± S.E.) cell death in SK-N-BE(2) and NGP cells, respectively, at 48 h post-infection, which increased to 65.8 ± 5.2% and 88.9 ± 0.6% by 72 h (Fig. 6*B*). However, although overexpression of TrkA in NGP cells induced cell death (86.7 ± 6.5%), TrkA expression in SK-N-BE(2) cells did not result in any statistically significant increase in cell death as compared with LacZ-infected or uninfected cells (TrkA: 4.9 ± 0.7%, uninfected: 4.4 ± 2.3% at 72 h) (Fig. 6*B*). These results suggest that TrkA requires a functional wild-type p53 to induce the death of NB cells.

We next determined whether expression of $p53^{C135S}$, a dominant inhibitory mutant of p53 (25), suppresses TrkA-mediated NB cell death. Pre-infection of NGP cells with $p53^{C135S}$ or LacZ 12 h prior to infection with TrkA adenoviruses prevented PARP cleavage (Fig. 6*C*) and completely protected NGP cells from TrkA-induced death (Fig. 6, *D* and *E*). Similarly, co-expression of E1B55K, which inhibits the activity of p53 (39), with TrkA potently suppressed TrkA-induced cell death (Fig. 6*F*). Taken together, these results indicate that expression of TrkA in NB cells results in the induction of apoptosis in a p53-dependent manner.

DISCUSSION

In this report, we show that expression of TrkAI in two *MYCN*-amplified NB cell lines from poor prognosis patient's tumors that do not express endogenous TrkA, induces apoptosis. TrkA potently suppressed Bcl-2 and enhanced p53 protein levels and activity. Overexpression of Bcl-X_L, a Bcl-2 functional homologue, or inhibition of p53 activity rescued NB cells from TrkA-mediated cell death, suggesting two mechanisms whereby TrkA induces apoptosis.

The induction of apoptosis was specific, because expression of equivalent levels of kinase-inactive TrkA and wild-type or kinase-inactive TrkB did not induce apoptosis. The recombinant adenovirus vector itself also had no effect on apoptosis, because heat-inactivated TrkA adenovirus or LacZ, GFP, Bcl-X_L, $p53^{C135S}$, and E1B55K adenoviruses had no affect on cell survival when expressed on their own. As an additional control, TrkA was overexpressed by recombinant adenovirus in newborn sympathetic neurons. In contrast to the apoptotic affects of TrkA in NB cells, TrkA expression in neurons enhanced NGF-independent and -dependent survival. TrkA therefore appears to be a specific inducer of apoptosis in NB cells. This result differs from previous studies showing that TrkA overexpression causes the growth arrest and differentiation, but not the death of NB cells (11, 12, 40, 41). An explanation for these different results may be the method used to express TrkA. In the previous studies, TrkA was expressed by stable transfection, which would select against apoptotic cells, whereas in our experiments, transient expression using recombinant adenovirus allowed for efficient infection and identification of any apoptotic cells.

Two mechanisms were identified whereby TrkA could induce apoptosis, the suppression of Bcl-2, and the increase in p53 levels. Several potential mechanisms might account for how TrkA suppresses the levels of Bcl-2, a key anti-apoptotic protein for many cell types (30). First, TrkA caused the up-regulation of p53, and p53 has been reported to repress *Bcl-2* transcription (42, 43). Therefore TrkA, through its effects on p53, could repress the *de novo* expression of the *Bcl-2* gene. A second mechanism by which TrkA could decrease the levels of Bcl-2 is by activation of caspase-3. Bcl-2 is a target of caspases (44), and cleavage of Bcl-2 by caspase-3 leads to an increase in caspase activation (45).

TrkA enhanced the expression of p53 in both the cytoplasm and nucleus. p53 expression was punctate, which is consistent with previous reports of p53 being sequestered in the cytoplasm in large clusters (35, 36, 46). The p53 immunostaining that we observed is likely specific, as the same results were obtained with three p53 antibodies, and the immunostaining was absent in SAOS cells, which are null for p53 (data not shown). p53 plays a crucial role in cancer cell death. Although human tumor cells that become resistant to cell death often have *p53* deletions or mutations (47), primary NB from patients that have not yet undergone therapy express wild-type p53 (32–34), with p53 sequestered in the cytoplasm (35, 36). In NB, p53 is required for cell death induced by chemotherapeutic drugs and flavonoids such as apigenin. These drugs increase p53 levels (48, 49), and inactivation of p53 by overexpression of dominant negative p53 or the papillomavirus *16E6* gene product, which causes the degradation of p53, prevents chemotherapeutic agent-induced cell death (50). Multidrug-resistant NB cell lines lack expression of functional p53, either due to p53 mutation or high expression of the endogenous p53 protein repressor, MDM2 (51, 52). The potential mechanisms whereby p53 levels increase in TrkA-expressing NB cells remain to be identified, and they include HDM2-induced ubiquitination or neddylation, or TAF-1-stimulated phosphorylation of p53 (53, 54). In addition, TrkA may signal to p53 in other ways, because it can bind both p53 and c-Abl, a p53 activator (55–57), and promote the deacetylation of p53 in PC12 cells (58). It is likely that the modulation of p53 and Bcl-2 levels by TrkA occurs at the post-transcriptional level, because several recent cDNA expression profiling studies on NB tumors or SH-SY5Y NB cells stably transfected with TrkA did not report changes in *p53* or *Bcl-2* genes expression (59, 60). We are currently characterizing the TrkA signals that lead to enhanced p53 expression.

There are several mechanisms whereby p53 can mediate cell death. p53 can induce the transcription of several pro-apoptotic genes, such as *Bax*, *Apaf-1*, *PUMA*, *NOXA*, and Forkhead transcription factors, ultimately resulting in the release of cytochrome *c* from the mitochondrion and the activation of caspase-9/3. Alternatively, p53 can act directly at the mitochondrion to induce the release of cytochrome *c*. In this study we show that

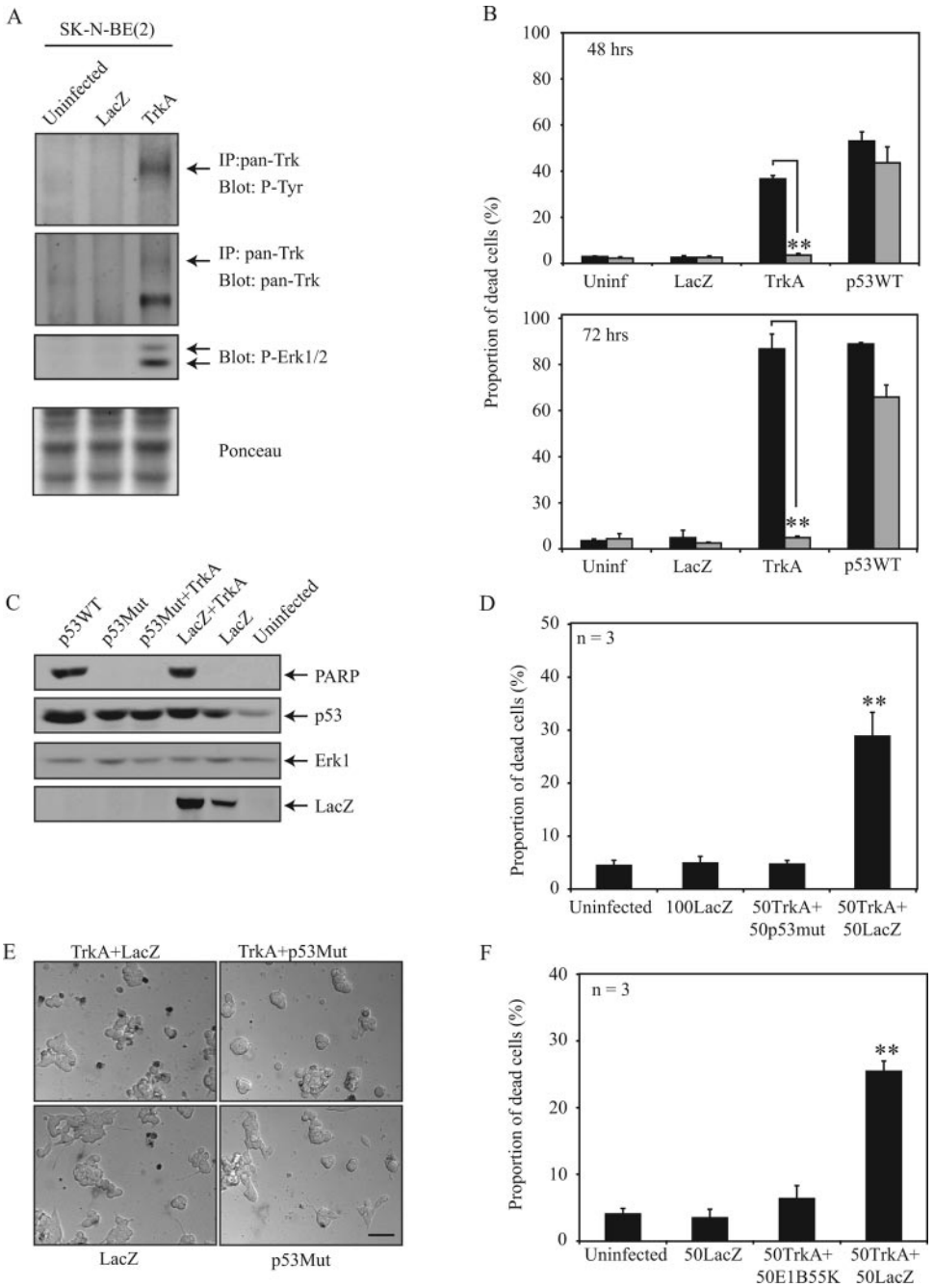

FIG. 6. **TrkA-induced NB cell death requires wild-type p53.** *A*, expression of TrkA in SK-N-BE (2) cells mutated for p53. Cells were infected with TrkA or LacZ adenoviruses, treated with NGF for 5 min, and lysates immunoprecipitated with anti-pan Trk and probed in Western blots with anti-Ptyr to visualize activated Trk (*top panel*), and reprobed with anti-Trk (*second panel*). Lysates were also probed with anti-phospho-Erk1/2 (*third panel*) to show that TrkA activates intracellular signaling proteins. The *bottom panel* shows the Ponceau S staining of the Western blot to show equivalent protein loading. *B*, TrkA fails to induce the apoptosis of SK-N-BE (2) cells. SK-N-BE (2) (*gray bars*) or NGP (*black bars*) were infected with TrkA, wild-type p53, or LacZ adenoviruses and survival assessed by Trypan blue exclusion assay (mean proportion of cell death with associated S.E., NGP $n = 2$, SK-N-BE (2) $n = 3$; **, $p < 0.01$, logistic regression). Wild-type p53 expression via adenovirus caused apoptosis in both cell lines. *C–E*, expression of $p53^{C135S}$ rescue NGP cells from TrkA-induced cell death. Cells were infected with TrkA, $p53^{C135S}$, or LacZ adenoviruses either alone or in combination for 36 h, and lysates probed in Western blots for anti-cleaved PARP, p53, LacZ, or Erk1 (*C*). *D* and *E*, 48 h after infection, survival was assessed by Trypan blue exclusion assay. Shown in *D* is the average of three independent experiment assays and S.E. (**, $p < 0.01$; Tukey). Representative pictures of the Trypan blue assay (*scale bar* = 50 μm) are shown in *E*. *F*, expression of E1B55K rescues NGP cells from TrkA-induced cell death. Cells were infected with TrkA, E1B55K, LacZ adenoviruses either alone or in combination for 48 h and survival assessed by Trypan blue exclusion assay. Shown is a representative experiment with the S.E. of the replicates.

following the increase in p53, the levels of $p21^{WAF-1}$, a p53 gene target, also increase, suggesting that p53 becomes active as a transcription factor. However, the levels of Bax were not elevated following TrkA expression, consistent with the report of Tweddle *et al.* (38) that NGP cells treated with gamma irradiation increase p53 and $p21^{WAF}$ but not Bax levels. In NB cells, it is likely that p53 induces apoptosis by a transcription-dependent mechanism, because the expression of the dominant negative $p53^{C135S}$ with a mutation in the DNA binding domain or E1B55K blocked TrkA-induced cell death.

TrkA and not TrkB caused the death of NB cells. The only difference we observed in the signaling proteins stimulated by these receptors was a much greater enhancement of sustained Erk1/2 phosphorylation by TrkA. However, suppression of

MEK/MAPK activity did not inhibit TrkA-mediated cell death, and TrkA did not increase p38 MAPK activity, which occurs in PC12 cells overexpressing TrkA (31, 61). This result is consistent with the reports of Chou *et al.* (61, 62) that TrkA-induced medulloblastoma apoptosis is independent of Erk1/2, JNK, and p38 MAPK. Therefore it is possible that TrkA induces a Ras and/or Raf-dependent, but MEK/Erk1/2-independent pathway that would be responsible for TrkA-induced apoptosis, as it has been suggested for MB (61).

TrkA expression has been suggested to play a crucial role in the spontaneous regression of NB tumors (63). Our results suggest that TrkA expression may cause malignant neuroblastoma tumors to regress not only by inducing differentiation as previously described (12, 13), but also by stimulating apoptosis. Both of these effects of elevated TrkA expression may explain why TrkA is a good prognosis marker for NB. Therefore, novel therapies base on the induction of TrkA expression/activity or drugs that will mimic TrkA activity might be efficacious to treat patients with aggressive neuroblastoma.

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REFERENCES

1. Aoyama, M., Asai, K., Shishikura, T., Kawamoto, T., Miyachi, T., Yokoi, T., Togari, H., Wada, Y., Kato, T., and Nakagawara, A. (2001) *Cancer Lett.* **164,** 51–60
2. Hoehner, J. C., Olsen, L., Sandstedt, B., Kaplan, D. R., and Pahlman, S. (1995) *Am. J. Pathol.* **147,** 102–113
3. Nakagawara, A., Azar, C. G., Scavarda, N. J., and Brodeur, G. M. (1994) *Mol. Cell. Biol.* **14,** 759–767
4. Nakagawara, A., Arima-Nakagawara, M., Scavarda, N. J., Azar, C. G., Cantor, A. B., and Brodeur, G. M. (1993) *N. Engl. J. Med.* **328,** 847–854
5. Kaplan, D. R., Matsumoto, K., Lucarelli, E., and Thiele, C. J. (1993) *Neuron* **11,** 321–331
6. Jaboin, J., Kim, C. J., Kaplan, D. R., and Thiele, C. J. (2002) *Cancer Res.* **62,** 6756–6763
7. Ho, R., Eggert, A., Hishiki, T., Minturn, J. E., Ikegaki, N., Foster, P., Camoratto, A. M., Evans, A. E., and Brodeur, G. M. (2002) *Cancer Res.* **62,** 6462–6466
8. Matsumoto, K., Wada, R. K., Yamashiro, J. M., Kaplan, D. R., and Thiele, C. J. (1995) *Cancer Res.* **55,** 1798–1806
9. Middlemas, D. S., Kihl, B. K., Zhou, J., and Zhu, X. (1999) *J. Biol. Chem.* **274,** 16451–16460
10. Scala, S., Wosikowski, K., Giannakakou, P., Valle, P., Biedler, J. L., Spengler, B. A., Lucarelli, E., Bates, S. E., and Thiele, C. J. (1996) *Cancer Res.* **56,** 3737–3742
11. Chen, J., Chattopadhyay, B., Venkatakrishnan, G., and Ross, A. H. (1990) *Cell Growth & Differ.* **1,** 79–85
12. Matsushima, H., and Bogenmann, E. (1993) *Mol. Cell. Biol.* **13,** 7447–7456
13. Lavenius, E., Gestblom, C., Johansson, I., Nanberg, E., and Pahlman, S. (1995) *Cell Growth & Differ.* **6,** 727–736
14. Kaplan, D. R., and Miller, F. D. (2000) *Curr. Opin. Neurobiol.* **10,** 381–391
15. Muragaki, Y., Chou, T. T., Kaplan, D. R., Trojanowski, J. Q., and Lee, V. M. (1997) *J. Neurosci.* **17,** 530–542
16. Ciccarone, V., Spengler, B. A., Meyers, M. B., Biedler, J. L., and Ross, R. A. (1989) *Cancer Res.* **49,** 219–225
17. Brodeur, G. M., Sekhon, G., and Goldstein, M. N. (1977) *Cancer* **40,** 2256–2263
18. Barnes, E. N., Biedler, J. L., Spengler, B. A., and Lyser, K. M. (1981) *In Vitro* **17,** 619–631
19. Atwal, J. K., Massie, B., Miller, F. D., and Kaplan, D. R. (2000) *Neuron* **27,** 265–277
20. Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F., and Kaplan, D. R. (1992) *Neuron* **9,** 883–896
21. Lucidi-Phillipi, C. A., Clary, D. O., Reichardt, L. F., and Gage, F. H. (1996) *Neuron* **16,** 653–663
22. Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A., and Kaplan, D. R. (1994) *Neuron* **12,** 691–705
23. Massie, B., Dionne, J., Lamarche, N., Fleurent, J., and Langelier, Y. (1995) *Biotechnology* (*New York*) **13,** 602–608
24. Mazzoni, I. E., Said, F. A., Aloyz, R., Miller, F. D., and Kaplan, D. (1999) *J. Neurosci.* **19,** 9716–9727
25. Narendran, A., Ganjavi, H., Morson, N., Connor, A., Barlow, J. W., Keystone, E., Malkin, D., and Freedman, M. H. (2003) *Exp. Hematol.* **31,** 693–701
26. Belliveau, D. J., Krivko, I., Kohn, J., Lachance, C., Pozniak, C., Rusakov, D., Kaplan, D., and Miller, F. D. (1997) *J. Cell Biol.* **136,** 375–388
27. Wartiovaara, K., Barnabe-Heider, F., Miller, F. D., and Kaplan, D. R. (2002) *J. Neurosci.* **22,** 815–824
28. Schwab, M., Alitalo, K., Klempnauer, K. H., Varmus, H. E., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. (1983) *Nature* **305,** 245–248
29. Brodeur, G. M. (2003) *Nat. Rev. Cancer* **3,** 203–216
30. Huang, Z. (2000) *Oncogene* **19,** 6627–6631
31. Yan, C., Liang, Y., Nylander, K. D., and Schor, N. F. (2002) *Cancer Res.* **62,** 4867–4875
32. Vogan, K., Bernstein, M., Leclerc, J. M., Brisson, L., Brossard, J., Brodeur, G. M., Pelletier, J., and Gros, P. (1993) *Cancer Res.* **53,** 5269–5273
33. Komuro, H., Hayashi, Y., Kawamura, M., Hayashi, K., Kaneko, Y., Kamoshita, S., Hanada, R., Yamamoto, K., Hongo, T., and Yamada, M. (1993) *Cancer Res.* **53,** 5284–5288
34. Imamura, J., Bartram, C. R., Berthold, F., Harms, D., Nakamura, H., and Koeffler, H. P. (1993) *Cancer Res.* **53,** 4053–4058
35. Moll, U. M., LaQuaglia, M., Benard, J., and Riou, G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4407–4411
36. Moll, U. M., Ostermeyer, A. G., Haladay, R., Winkfield, B., Frazier, M., and Zambetti, G. (1996) *Mol. Cell. Biol.* **16,** 1126–1137
37. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) *Cell* **90,** 809–819
38. Tweddle, D. A., Malcolm, A. J., Cole, M., Pearson, A. D., and Lunec, J. (2001) *Am. J. Pathol.* **158,** 2067–2077
39. Aloyz, R. S., Bamji, S. X., Pozniak, C. D., Toma, J. G., Atwal, J., Kaplan, D. R., and Miller, F. D. (1998) *J. Cell Biol.* **143,** 1691–1703
40. Lucarelli, E., Kaplan, D., and Thiele, C. J. (1997) *Eur. J. Cancer* **33,** 2068–2070
41. Poluha, W., Poluha, D. K., and Ross, A. H. (1995) *Oncogene* **10,** 185–189
42. Budhram-Mahadeo, V., Morris, P. J., Smith, M. D., Midgley, C. A., Boxer, L. M., and Latchman, D. S. (1999) *J. Biol. Chem.* **274,** 15237–15244
43. Wu, Y., Mehew, J. W., Heckman, C. A., Arcinas, M., and Boxer, L. M. (2001) *Oncogene* **20,** 240–251
44. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
45. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) *Science* **278,** 1966–1968
46. Nikolaev, A. Y., Li, M., Puskas, N., Qin, J., and Gu, W. (2003) *Cell* **112,** 29–40
47. Levine, A. J. (1997) *Cell* **88,** 323–331
48. Fulda, S., Sieverts, H., Friesen, C., Herr, I., and Debatin, K. M. (1997) *Cancer Res.* **57,** 3823–3829
49. Torkin, R., Lavoie, J. F., Kaplan, D. R., and Yeger, H. (2005) *Mol. Cancer Ther.* **4,** 1–11
50. Cui, H., Schroering, A., and Ding, H. F. (2002) *Mol. Cancer Ther.* **1,** 679–686
51. Keshelava, N., Zuo, J. J., Chen, P., Waidyaratne, S. N., Luna, M. C., Gomer, C. J., Triche, T. J., and Reynolds, C. P. (2001) *Cancer Res.* **61,** 6185–6193
52. Tweddle, D. A., Malcolm, A. J., Bown, N., Pearson, A. D., and Lunec, J. (2001) *Cancer Res.* **61,** 8–13
53. Brooks, C. L., and Gu, W. (2003) *Curr. Opin. Cell Biol.* **15,** 164–171
54. Li, H. H., Li, A. G., Sheppard, H. M., and Liu, X. (2004) *Mol. Cell* **13,** 867–878
55. Brown, A., Browes, C., Mitchell, M., and Montano, X. (2000) *Oncogene* **19,** 3032–3040
56. Koch, A., Mancini, A., Stefan, M., Niedenthal, R., Niemann, H., and Tamura, T. (2000) *FEBS Lett.* **469,** 72–76
57. Yano, H., Cong, F., Birge, R. B., Goff, S. P., and Chao, M. V. (2000) *J. Neurosci. Res.* **59,** 356–364
58. Vaghefi, H., and Neet, K. E. (2004) *Oncogene* **23,** 8078–8087
59. Schulte, J. H., Schramm, A., Klein-Hitpass, L., Klenk, M., Wessels, H., Hauffa, B. P., Eils, J., Eils, R., Brodeur, G. M., Schweigerer, L., Havers, W., and Eggert, A. (2005) *Oncogene* **24,** 165–177
60. Krasnoselsky, A. L., Whiteford, C. C., Wei, J. S., Bilke, S., Westermann, F., Chen, Q. R., and Khan, J. (2005) *Oncogene* **24,** 1533–1541
61. Chou, T. T., Trojanowski, J. Q., and Lee, V. M. (2000) *J. Biol. Chem.* **275,** 565–570
62. Chou, T. T., Trojanowski, J. Q., and Lee, V. M. (2001) *J. Biol. Chem.* **276,** 41120–41127
63. Nakagawara, A. (2001) *Cancer Lett.* **169,** 107–114