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

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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-xL 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.

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Neuroblastoma (NB) is the most frequent solid extracranial tumor of childhood and likely arises from sympathoadrenergic 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 JNK, c-Jun NH2-terminal kinase, Z-VAD-fmk, benzoylcyxarnil-VAL-FD 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; Ptpy, phosphotyrosine; m.o.i., multiplicity of infection; PARP, poly(ADP-ribose) polymerase; TTA, tetracycline-responsive transcriptional activator.

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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).
proteins were visualized using a chemiluminescence ECL™ kit (Amer-
were electrophoresed on a 7% to 15% gradient or 10% SDS-PAGE, and
phorylation in Western blots then stripped and reprobed for total pro-
Trk proteins were immunoprecipitated and assessed for tyrosine phos-
trophic factor (100 ng/ml), and U0126 MEK and Z-VAD-fmk were added
h. Cells were infected with adenovirus at the indicated multiplicity of
poly-D-lysine (for survival assays and immunohistochemistry) for 18–24
flasks coated with either 4% rat tail collagen (for biochemistry) or
100 °C for 5 min. For infections, NB cells were counted and plated in
infections in 15N and NGP cell lines and survival deter-
mained by Trypan blue exclusion (which measures both necrosis and apoptosis) and MTT assay (for mitochondrial function) determined 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. 1D, p < 0.05, Dunnett).
In contrast, the expression of TrkB, KDTrkA, KDTrkB or LacZ had no significant effect on cell survival. The cell death-induc-
ing 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 in-
fecction of NGP cells. This treatment abolished TrkA-induced cell death (Fig. 1G). To ascertain whether TrkA caused apop-
totic 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. 1H). 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. 1I), 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. 1J), 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.

**RESULTS**

TrkA was expressed by recombinant adenovirus in two hu-
man NB cell lines derived from malignant tumors, 15N and
NGP (16, 17). Both of these cell lines exhibit MYCN amplifica-
and do not express detectable endogenous TrkA or TrkB (16, 28). Recombinant adenoviruses expressing wild-type hu-
man 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 pro-
moter (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 ki-
rase-active TrkA or TrkB, as determined by probing anti-pan
Trk immunoprecipitates of lysates from infected cells with
anti-phosphorytosine (Ptyr). In cells infected with the adenovi-
ruses 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. 1A). The
infection rate was determined to be 50% or more by immu-
nohistochemistry at 27 h post-infection. At this time post-infec-
tion, TrkA expressed in NB cells is lower than the endogenous
levels of TrkA in cultured sympathetic neurons (Fig. 1C), indi-
cating 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. 1B), 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 determin-
estimated by Trypan blue exclusion (which measures both necrosis and apoptosis) and MTT assay (for mitochondrial function) determined at
72 h post-infection. The survival of TrkA-infected cells was reduced by 50% as compared with uninfected cells as assessed by
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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.
expression of Bcl-XL should suppress TrkA-induced cell death. We hypothesized that over-anti-apoptotic stimuli (30). If TrkA induces caspase-3 activation and PARP induced by TrkA (Fig. 2). Z-VAD-fmk prevented the appearance of cleaved caspase-3 and cleaved PARP induced by TrkA (Fig. 3). Cleavage of PARP was observed by 24 h and increased up to 35 h post-TrkA infection (Fig. 2B). 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 for 36 h. Z-VAD-fmk prevented the appearance of cleaved caspase-3 and PARP induced by TrkA (Fig. 2E, lanes 1 and 2) and almost completely suppressed cell death induced by TrkA expression (Fig. 2F).

Overexpression of anti-apoptotic Bcl-2 family member proteins such as Bcl-XL 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-XL should suppress TrkA-induced cell death. NGP cells were infected with either Bcl-XL or control GFP-expressing adenovirus, together with TrkA. The overexpression of Bcl-XL was confirmed by Western blotting (Fig. 3A, first panel). Bcl-XL expression prevented TrkA-induced caspase-3 activation and PARP cleavage (Fig. 3A) 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. 3B). 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. 3C). TrkA expression did not alter the levels of Bax (Fig. 3D) or Bcl-XL (Fig. 3A, 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-\(\gamma\) (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-\(\gamma\). Although TrkA and TrkB activation resulted in the phosphorylation of both Akt and PLC-\(\gamma\) to similar extents (Fig. 4, A and B), TrkA expression resulted in much more robust Erk1/2 phosphorylation (Fig. 4C). 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 \( \mu \)M) for 36 h suppressed the phosphorylation of Erk2, but not of Akt, indicating that U0126 acts selectively in these experiments (Fig. 4D). U0126 treatment, however, did not inhibit cell death induced by TrkA (48 h post-infection) in NGP cells as determined by MTT (Fig. 4E) or Trypan blue exclusion assay (Fig. 4F). 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. 4G), indicating that these JNK isoforms are likely not involved in TrkA-mediated NB cell death. We also assessed p38 MAPK activity,
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
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. 5C). In addition, that increase was determined to

**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, p21WAF, or Erk1/2 (as a loading control). B, lysates were probed in Western blots with anti-LacZ, p53, p21WAF, 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).
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 p21WAF-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. 5F). The increases in p53 levels in TrkA-expressing NB cells were confirmed by immunostaining NGF 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. 5G). 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 NGF (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-PY (Fig. 6A, first two panels) and by probing with anti-phosphorylation and activation specific Erk1/2 (Fig. 6A, third panel). Although overexpression of wild-type p53 was sufficient to induce p21WAF-1 expression in both cell lines, TrkA was unable to elevate p21WAF-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 NGF cells, respectively, at 48 h post-infection, which increased to 65.8 ± 5.2% and 88.9 ± 0.6% by 72 h (Fig. 6B). However, although overexpression of TrkA in NGF 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. 6B). These results suggest that TrkA requires a functional wild-type p53 to induce the death of NB cells.

We next determined whether expression of p53C135S, a dominant inhibitory mutant of p53 (25), suppresses TrkA-mediated NB cell death. Pre-infection of NGF cells with p53C135S or LacZ 12 h prior to infection with TrkA adenoviruses prevented PARP cleavage (Fig. 6C) and completely protected NGF cells from TrkA-induced death (Fig. 6D and E). Similarly, co-expression of E1B55K, which inhibits the activity of p53 (39), with TrkA potentially suppressed TrkA-induced cell death (Fig. 6F). 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 TrkA 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, 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-XL, p53C135S, 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 effects 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 E6/E6 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 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-Abi, 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...
following the increase in p53, the levels of p21WAF, a p53 gene
target, also increase, suggesting that p53 becomes active as a
transcription factor. However, the levels of Bax were not ele-
vated following TrkA expression, consistent with the report of
Tweddle et al. (38) that NGP cells treated with gamma irradi-
ation increase p53 and p21WAF but not Bax levels. In NB cells,
it is likely that p53 induces apoptosis by a transcription-de-
pendent mechanism, because the expression of the dominant
negative p53C135S 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

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 p53C135S rescue NGP cells from TrkA-induced cell death. Cells were infected with TrkA, p53C135S, 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.
MEKMAPK 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 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 based 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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