TrkA alternative splicing: A regulated tumor-promoting switch in human neuroblastoma

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Summary

We identify a novel alternative TrkA splice variant, TrkAIII, with deletion of exons 6, 7, and 9 and functional extracellular IG-C1 and N-glycosylation domains, that exhibits expression restricted to undifferentiated early neural progenitors, human neuroblastomas (NBs), and a subset of other neural crest-derived tumors. This NGF-unresponsive isoform is oncogenic in NIH3T3 cells and promotes tumorigenic NB cell behavior in vitro and in vivo (cell survival, xenograft growth, angiogenesis) resulting from spontaneous tyrosine kinase activity and IP3K/Akt/NF-κB but not Ras/MAPK signaling. TrkAIII antagonizes NGF/TrkAI signaling, which is responsible for NB growth arrest and differentiation through Ras/MAPK, and its expression is promoted by hypoxia at the expense of NGF-responsive receptors, providing a mechanism for converting NGF/TrkA/Ras/MAPK antioncogenic signals to TrkAIII/IP3K/Akt/NF-κB tumor-promoting signals during tumor progression.

Introduction

Neurotrophin tyrosine kinase receptor type 1 (TrkA), a member of the tyrosine kinase neurotrophin receptor family that includes TrkB and TrkC, is the preferred receptor for nerve growth factor (NGF) and is critical for development and maturation of central and peripheral nervous systems, regulating proliferation, differentiation, and programmed cell death (Bibel and Barde, 2000; Oppenheim, 1991; Hempstead et al., 1991; Martin-Zanca et al., 1989; Klein et al., 1991; Kaplan et al., 1991; Meakin and Shooter, 1991).

The 25 kb human *TrkA* gene maps to chromosome 1q21-q22 (Weier et al., 1995), is organized into 17 exons (Greco et al., 1996), with exon 9 alternatively spliced, producing TrkAI and II isoforms (Barker et al., 1993), and encodes a transmembrane receptor that is comprised of extracellular, transmembrane, and intercellular domains. The extracellular domain contains two cysteine-rich regions interrupted by a leucine-rich domain and two immunoglobulin-like IGC1 and IGC2 regions (Urfer et al., 1995; Windisch et al., 1995; Holden et al., 1997; Arevalo et al., 2000). Transmembrane and juxtamembrane domains are critical for signal internalization and transduction (Peng et al., 1995; Monshipouri et al., 2000). The intercellular domain exhibits tyrosine kinase (Tk) activity upon dimerization (Kaplan et al., 1991; Meakin and Shooter, 1991) and initiates intracellular signal transduction by autophosphorylating Y490, Y674/675, Y751, and Y785 tyrosine residues, which act as phosphorylation-dependent binding sites for Shc (Y490), Grb-2/SOS (Y674/675), and FRS-2 (Y490) adaptor proteins, the inositol phosphate 3 kinase (IP3K) subunit p85α (Y751), and PLCγ (Y785). These interactions induce signal transduction through Ras/MAPK, inositol phosphate, and/or PKC, which mediate NGF effects on proliferation, differentiation, and apoptosis (Obermeier et al., 1993a, 1993b, 1994; Kaplan and Stephens, 1994; Yao and Cooper, 1995; Green and Kaplan, 1995; Segal et al., 1996; Cunningham et al., 1997; Meakin et al., 1999).

Oncogenic TrkA activation by point mutation, deletion, or novel chimera formation due to chromosomal rearrangements results in spontaneous ligand-independent receptor dimerization and activation, constitutive adaptor protein binding, chronic signal transduction, and cellular transformation. Activated TrkA oncogenes have been associated with several human malignancies, including colon, thyroid, and prostate carcinomas and acute myeloid leukemia (Oskam et al., 1988; Martin-Zanca et al., 1986; Coulier et al., 1990; Borello et al., 1994; George et

SIGNIFICANCE

Identification of the novel TrkAIII splice variant unveils an alternative splice mechanism for inducing ligand-independent TrkA signaling and antagonizing signals from NGF-responsive receptors. The restricted pattern of TrkAIII expression that is observed suggests a mechanism of physiological relevance to normal neural progenitors, conserved but subverted to pathological importance in NB, that challenges current concepts of an exclusively tumor-suppressing role for TrkA in this tumor type. Stimulation of alternative TrkAIII splicing by hypoxia, as well as the capacity of TrkAIII to promote a more angiogenic and stress-resistant phenotype, suggests a role in protection against hypoxic conditions, while providing an important epigenetic mechanism to promote TrkAIII involvement in tumor progression and a potential target for therapeutic intervention.

al., 1998; Greco et al., 1992, 1995; Butti et al., 1995; Reuther et al., 2000; Arevalo et al., 2001).

Neuroblastoma (NB), a highly aggressive malignant childhood tumor of neural crest origin (reviewed in Pahlman and Hedborg, 2000), associates with noncoding *TrkA* gene polymorphisms and point mutations (Scaruffi et al., 1999) but not with genetic oncogenic TrkA activation. NB cell lines exhibit general NGF unresponsiveness (Azar et al., 1990), and TrkA expression exhibits an inverse relationship to NB aggressiveness, consistent with a potential marker of good prognosis (Nakagawara et al., 1992; Nakagawara, 2001). A potential tumor-suppressing role for TrkA in NB is supported by TrkA gene transduction in NB cells, which restores NGF responsiveness, characterized by TrkA activation and binding of phosphorylated Shc, Grb-2/SOS, FRS-2, PLCγ-1, and p85α and signal transduction through IP, Ras/MAPK, and PKC, and results in differentiation, growth arrest, apoptosis, and inhibition of angiogenesis (Matsushima and Bogenmann, 1990; Azar et al., 1990; Lavenius et al., 1995; Yao and Cooper, 1995; Lucarelli et al., 1997; Edsjo et al., 2001; Sugimoto et al., 2001; Eggert et al., 2002).

Here, we challenge the concept of an exclusively tumor-suppressing, nononcogenic role for TrkA in NB by unveiling a novel hypoxia-regulated mechanism for oncogenic TrkA activation in NB cells characterized by generation of a novel constitutively active TrkAIII splice variant that exhibits oncogenic properties, antagonizes antioncogenic NGF/TrkAI signaling, and is expressed by primary human NBs.

Results

Identification, cloning, and sequence analysis of the novel TrkAIII splice variant expressed by SH-SY5Y cells

Direct sequence comparison of full-length coding TrkA cDNAs cloned from human SH-SY5Y NB cells, by reverse transcriptase-PCR (RT-PCR) using *TrkA*-specific primers (Experimental Procedures), revealed a novel TrkA isoform lacking exons 6, 7, and 9 sequence, other than TrkAI and TrkAII (Martin-Zanca et al., 1989; Barker et al., 1993), which we have named TrkAIII (Figure 1A). Omission of exons 6, 7, and 9 sequence did not introduce a frameshift or novel stop codon. Deletions or point mutations were not detected in *TrkA* gene sequence, examined by direct PCR sequencing of products generated from purified SH-SY5Y genomic DNA, using primers (see the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/) spanning the intron/exon 5 to exon 8/intron splice junctions (data not shown) (Greco et al., 1996). TrkA exon 6–7 omission predicts loss of amino acids 192–284, encoding extracellular immunoglobulin-like IG-C1 and several functional N-glycosylation domains (Martin-Zanca et al., 1989) and a valine addition at the novel exon 5/8 splice junction (Figure 1A).

In Northern blots, SH-SY5Y cells expressed two TrkA species of approximately 2.8 and 3.2 kb, both of which hybridized to a *TrkA* exon 8 probe, with the larger but not smaller species hybridizing also with a *TrkA* exon 6–7 probe (Figure 1B). Western blots of anti-TrkA immunoprecipitates revealed low-level expression of two TrkA species of approximately 140 kDa and 100 kDa, in SH-SY5Y cells, consistent with TrkAI/II (140 kDa) and TrkAIII (100 kDa) (Figure 1B), at an approximate ratio of 3:1 (Figures 1B and 8B; see Figure 3B for transfected TrkAI and TrkAIII).

TrkAIII expression is relatively specific but not exclusive to neuroblastoma

TrkAIII mRNA expression in human primary NB samples was confirmed by RT-PCR/Southern blot using *TrkA*-specific primers spanning cDNA sequences including exons 5 to 8 (see the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/). Primers generated two RT-PCR products of approximately 850 bp and 570 bp from 1 μg total RNAs, determined to be within the linear range by comparative 15-, 25-, 35-, and 50-cycle RT-PCR amplification (Figure 2 and data not shown). In Southern blots, both products hybridized to a *TrkA* exon 8 probe, whereas only the 850 bp product hybridized to a *TrkA* exon 6–7 probe, consistent with TrkA (I/II) identity for the 850 bp product and TrkAIII identity for the 570 bp product (Figure 2A). Confirmation that products reflected individual mRNA species was obtained using additional primer sets (see the Supplemental Data) spanning a similar region, which also generated two major RT-PCR products consistent with TrkAI/II and TrkAIII (data not shown). TrkAIII identity was further confirmed by direct sequence analysis of several independent 570 bp products (data not shown). The NB RNAs available for this study were insufficient for Northern blot analysis. However, human primary NBs have previously been shown to contain an additional lower-sized species other than 3.2 kb TrkAI by Northern blotting (Nakagawara et al., 1992), consistent with TrkAIII expression.

Densitometric analysis of the ratio between TrkAI/II and TrkAIII RT-PCR products (i.e., the TrkA I/II densitometric value divided by the TrkAIII densitometric value) in NB samples grouped by disease stage (I–IV, international NB staging system) revealed that TrkAI/II to TrkAIII ratios in stage I and II tumors (5.3 ± 3.89 and 7.0 ± 3.6, respectively) were higher those in stage III and IV tumors (1.3 ± 2.0 and 1.4 ± 1.4, respectively) (Figure 2B). Indeed, the mean ($\pm$SD) TrkAI/II to TrkAIII densitometric ratio in combined low stage (I and II) samples of 6.21 ± 3.64 ($n = 11$) was significantly greater ($p < 0.001$, Student's t test; $p < 0.02$, Mann-Whitney test) than the ratio in combined high stage (III and IV) samples (1.335 ± 1.66; $n = 13$). Accordingly, TrkAIII predominated over TrkA (I/II) RT-PCR products in one of eleven stage I and II tumors and six out of thirteen stage III and IV tumors (Figure 2B). Several high stage tumors exhibited relatively low-level TrkA(I/II) expression, with three also exhibiting Nmyc amplification (Figure 2A), supporting reports of an inverse relationship between Nmyc amplification and TrkA(I/II) expression (Nakagawara et al., 1992; Nakagawara, 2001). Together, these data suggest that, in contrast to TrkA(I/II), TrkAIII expression may relate to disease progression.

In other neural crest-derived tumor tissues, comparatively low-level expression of TrkAIII together with TrkA(I/II) was detected in a medullary thyroid tumor and in two of three prostate tumors, whereas neither TrkA(I/II) nor TrkAIII was detected in melanomas. In neural crest-unrelated tumors, TrkA(I/II) but not TrkAIII expression characterized six human pituitary adenomas, whereas neither was detected in tumors of the breast, colon, and stomach (Figure 2C).

In nontumor tissues, TrkA(I/II) without TrkAIII expression characterized normal human fetal and adult brain- and neural crest-derived adrenal medulla and dorsal root ganglia, whereas neither TrkA(I/II) nor TrkAIII was detected in nonneural normal breast, colon, and stomach tissues (Figure 2C).

To further investigate TrkAIII in cell subsets, expression was

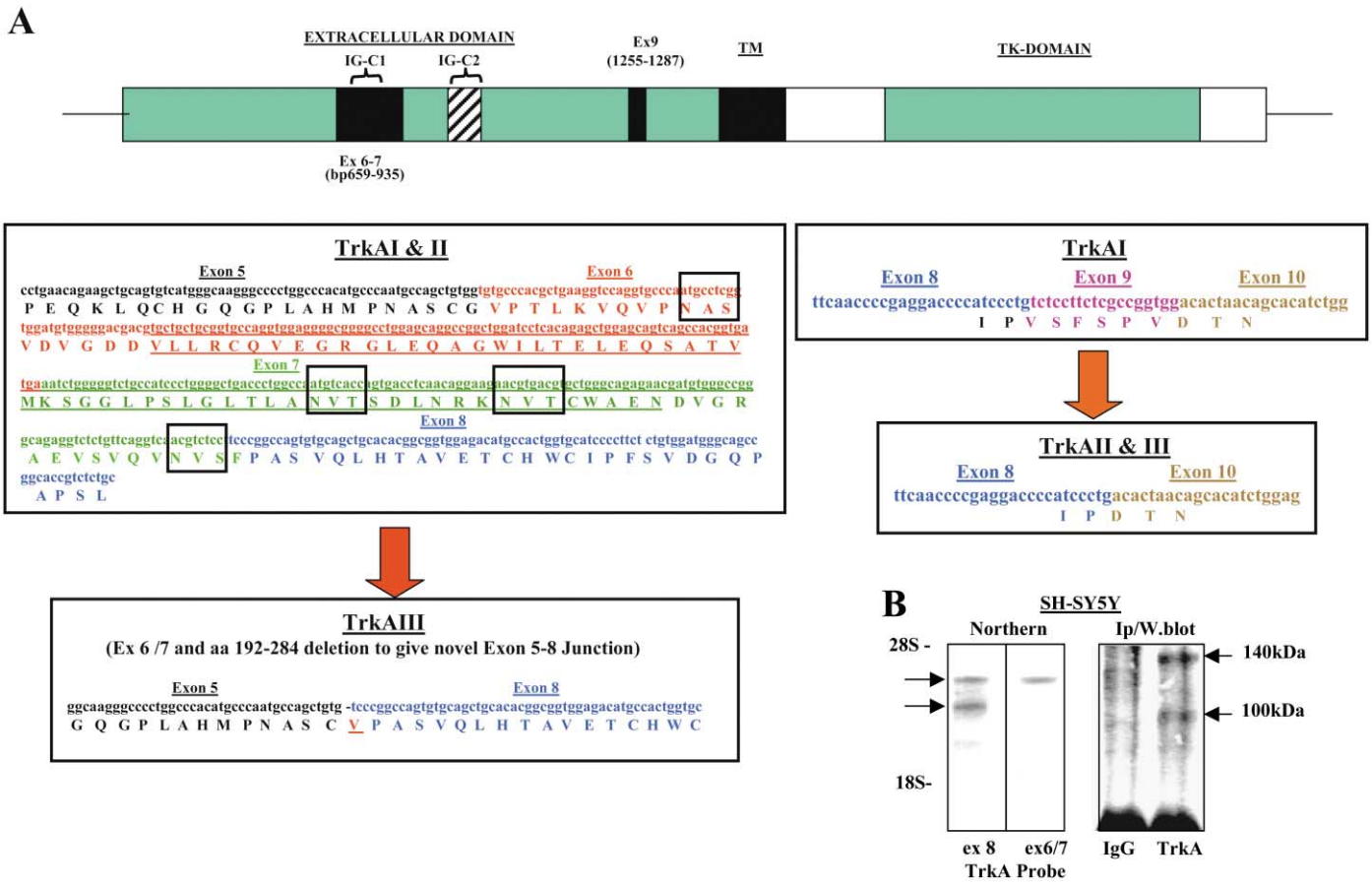

Figure 1. TrkAIII is devoid of exons 6, 7, and 9

A: Schematic TrkAI representation indicating extracellular IG-C1 and -C2, transmembrane (TM) tyrosine kinase (TK) domains, and relative exons 6, 7 (Ex 6–7), and 9 (Ex 9) positions. Partial exon 5 (black), 8 (blue), 10 (brown), complete exon 6 (red), 7 (green), 9 (purple), and novel unique TrkAIII exon 5/8 and exon 8/10 splice junction (TrkAII and AIII) cDNA and amino acid sequences are provided (IG-C1 domain is underlined; N-glycosylation sites are boxed).
B: Northern blot (left) of SH-SY5Y TrkA mRNA species (arrows) hybridizing to TrkA exon 8 (ex 8) and exon 6–7 (ex6/7) probes (28S and 18S indicated). Western blot (right) of 140 and 100 kDa TrkA species immunoprecipitated from SH-SY5Y cells (2 mg protein) by anti-TrkA but not preimmune IgG.

analyzed in both established and primary normal and tumor cell cultures. In tumor cell cultures, TrkA(I/II) and TrkAIII coexpression characterized the human (IMR32, SH-SY5Y, SK-N-AS, KCNR, SK-N-SH [S and N], LA-N-5, SK-N-BE) and mouse (Neuro2a) NB cell lines, TT medullary thyroid cancer, PC12 pheochromocytoma, and human PC-3 prostate cancer cells (Figure 2D). TrkA(I/II) without TrkAIII expression characterized human A-549 nonsmall cell lung carcinoma and M14 melanoma cells, whereas human MDA-MB-231 breast cancer cells expressed neither TrkA(I/II) nor TrkAIII, confirming data from primary tumors. In "normal" cell cultures, TrkA(I/II) and TrkAIII coexpression characterized normal human neural stem cell neurospheres, representing a very early developmental stage that precedes neuronal, astroglial, or oligodendroglial lineage choice, as well as mouse neural crest-derived TC-1S progenitor cells, which can undergo either neurogenic or myogenic lineage choice in response to external signals (Giannini et al., 2001). In contrast, TrkA(I/II) without TrkAIII expression characterized primary cultures of mouse cerebellar granule neurons (Figure 2D), displaying morphological features of neuronal differentiation (neurite extension) and expression of neuronal markers βIII-tubulin (Figure 2E) and NeuN (data not shown). Neither TrkA(I/II) nor TrkAIII expression was detected in mouse NIH3T3 or human MRC-5 fibroblasts (data not shown). These observations suggest relatively restricted TrkAIII expression to neural crest-derived neuroblastic tumors and normal pluripotent neural stem and neural crest progenitors.

TrkAIII exhibits spontaneous Tk and IP3K activities

Functional characterization of TrkAIII was made in direct comparison with empty pcDNA3.1 vector, TrkAI, and Trk-T3 oncogene (positive oncogenic control; Greco et al., 1995) stable transfected SH-SY5Y cells. Several clones were isolated by zeocin resistance, and expression was confirmed by indirect immunofluorescence, immunoprecipitation/Western blotting (Figures 3A and 3B), and RT-PCR (data not shown). Immunoprecipitation of TrkA isoforms from stable transfectants identified 140 kDa and 110 kDa TrkAI species, the latter representing differentially glycosylated TrkAI (Martin-Zanca et al., 1989), a single 100 kDa TrkAIII and a single 68 kDa Trk-T3 species, respectively (Figure 3B, left panel). Endogenous TrkAIII expression was not detected in these blots due to the low lysate concentration and brevity

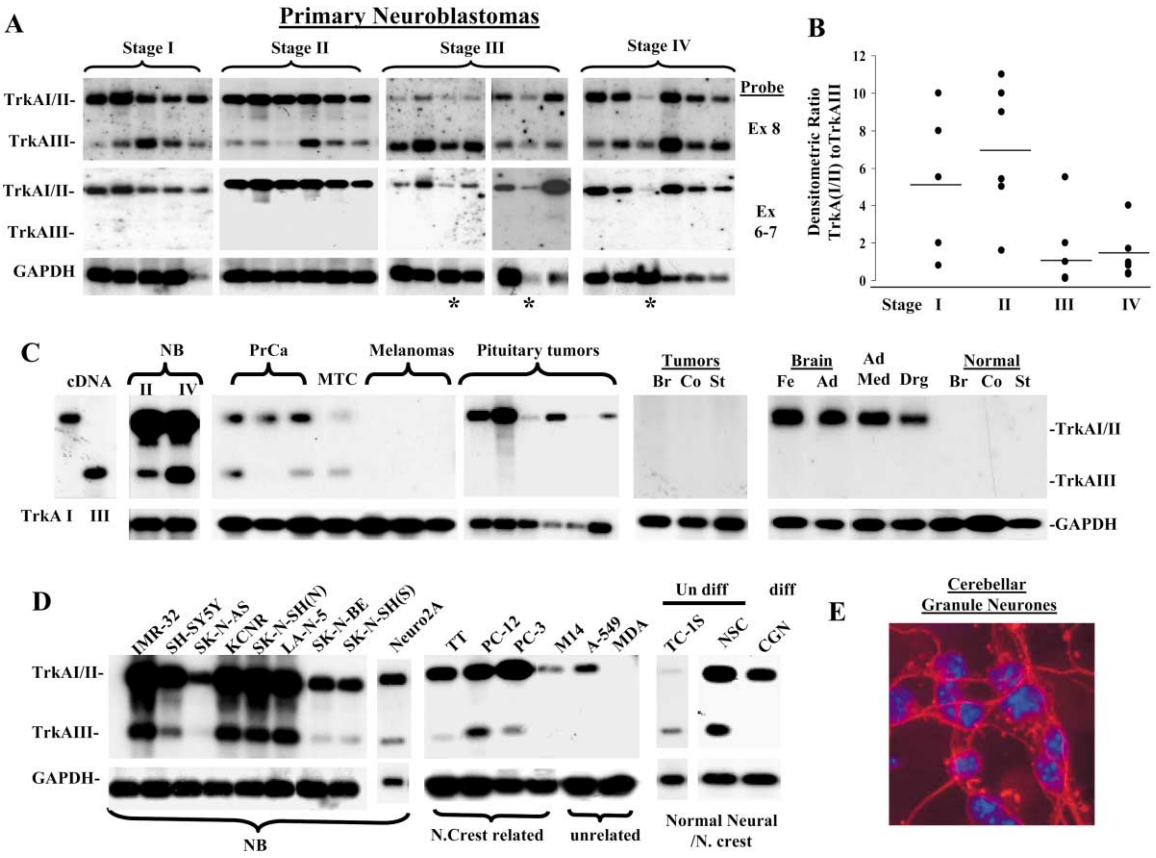

Figure 2. Human NBs, NB cells, and neural stem cells express TrkAIII

A: Southern blots of TrkA(I/II), TrkAIII, and GAPDH RT-PCR products from total RNAs (1 μg) from different stage (I–IV) primary human NBs, hybridized with TrkA exon 8 (upper panels), TrkA exon 6–7 (middle panels), or GAPDH probes (lower panels; asterisks denote Nmyc amplification).
B: Distribution (dots) and mean (horizontal line) densitometric TrkA(I/II)/TrkAIII ratios in Southern blots of NB samples grouped according to disease stage.
C: Southern blots of TrkAI and TrkAIII cDNAs (PCR) and TrkA(I/II), TrkAIII, and GAPDH RT-PCR products from human stage II and stage IV NBs; prostate (PrCa), medullary thyroid (MTC), melanoma, pituitary, breast (Br), colon (Co), and stomach (St) tumors; fetal (Fe) and adult (Ad) brain; adrenal medulla (Ad Med) and dorsal root ganglia (Drg); and normal breast (Br), colon (Co), and stomach (St) tissues.
D: Southern blots of TrkA(I/II), TrkAIII, and GAPDH RT-PCR products from human IMR-32, SH-SY5Y, SK-N-AS, KCNR, SK-N-SH (N and S), LA-N-5, SK-N-BE, and mouse Neuro2a NB cells; neural crest-derived/related medullary thyroid (TT), pheochromocytoma (PC-12), prostate (PC-3), melanoma (M14); unrelated lung carcinoma (A-549) and breast (MDA-MB-231) tumor cells; undifferentiated neural crest-derived progenitors (TC-1S) and neural stem cells (NSC); and differentiated cerebellar granule neurons (CGN).
E: Immunofluorescent micrograph of differentiated cerebellar granule cell staining for the neuronal marker βIII-tubulin (red).

of the ECL reaction that is required to detect overexpressed TrkA isoforms.

TrkA isoforms immunoprecipitated from SH-SY5Y transfectants were assayed for constitutive tyrosine phosphorylation using anti-TrkA phospho-tyrosine-specific antibodies. In contrast to TrkAI, which did not exhibit spontaneous tyrosine residue phosphorylation, TrkAIII and the Trk-T3 oncogene exhibited spontaneous phosphorylation of tyrosines Y490, Y674/675, and Y785 (Figure 3B), consistent with spontaneous receptor activity and suggesting potential for signal transduction (Kaplan et al., 1991; Obermeier et al., 1993a, 1993b, 1994; Meakin and Shooter, 1991; Meakin et al., 1999; Loeb et al., 1994; Segal et al., 1996; Cunningham et al., 1997). Spontaneous receptor activity associated with TrkAIII and Trk-T3 was confirmed by rabbit enolase Tk assay, which detected spontaneous Tk activity in similar concentrations of immunoprecipitated TrkAIII and Trk-T3 but not TrkAI (Figure 3C). Furthermore, TrkAIII and Trk-T3 exhibited higher IP3K activity than equivalent concentrations of TrkAI or pcDNA control transfectant immunoprecipitates (Figure 3D).

TrkAI and TrkAIII but not Trk-T3 exhibited membrane association in SH-SY5Y cells, assessed by immunoprecipitation of washed 100,000 × g cell membrane fractions purified from stable transfectants (data not shown). Transient transfection of myc-tagged TrkAIII into TrkAI stable transfectants followed by anti-myc antibody immunoprecipitation did not result in TrkAI pull-down, indicating that TrkAIII does not interact with TrkAI and implying that TrkAIII membrane association is not dependent upon interaction with TrkAI (data not shown).

TrkAIII induces NF-κB transcription factor activity

To confirm signal transduction from spontaneously active TrkAIII, NF-κB transcription factor activity was assessed as a transcriptional endpoint for IP3K/Akt signaling (Ozes et al., 1999). Electromobility shift assays (EMSAs) revealed enhanced NF-κB site binding activity in nuclear extracts from TrkAIII and

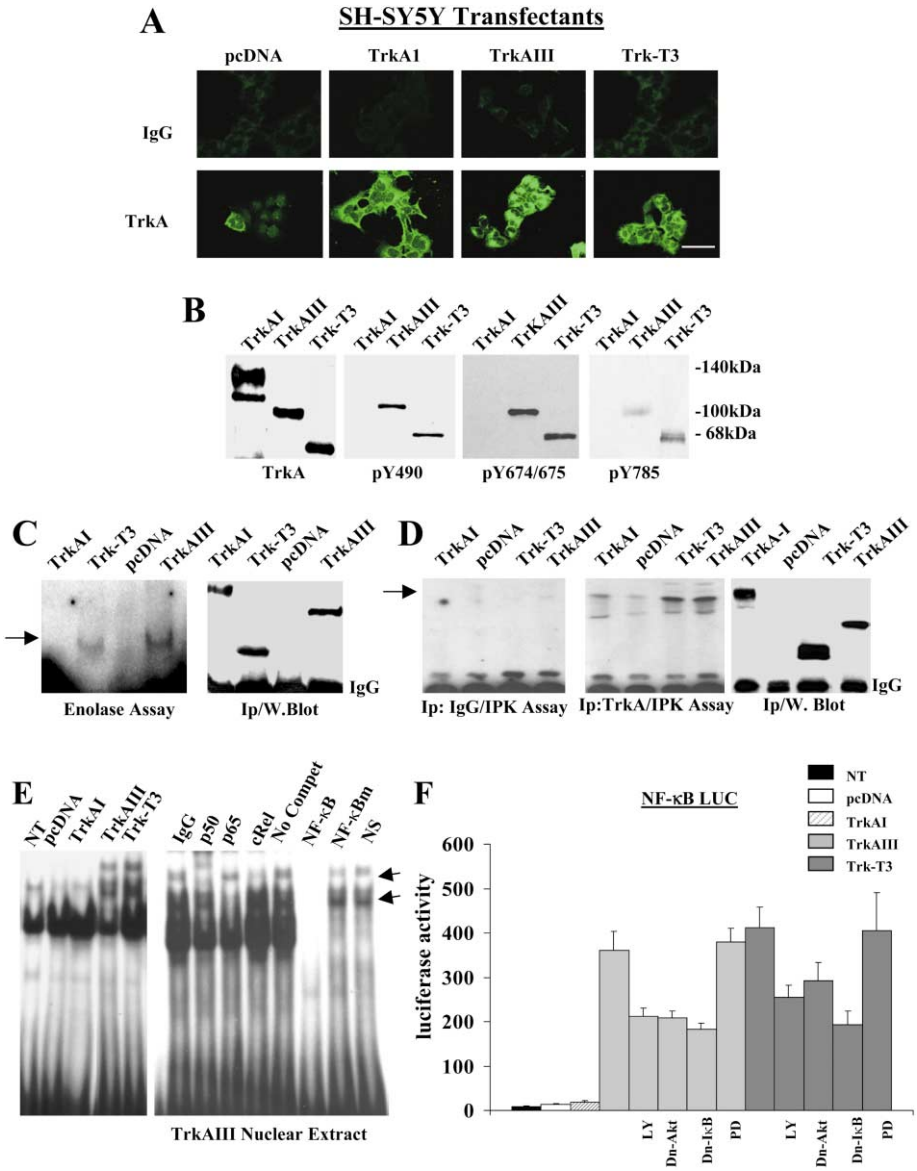

Figure 3. TrkAIII is active and signals through IP/Akt/NF-κB

A: Indirect immunofluorescence of TrkA isoform expression in pcDNA, TrkAI, TrkAIII, and Trk-T3 SH-SY5Y transfectants (scale bar, 50 μm).

B: Western blots showing phospho-Y490, -Y674/675, and -Y785 immunoreactivity of TrkA isoform immunoprecipitates from untreated SH-SY5Y transfectants.

C: ^{32}P-rabbit enolase kinase assay showing enolase (arrow) kinased by TrkAIII and Trk-T3 but not TrkAI immunoprecipitates from SH-SY5Y transfectants (relative isoform levels on right).

D: ^{32}P-inositol kinase assay showing increased kinased inositol levels (arrow) in TrkAIII and Trk-T3 compared to TrkA-I immunoprecipitates (middle panel) (preimmune IgG controls are on the left; relative isoform levels are on the right).

E: EMSA of specific NF-κB complexes (arrows) in nontransfected (NT), pcDNA, TrkAI, TrkAIII, and Trk-T3 SH-SY5Y transfectant nuclear extracts, complex immunoreactivity to anti-p50 and -p65 but not cRel antibodies, and a competition assay showing specific complex competition (TrkAIII extracts only) by cold but not mutated NF-κB (NF-κBm) or nonspecific (NS) oligonucleotides.

F: Histogram of reporter gene assays showing increased NF-κB activity in TrkAIII and Trk-T3 compared to nontransfected (NT), pcDNA, and TrkAI stable SH-SY5Y transfectants and the inhibitory effect of LY-294002 (LY), dominant-negative Akt (dn-Akt), and dominant-negative I-κBα (dn-IκB) but not PD98059 (PD) on NF-κB reporter gene activity in TrkAIII and Trk-T3 transfectants. Results are shown as mean (±SD) luciferase activity in three independent assays performed in duplicate.

Trk-T3, compared to pcDNA control and TrkAI transfectants (Figure 3E). Specific NF-κB site binding complexes, which were identified by competition assay in which 50-fold excess of unlabeled NF-κB but not nonspecific competitor or mutated NF-κB oligonucleotide competed for binding, contained both p65 and p50 proteins, determined using specific antibodies (Figure 3E, TrkAIII transfectants only). NF-κB function in TrkAIII and Trk-T3 transfectants was confirmed by NF-κB element luciferase reporter gene assay (NF-κB LUC), which detected significantly elevated activity in both ($p < 0.001$), compared to pcDNA and TrkAI transfectants (Figure 3F). NF-κB activity in TrkAIII and Trk-T3 transfectants was significantly inhibited by the IP3K inhibitor LY294002 (25 μM for 24 hr) ($p < 0.001$ for both), by dominant-negative Iκ-Bα (Duffey et al., 1999) ($p < 0.001$ for both) and by K179M mutated Akt (Franke et al., 1995) ($p < 0.001$ for both), but not by the MEK inhibitor PD98059 (2 μM for 48 hr), confirming NF-κB, IP3K, and Akt but not Ras/MAPK involvement (Figure 3F).

TrkAIII binds Shc, p85α, and PLCγ but does not respond to NGF

In contrast to TrkAI transfectants, which upon NGF treatment (100 ng/ml for 10 min) exhibited induction of TrkAI Y490, Y674/675, and Y785 phosphorylation, the spontaneous tyrosine phosphorylation exhibited by TrkAIII and Trk-T3 was not affected by NGF (Figure 4A). NGF activation of TrkAI induced receptor phospho Shc, Grb-2, Frs-2, p85α, and PLCγ1 binding (Figure 4B), consistent with reports that phosphorylated Y490, Y674/675, and Y785 TrkA residues act as binding sites for signal-transducing adaptor proteins (Obermeier et al., 1993a, 1993b, 1994; Loeb et al., 1994; Segal et al., 1996; Meakin et al., 1999). In contrast to TrkAI, TrkAIII and Trk-T3 constitutively bound nonphosphorylated Shc and p85α, TrkAIII but not Trk-T3 bound PLCγ1, and neither isoform exhibited constitutive Grb-2 or Frs-2 binding (Figure 4B). NGF (100 ng/ml for 30 min) did not alter this pattern of binding to either TrkAIII or Trk-T3 (Figure 4B). Thus, spontaneously active TrkAIII and TrkT3 bind signal-trans-

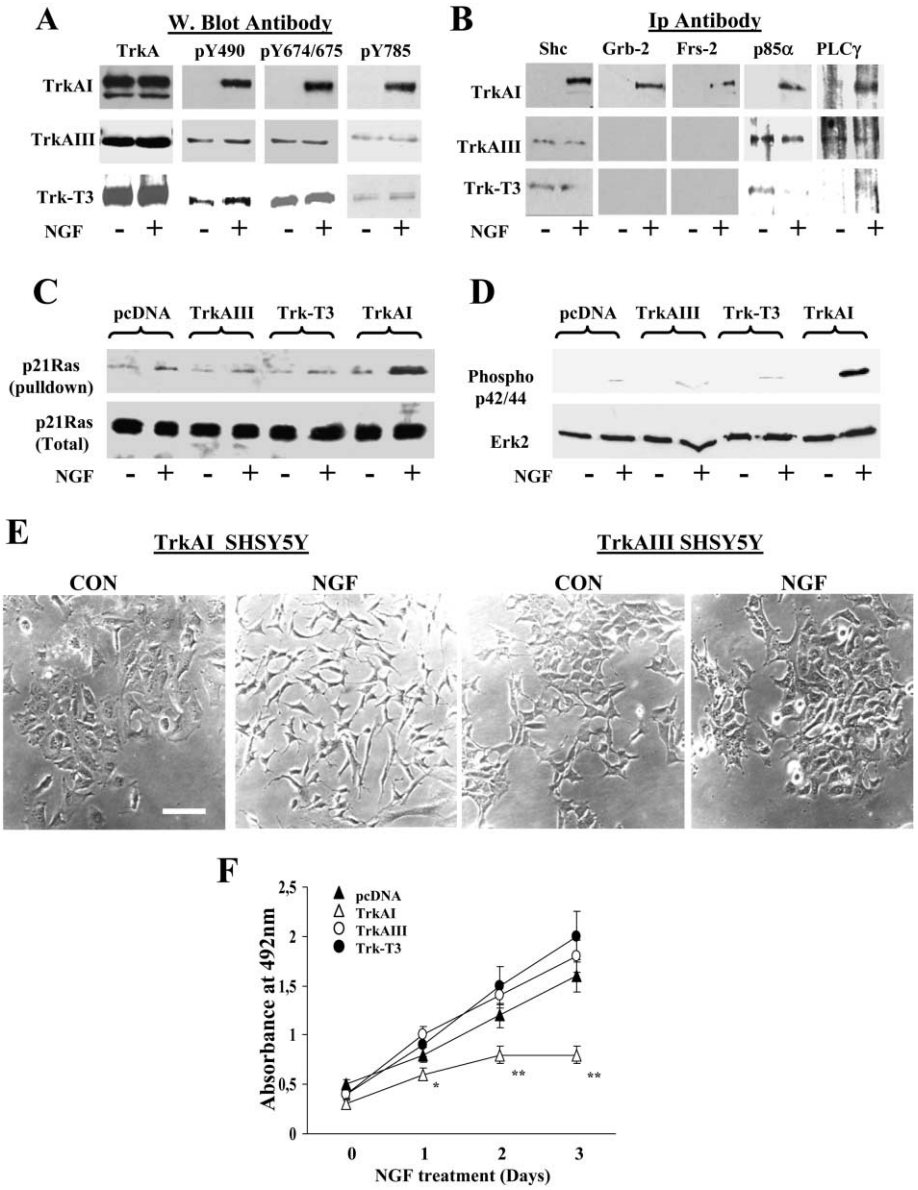

Figure 4. TrkAIII binds Shc, PLCγ, and p85α and does not respond to NGF

A: Western blots demonstrating phospho-Y490 (pY490), -Y674/675 (pY674/675), and -Y785 (pY785) immunoreactivity of TrkAI, TrkAIII, and Trk-T3 immunoprecipitates from untreated (−) and NGF-treated (+) (100 ng/ml for 10 min) SH-SY5Y transfectants.

B: Western blot showing comparative differences in TrkA isoform association with Shc, Grb-2, Frs-2, p85α, and PLC-γ1 immunoprecipitates from untreated (−) and NGF-treated (+) (100 ng/ml for 30 min) SH-SY5Y transfectants.

C and D: Western blots demonstrating total (lower panel) and active (upper panel) Ras levels (**C**) and total (lower panel) and phosphorylated (upper panel) p44/p42 Erk1/2 levels (**D**) in untreated (−) and NGF-treated (+) (100 ng/ml, 10 min for ras and 30 min for Erk) SH-SY5Y transfectant extracts.

E: Phase contrast demonstration of NGF (100 ng/ml for 48 hr) induction of neuritogenesis in TrkAI but not TrkAIII SH-SY5Y transfectants (scale bar, 50 μm).

F: MTS assays showing NGF (100 ng/ml) inhibition of TrkAI (open triangle) but not TrkAIII (open circle), Trk-T3 (closed circle), or pcDNA (closed triangle) SH-SY5Y transfectant proliferation over 3 days. Data are shown as mean absorbance at 492 nm in quadruplet cultures for each time (*$p < 0.05$; **$p < 0.001$; Student's t test).

ducing adaptor proteins in a manner that differs from NGF-activated TrkAI and do not respond to NGF.

In a Ras binding domain pull-down assay, neither TrkAI, TrkAIII, nor Trk-T3 transfectants exhibited above background Ras activity, suggesting that within the context of SH-SY5Y cells spontaneously active TrkAIII and Trk-T3 do not activate Ras (Figure 4C). Treatment with NGF (100 ng/ml for 10 min) stimulated above background Ras activity in TrkAI but not TrkAIII or Trk-T3 transfectants (Figure 4C), further confirming that neither TrkAIII nor Trk-T3 responds to NGF.

Phosphorylation of MAPK homologs p44/p42 Erk1/2 or p38 is induced by Ras activation (Kolch, 2000). Consistent with an incapacity to activate Ras, TrkAIII and Trk-T3 transfectants did not exhibit constitutive Erk1/2 or p38 phosphorylation (Figure 4D, Erk1/2 only). Furthermore, NGF (100 ng/ml for 30 min), which induced relatively high-level p44/p42 Erk phosphorylation in TrkAI transfectants, did not stimulate Erk phosphorylation above low background levels in TrkAIII and TrkT3 transfectants, further confirming lack of TrkAIII and Trk-T3 NGF responsiveness (Figure 4D). NGF did not induce p38 phosphorylation in any transfectant (data not shown). TrkAIII is devoid, therefore, of Ras/MAPK activating capacity even in the presence of NGF.

TrkAI gene transduction restores NGF responsiveness to NB cells, inducing growth arrest differentiation and/or apoptosis (Matsushima and Bogenmann, 1990; Lavenius et al., 1995; Lucarelli et al., 1997; Hallberg et al., 1998; Meakin et al., 1999; Kolch, 2000; Edsjo et al., 2001; Sugimoto et al., 2001; Eggert et al., 2002). In this study, NGF (100 ng/ml for 72 hr) induced neuritogenesis in TrkAI but not in pcDNA control, TrkAIII, or Trk-T3 transfectants, none of which exhibited spontaneous neuritogenesis in the absence of NGF (Figure 4E; only TrkAI and TrkAIII are shown).

No significant difference in proliferation rate or doubling time was observed between pcDNA, TrkAI, TrkAIII, and Trk-T3

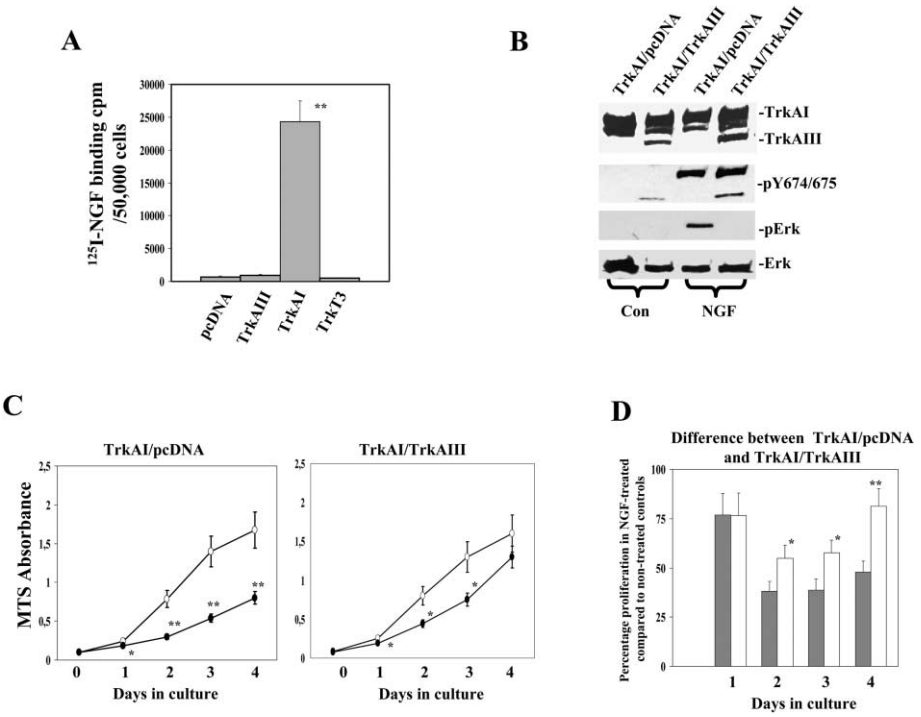

Figure 5. TrkAIII does not bind NGF but interferes with NGF/TrkAI signaling

A: Cellular ^{125}I-NGF binding assay demonstrating mean (±SD) cpm/50,000 cells ^{125}I-NGF bound by pcDNA, TrkAIII, TrkAI, and Trk-T3 SH-SY5Y transfectants, in duplicate assays performed in triplicate.

B: Western blot of TrkAI and TrkAIII (upper panel), phospho Y674/675 TrkA (second panel), phospho p44/p42 Erk (third panel), and total Erk (bottom panel) immunoreactivity in lysates (100 μg/lane) from TrkAI/pcDNA3.1 and TrkAI/TrkAIII SH-SY5Y cotransfectants in the absence (Con) or presence of NGF (100 ng/ml for 10 min).

C: MTS assay showing differences in TrkAI/pcDNA and TrkAI/TrkAIII SH-SY5Y cotransfectant proliferation in the absence (open circles) or presence (closed circles) of NGF (100 ng/ml), shown as mean (±SD) absorbance at 492 nm in duplicated experiments performed in quadruplet.

D: Histogram of the effects of NGF (100 ng/ml) on TrkAI/pcDNA (gray) and TrkAI/TrkAIII (white) cotransfectant proliferation over 4 days, expressed as percentage (±SD) difference in proliferation compared to untreated controls (arbitrarily 100% for cell line at each time point) (*$p < 0.05$; **$p < 0.001$; Student's t test).

transfectants, determined by cell counting and MTS proliferation assay (data not shown). NGF (100 ng/ml for 1–3 days) significantly inhibited the proliferation of TrkAI but not pcDNA, TrkAIII, or Trk-T3 transfectants, determined by MTS assay (Figure 4F) ($p < 0.001$ for all three time points). Therefore, spontaneously active TrkAIII, unlike TrkAI, promotes neither SH-SY5Y differentiation nor growth arrest in the absence or presence of NGF.

TrkAIII does not bind NGF and antagonizes NGF/TrkAI signaling

Because TrkAIII does not respond to NGF, we examined its binding capacity in a cellular ^{125}I-NGF binding assay. In contrast to TrkAI transfectants, which bound significantly more ^{125}I-NGF than pcDNA control transfectants ($p < 0.001$), neither TrkAIII nor Trk-T3 transfectants bound significantly more NGF than pcDNA controls (Figure 5A), indicating that TrkAIII does not bind NGF in SH-SY5Y cells.

Because TrkAI and TrkAIII display differences in signaling and differ in ratio as a function of NB disease stage, we examined whether TrkAIII may influence TrkAI-dependent cellular functions. TrkAIII was transiently transfected into TrkAI stable SH-SY5Y transfectants, altering the TrkAI/TrkAIII ratio from almost exclusively TrkAI to approximately 3 to 1 (Figure 5B). Under these conditions, characterized by relatively lower levels of TrkAIII compared to TrkAI expression, TrkAIII but not TrkAI exhibited constitutive Y674/675 phosphorylation (Figure 5B). This suggests that receptor overexpression alone is unlikely to be responsible for spontaneous TrkAIII activation, by comparison to TrkAI. Furthermore, TrkAIII did not induce phosphorylation of TrkAI (Figure 5B).

NGF (100 ng/ml) treatment of pcDNA/TrkAI (control) and TrkAIII/TrkAI cotransfectants induced TrkAI Y674/675 phosphorylation in both, indicating that TrkAIII does not interfere with NGF activation of TrkAI (Figure 5B). In contrast, NGF induced Erk phosphorylation in pcDNA/TrkAI but not TrkAIII/TrkAI cotransfectants, indicating that TrkAIII interferes with NGF/TrkAI induction of Erk phosphorylation (Figure 5B).

Because the MAPK/ERK pathway is involved in NGF/TrkAI-induced NB cell growth inhibition (Edsjo et al., 2001), we investigated the capacity of NGF to inhibit proliferation in pcDNA/TrkAI compared to TrkAIII/TrkAI cotransfectants. NGF (100 ng/ml) inhibited the proliferation of pcDNA/TrkAI cotransfectants over a 4 day time course (Figure 5C; $p < 0.02$ for all time points), whereas TrkAIII/TrkAI cotransfectants exhibited altered behavior in response to NGF, which inhibited proliferation to a significantly lesser extent from 1–3 days or not at all at 4 days (Figures 5C and 5D).

TrkAIII protects SH-SY5Y cells from doxorubicin-induced death

TrkAIII induces Akt/NF-κB pathway activation, suggesting that TrkAIII may protect SH-SY5Y cells from chemotherapy-induced apoptosis, as reported for BDNF/TrkB interaction in SH-SY5Y cells (Jaboin et al., 2002). Indeed, TrkAIII expression significantly protected SH-SY5Y cells from doxorubicin-induced death, compared to pcDNA and TrkAI transfectants. Both pcDNA and TrkA-I transfectants exhibited similar reductions in viable cell number 48 hr after treatment with 0.1 μM (60%–62%), 0.5 μM (76%–77%), and 1.0 μM doxorubicin (84%–86%), whereas TrkAIII transfectants exhibited significantly increased resistance: mean reductions in cell viability of 40% with 0.1 μM, 58% with 0.5 μM, and 74% with 1.0 μM doxorubicin were recorded ($p < 0.05$ for all three doses compared to either control or TrkAI transfectants) (data are shown in the text only).

TrkAIII transforms NIH3T3 cells and promotes aggressive SH-SY5Y behavior

TrkAIII oncogenic potential was assessed in transfected NIH3T3 fibroblasts. In contrast to control and TrkAI stable NIH3T3

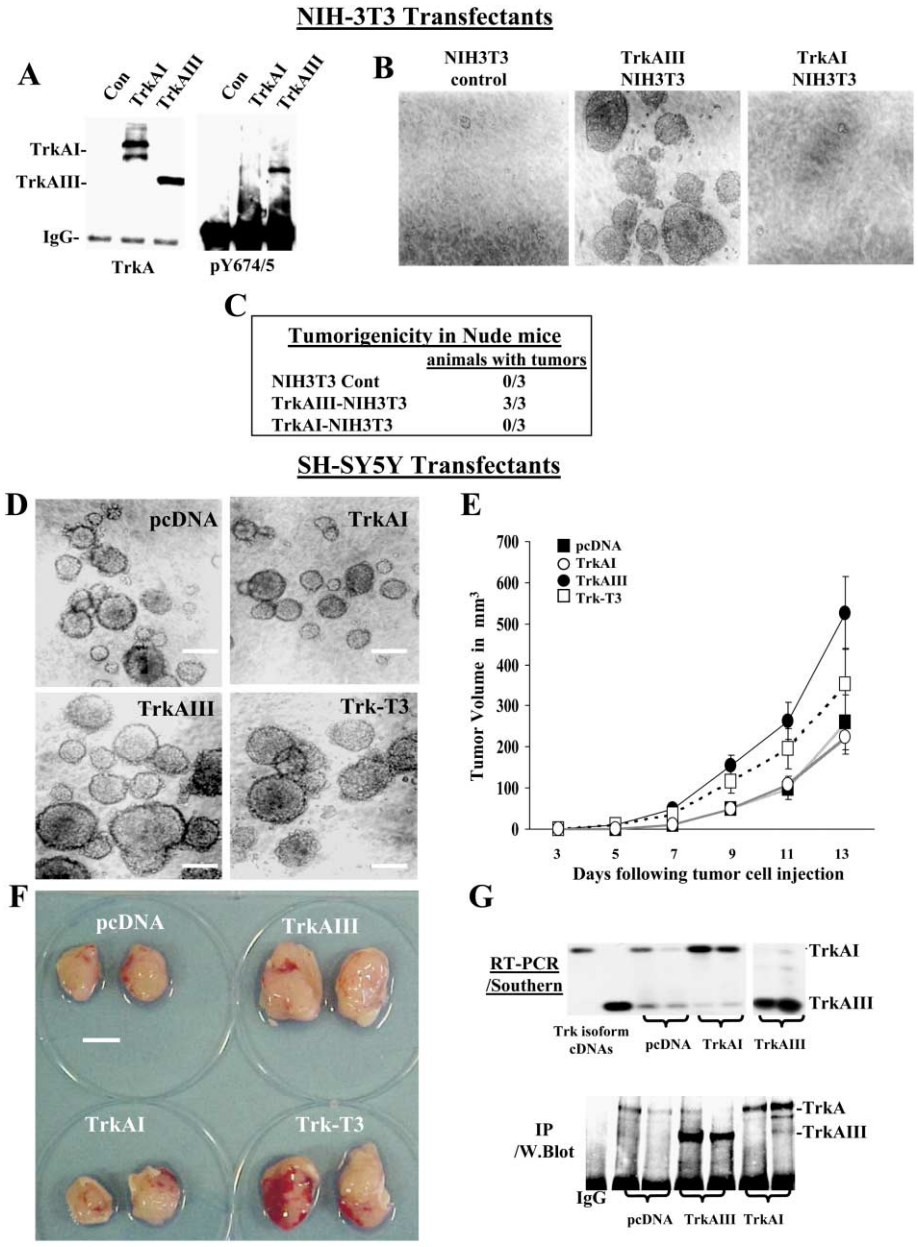

Figure 6. TrkAIII transforms NIH-3T3 cells and promotes aggressive SH-SY5Y behavior

A: Western blot demonstrating TrkAIII but not TrkAI Y674/675 phosphorylation (right panel) in anti-TrkA but not preimmune IgG immunoprecipitates (left panel) from NIH-3T3 transfectants.

B: Phase contrast demonstration of soft agar growth by TrkAIII but not control or stable TrkAI NIH3T3 transfectants.

C: Tumorigenic activity exhibited by TrkAIII but not control or TrkAI NIH-3T3 transfectants in nude mice.

D: Phase contrast demonstration of increased soft agar growth exhibited by TrkAIII and Trk-T3 compared to pcDNA and TrkAI SH-SY5Y transfectants (scale bar, 1 mm).

E: Histogram of mean (±SD) tumor volumes (mm^3) of xenograft tumors formed by TrkAIII (closed circles), Trk-T3 (open squares), pcDNA (closed squares), and TrkAI (open circles) SH-SY5Y transfectants in nude mice over 13 days (five animals per group, repeated twice).

F: Photographic demonstration of comparative size differences of duplicate TrkAIII, Trk-T3, pcDNA, and TrkAI transfectant-derived tumors (day 13) (scale bar, 10 mm).

G: RT-PCR/Southern and IP/Western blots of TrkA isoform expression in pcDNA, TrkAI, and TrkAIII SH-SY5Y tumors in **F**.

transfectants, TrkAIII stable NIH3T3 transfectants exhibited constitutive TrkAIII Y674/675 phosphorylation (Figure 6A), formed large tumor-like spheroids in 14 day soft agar substrate-independent growth assay (Figure 6B), and formed tumors within 14 days of subcutaneous injection into nude mice (Figure 6C), confirming TrkAIII oncogenic activity. Tumor formation was considered at volumes above 12 mm^3, and at 14 days TrkAIII transfectants produced tumors with a mean tumor volume of 116 mm^3 (±68.9). Neither control nor TrkAI transfectants produced tumors.

In SH-SY5Y cells, which already exhibit substrate-independent growth capacity and are tumorigenic in nude mice, both TrkAIII and Trk-T3 stable transfection promoted the growth of larger tumor-like spheroids in a 14 day soft agar substrate-independent growth assay than either nontransfected (data not shown) or pcDNA- or TrkAI-transfected counterparts (Figure 6D). In nude mice, TrkAIII and Trk-T3 SH-SY5Y transfectants initiated tumor formation within 5 days (determined at a tumor volume of 12 mm^3), whereas pcDNA and TrkAI transfectants did not initiate tumors for a further 2 days. Subsequent measurements at 7, 9, 11, and 13 days revealed significant differences in tumor size for both TrkAIII and Trk-T3 versus pcDNA and TrkAI transfectants (Figure 6E; $p < 0.05$ for both TrkAIII and Trk-T3 tumors at all time points): mean tumor volumes recorded at sacrifice (13 days) were 526 ± 89 mm^3 in TrkAIII transfectants ($n = 5$), 354 ± 60 mm^3 in Trk-T3 transfectants ($n = 5$), 225 ± 60 mm^3 in TrkAI transfectants ($n = 5$), and 260 ± 49 mm^3 in pcDNA3.1 transfectants ($n = 5$) (Figure 6E). These differences are reflected in Figure 6F, demonstrating tumors in which TrkA isoform expression was confirmed by RT-PCR, immunoprecipitation/Western blot (Figure 6G; Trk-T3 not shown), and immunohistochemistry (Figure 7A). Note that endogenous TrkAIII ex-

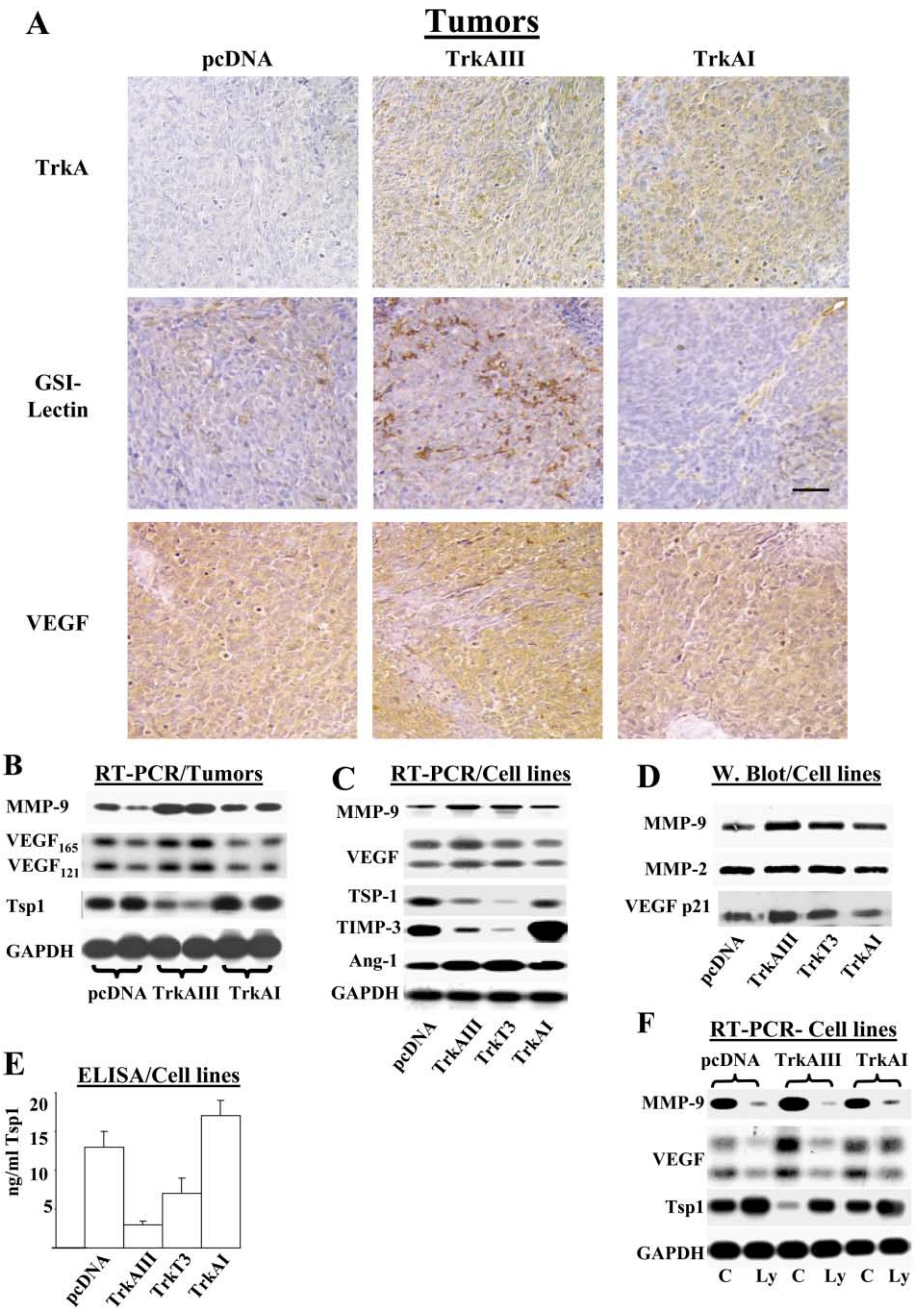

Figure 7. TrkAIII increases tumor vascularity and alters the angiogenic phenotype

A: Light micrographs of TrkA isoform, VEGF, and GSI lectin neovascular staining in sections of 13 day pcDNA, TrkAIII, and TrkAI SH-SY5Y tumors (scale bar, 100 μm).

B: RT-PCR/Southern blot comparison of MMP-9, VEGF, Tsp1, and GAPDH expression in duplicate 13 day pcDNA, TrkAIII, and TrkAI SH-SY5Y tumors.

C: RT-PCR/Southern blot comparison of steady-state MMP-9, VEGF, Tsp1, TIMP-3, Ang1, and GAPDH expression by pcDNA, TrkAIII, Trk-T3, and TrkAI SH-SY5Y transfectants in vitro.

D and E: Western blot of MMP-9, MMP-2, and p21VEGF (**D**) and ELISA of Tsp1 levels (**E**) in 48 hr serum-free conditioned medium from pcDNA, TrkAIII, TrkT3, and TrkAI SH-SY5Y transfectants in vitro.

F: RT-PCR/Southern blot showing the effects of LY294002 (Ly) (25 μM for 48 hr) on MMP-9, VEGF, Tsp1, and GAPDH expression by pcDNA, TrkAIII, and TrkAI transfectants in vitro.

pression was also detected in tumors derived from pcDNA and TrkAI transfectants (Figure 6G).

TrkAIII promotes tumor growth in association with increased tumor vascularity and an altered angiogenic phenotype

Consistent with a potential role for angiogenesis in TrkAIII promotion of SH-SY5Y cell tumorigenicity, 13-day-old tumors from TrkAIII transfectants exhibited a significantly elevated mean (±SD) number of GSI-lectin-stained vessels per 40× magnification microscopic tumor field (ten counts per tumor section) of 36.1 ± 10.81 ($p < 0.001$) when compared to 13-day-old pcDNA (mean vessel count of 13.9 ± 4.43) and TrkAI counterparts (mean vessel count of 12.9 ± 5.42) (data in text only). Vessel counts did not significantly differ between pcDNA and TrkAI tumors. This relative difference in tumor vascular density is reflected in Figure 7A, in which TrkA isoform, vascular lectin, and VEGF staining are compared between representative pcDNA, TrkAIII, and TrkAI transfectant-derived tumor sections. Thus, TrkAIII transfectants produce significantly more vascular tumors than control and TrkAI counterparts.

RT-PCR assessment of angiogenic factor and inhibitor expression in tumor extracts revealed an association between TrkAIII tumors and a relative increase in MMP-9 and VEGF angiogenic factor expression and a relative decrease in the expression of the angiogenic inhibitor thrombospondin-1 (Tsp1),

when compared to less vascular pcDNA and TrkAI tumor counterparts (Figure 7B). A similar pattern of elevated MMP-9 and VEGF but reduced Tsp1 expression, assessed by RT-PCR (Figure 7C), Western blot (Figure 7D), and ELISA (Figure 7E), characterized TrkAIII and Trk-T3 compared to pcDNA and TrkAI SH-SY5Y transfectants in vitro. TrkAIII and Trk-T3 transfectants also exhibited a relative increase in Ang1 and decrease in TIMP-3 expression compared to control and TrkAI transfectants (Figure 7C), whereas no differences in Ang2, bFGF, MMP-2, TIMP-1, or TIMP-2 expression were detected (data not shown).

Consistent with a potential role for IP3K but not Ras signaling in TrkAIII regulation of the angiogenic phenotype, the IP3K inhibitor LY294002 (25 μM for 48 hr), but not the MEK inhibitor PD98059 (2 μM for 48 hr), inhibited MMP-9 and VEGF expression but stimulated that of Tsp1 to a greater extent in TrkAIII compared to pcDNA control and TrkAI transfectants (Figure 7F). This implicates IP3K in regulation of the MMP-9/VEGF/Tsp1 equilibrium in SH-SY5Y cells and provides a mechanism by which TrkAIII may alter the angiogenic phenotype.

TrkAIII expression is regulated by hypoxia

Hypoxia is an important epigenetic regulator of aggressive tumor behavior, including NB (Harris, 2002; Jogi et al., 2002; Pennacchietti et al., 2003). In this study, hypoxia induced by $CoCl_2$ (150 μM for 24 hr) (Miyazaki et al., 2002) selectively increased TrkAIII relative to TrkA(I/II) expression in human and mouse NB cell lines (KCNR, SK-N-BE, SH-SY5Y, Neuro2a), PC-12 pheochromocytoma, and TT medullary thyroid carcinoma cells (Figure 8A) and stimulated TrkAIII relative to TrkAI protein levels in SH-SY5Y cells (Figure 8B). Hypoxia did not enhance TrkAIII expression by PC-3 prostate cells or induce expression in TrkAIII-negative M14 melanoma, A-549 lung, and MDA-MB-231 breast tumor cells. Hypoxia stimulation of TrkAIII expression was also observed in undifferentiated human neural stem cells and neural crest-derived TC-1S progenitors, which exhibit constitutive TrkAIII expression, whereas cerebellar granule cells differentiating along the neuronal lineage (Figure 2E) were negative for TrkAIII expression even under hypoxic conditions (Figure 8A). Therefore, hypoxia promotion of alternative TrkAIII splicing is restricted to NB, a subset of neural crest-derived tumor cells and pluripotent early neural stem cells and neural crest progenitors.

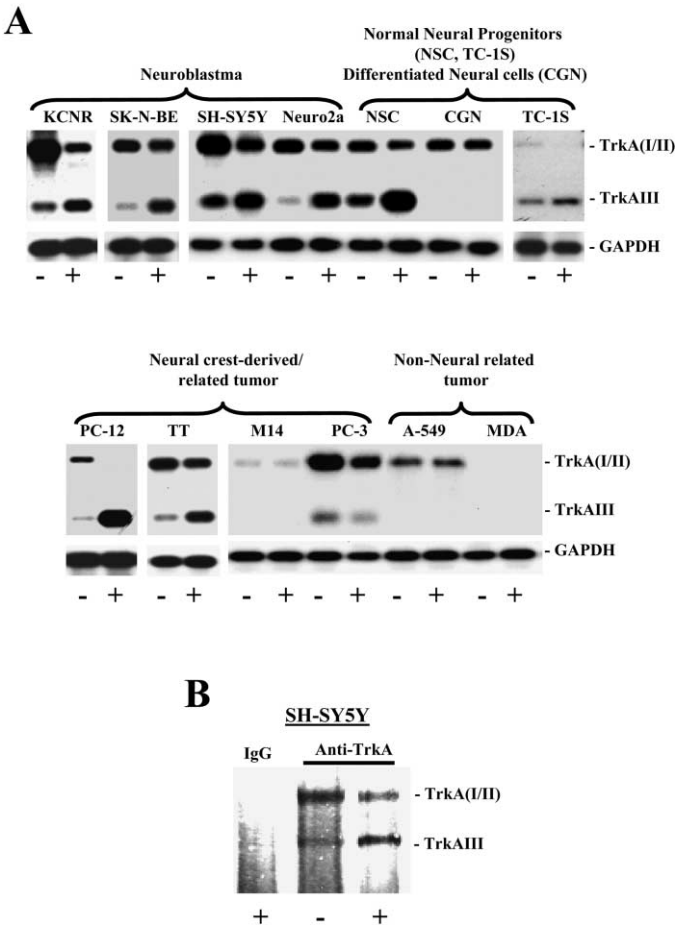

Figure 8. Hypoxia stimulates TrkAIII expression in NB cells and neural progenitors

A: RT-PCR/Southern blot of normoxia (−) and hypoxia (+) effects on TrkA(I/II), TrkAIII, and GAPDH expression by (upper panel) human KCNR, SK-N-BE, SH-SY5Y, and mouse Neuro2a NB cells, undifferentiated normal human neural stem cells (NSC), differentiated cerebellar granule neurons (CGN), and undifferentiated neural crest-derived progenitors (TC-1S); and (lower panel) neural crest-derived/related pheochromocytoma (PC-12), medullary thyroid cancer (TT), melanoma (M14), prostate cancer (PC-3), and nonneural related lung (A-549) and breast (MDA-MB-231) tumor cells.
B: IP/Western blot showing relative differences in TrkAIII and TrkA(I/II) expression by SH-SY5Y cells cultured under normoxic (−) or hypoxic (+) conditions.

Discussion

In this study, we unveil a hypoxia-regulated mechanism for determining aggressive NB behavior characterized by a "switch" to alternative TrkA splicing and expression of a novel, constitutively active, tumor-promoting TrkAIII variant that lacks exons 6, 7, and 9. TrkAIII, expressed at the expense of NGF-responsive TrkA receptors that promote NB cell differentiation and growth arrest, exhibits spontaneous activity, induces chronic signal transduction through IP/Akt but not Ras/MAPK, activates transcription factor NF-κB, antagonizes NGF/TrkAI signaling, transforms NIH3T3 cells, and promotes aggressive NB cell behavior associated with a more resistant and angiogenic phenotype, consistent with a potential oncogenic/tumor-promoting function. Elevated TrkAIII expression in advanced stage NBs suggests a potential role in NB pathogenesis.

TrkAIII expression characterizes neural stem cells and an aggressive NB phenotype

TrkAIII, cloned from SH-SY5Y NB cells and characterized as a novel splice variant in addition to TrkAI and TrkAII (Barker et al., 1993), encodes a cell membrane-associated TrkA isoform with deletion of extracellular IG-C1 and related N-glycosylation domains of functional importance (Martin-Zanca et al., 1989; Watson et al., 1999; Arevalo et al., 2000) with an extracellular valine addition at the novel exon 5/8 splice junction.

TrkAIII identification raises questions concerning specificity of expression. In a variety of neural crest-derived and unrelated tumors and cell lines, TrkAIII exhibited relatively restricted expression to NBs and, at a relatively low level, medullary thyroid carcinoma, prostate tumors and cells, and pheochromocytoma cells, suggesting relative specificity for tumors and cells of neural crest origin or those that exhibit neuroendocrine features

(Rumpold et al., 2002; Barakat et al., 2004). TrkAIII was also expressed by normal undifferentiated human neural stem cells at a preneuronal astroglial and oligodendroglial developmental stage and murine neural crest-derived progenitors but not by differentiation-committed cerebellar granule neurons; normal neural tissues including fetal and adult brain (probably reflecting low stem cell content); dorsal root ganglia and adrenal medulla; normal nonneural stomach, lung, colon, and breast tissues; or normal fibroblasts. This suggests that expression is restricted to undifferentiated neural stem/crest progenitors but not cells differentiated along the neuronal lineage (i.e., granule neurons) or nonneural tissues and supports the possibility of a tissue-specific rather than tumor-specific alternative splice mechanism, despite unavailability of normal human embryo neural crest tissue, that within the tumor context may represent a stem/progenitor cell condition related to a particular phase of neural progenitor neoplastic transformation of relevance to NB, medullary thyroid cancer, and pheochromocytoma, which share neural crest origin, and prostate cancer, which exhibits neural crest/neuroendocrine behavior (Rumpold et al., 2002; Barakat et al., 2004).

In NBs, TrkAIII expression varied as a ratio to other variants as a function of disease stage and predominated in more high stage (III and IV) than low stage (I and II) tumors, with a significant increase in the TrkAIII/TrkA(I/II) expression ratio observed in combined high stage (III and IV) compared to low stage (I and II) NBs. This suggests that TrkAIII expression may be linked to more aggressive NB behavior. Although this altered equilibrium was statistically significant, greater numbers would be required to firmly establish any potential prognostic value. Since most approaches in the past would not have identified TrkAIII, our data are also in agreement with reports that low stage (I and II) and a majority (over 60%) of advanced stage (III and IV) NBs exhibit high TrkAI expression, with low expression associated with high stage Nmyc-amplified disease (Nakagawara et al., 1992; Nakagawara, 2001). Although RT-PCR detected TrkA in all NB samples, three high stage Nmyc-amplified NBs exhibited low TrkAI expression, supporting this inverse relationship.

TrkAIII is a tumor promoter and antagonist of antioncogenic NGF/TrkAI signaling

In SH-SY5Y cells, TrkAIII exhibits spontaneous activity (tyrosine phosphorylation, Tk, and IP3K activity); interacts with known TrkA signal transducers Shc, PLC-γ, and the IP3K subunit p85α (Obermeier et al., 1993a, 1993b, 1994; Segal et al., 1996; Meakin et al., 1999); and constitutively signals through IP3K/Akt but not Ras/MAPK to activate the transcription factor NF-κB. It is unlikely that this spontaneous activity reflects enforced receptor overexpression, reported to activate TrkAI in some cells (Lee and Chao, 2001), since TrkAI activation did not occur under similar conditions, and TrkAIII/TrkAI coexpression at an approximate ratio of 1 to 3 was accompanied by TrkAIII but not TrkAI activation. It is more likely, therefore, that activation reflects exon 6–7 omission, since IG-C1 and related N-glycosylation domains, deleted in TrkAIII, prevent ligand-independent TrkAI dimerization and activation (Watson et al., 1999; Arevalo et al., 2000).

TrkAIII induction of IP/Akt but not Ras/MAPK signaling in SH-SY5Y cells is consistent with TrkAIII binding of p85α but not phospho Shc, Grb-2, or Frs-2, which are required for NGF/TrkAI activation of Ras/MAPK (this study; Hallberg et al., 1998; Meakin et al., 1999). It is unlikely that this reflects a malfunction in postreceptor signaling, since NGF activated Ras/MAPK in TrkAI transfectants in association with induction of phospho Shc, Grb-2, or Frs-2 binding. Possible explanations include TrkAIII-associated IP3K activity below the Ras/MAPK activation threshold (Hallberg et al., 1998) or direct inhibition of Raf-MEK-ERK by constitutive IP/Akt signaling (Moelling et al., 2002). The latter possibility is supported by TrkAIII antagonism of NGF/TrkAI-induced Erk phosphorylation. Alternatively, reduced TrkAI extracellular N-glycosylation alters membrane localization and induces receptor but not Ras-MAPK activation (Watson et al., 1999), suggesting that deletion of N-glycosylation domains from TrkAIII may induce a similar activation.

The biological activity of TrkAIII differs dramatically from that of TrkAI. TrkAI does not exhibit spontaneous ligand-independent activity, is not oncogenic in NIH3T3 cells, and does not promote SH-SY5Y tumorigenic behavior, but it restores NGF responsiveness, inducing differentiation and growth arrest consistent with a potential antioncogenic role in NB (this study; Matsushima and Bogenmann, 1990; Lavenius et al., 1995; Lucarelli et al., 1997; Hallberg et al., 1998; Meakin et al., 1999; Kolch, 2000; Edsjo et al., 2001; Sugimoto et al., 2001; Eggert et al., 2002). In contrast, TrkAIII exhibits spontaneous activity in SH-SY5Y cells but does not induce morphological differentiation or growth arrest and does not restore NGF responsiveness to SH-SY5Y cells but promotes their tumorigenic behavior and is oncogenic in NIH3T3 cells. These differences reflect spontaneous TrkAIII but not TrkAI activity, TrkAIII signaling through IP/Akt/NF-κB but not Ras/MAPK, and the incapacity of TrkAIII but not TrkAI to bind and respond to NGF. Furthermore, TrkAIII antagonizes NGF/TrkAI signaling and inhibition of NB cell proliferation, which may help explain the NGF unresponsiveness exhibited by many NB cell lines (Azar et al., 1990), and suggests that altering the TrkAI/TrkAIII equilibrium in favor of TrkAIII may compromise antioncogenic NGF/TrkAI signals in NB. The oncogenic activity exhibited by TrkAIII in NIH3T3 cells is supported by a report that engineered deletion of IG-C1 from rat TrkAI, similar to naturally expressed TrkAIII, also induces oncogenic activation (Arevalo et al., 2000). However, TrkAIII is also expressed by nontumorigenic neural crest progenitors and neural stem cells, suggesting dependence for oncogenic activity upon additional "cooperating" factors, despite being associated with anchorage-independent growth and a dedifferentiated phenotype.

Potential mechanisms through which TrkAIII promotes NB cell tumorigenic behavior may include induction of a more stress-resistant phenotype, suggested by TrkAIII partial protection of SH-SY5Y cells from doxorubicin-induced death, which is likely to involve IP/Akt/NFkB signaling (Jaboin et al., 2002), and angiogenesis, suggested by the significantly increased vascularity exhibited by TrkAIII transfectant-derived tumors. This was associated with an altered MMP-9/VEGF/Tsp-1 angiogenic equilibrium characterized by increased MMP-9 and VEGF but reduced Tsp-1 expression. This equilibrium is emerging as an important determinant of tumor angiogenesis, since MMP-9 triggers VEGF-mediated angiogenesis and Tsp-1 inhibits both MMP-9 activation and VEGF activity (Bergers et al., 2000; Rodriguez-Manzaneque et al., 2001). Inhibitor studies implicated IP3K in promoting high MMP-9 and VEGF but low Tsp1 expression in SH-SY5Y cells, providing a mechanism through which TrkAIII may exert its proangiogenic effect. The potential importance of

this mechanism in NB pathogenesis is further supported by the relatively increased MMP-9 and VEGF but reduced Tsp-1 expression exhibited by advanced stage NBs (Ribatti et al., 2001; Fakhari et al., 2002; Yang et al., 2003).

Hypoxia promotes alternative TrkAIII splicing in neural progenitors and transformed counterparts

Although the molecular mechanisms that regulate alternative TrkAIII splicing remain to be elucidated, hypoxia was found to stimulate alternative TrkAIII splicing in a variety of cell types, including NB cells, a subset of neural crest-derived tumor cells (medullary thyroid and pheochromocytoma), normal neural stem cells, and neural crest-derived progenitors but not in neural crest-derived melanoma cells, differentiation-committed cerebellar granule neurons, or a variety of nonneural normal (MRC-5 and NIH3T3 fibroblasts) or tumor (PC-3 prostate, A-549 lung, MDA-MB-231 breast) cells. This suggests a possible hypoxia protection mechanism of potential physiological relevance to normal non-differentiation-committed neural/neural crest progenitors that is lost upon differentiation commitment and absent in unrelated tissues but conserved by transformed NB, medullary thyroid cancer, and pheochromocytoma counterparts, possibly reflecting a stem/progenitor cell condition. Pathological subversion of this mechanism by tumors may therefore provide a novel way for hypoxia to promote aggressive behavior, in addition to Met-mediated invasive growth, dedifferentiation, and increased angiogenesis (Harris, 2002; Jogi et al., 2002; Pennacchietti et al., 2003), and may help to explain, together with dedifferentiation, the increased TrkAIII expression exhibited by advanced stage NBs.

In conclusion, we propose that alternative TrkA splicing, which generates the novel TrkAIII variant, represents a regulated tumor-promoting switch of relevance to NB. TrkAIII expression that is associated with a stem cell-like phenotype and promoted by tumor hypoxia would interfere with antioncogenic NGF/TrkAI signaling through Ras/MAPK, promote NGF-independent signaling through IP/Akt, and induce a more stress-resistant, angiogenic, and tumorigenic NB phenotype. A potential mechanism for regulating NB progression based on alternative TrkAIII splicing rather than genetic TrkA abnormality would theoretically permit reversal by reestablishing regular TrkA (I/II) splicing, which would represent a potential therapeutic goal.

Experimental procedures

Complete details of all cell lines, reagents, and experimental procedures used are presented as Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

TrkA isoform cDNA cloning

Briefly, purified mRNA from SH-SY5Y cells was subjected to RT-PCR using the TrkA-specific primers 5′-ATGCTGCGAGGCGGACGGCGC-3′ and 5′-CTAGCCCAGGACATCCAGGTA-3′, which span the complete human TrkAI cDNA coding sequence. Products were gel purified, cloned into TA PCR cloning vectors, and sequenced using an ABI prism automated DNA sequencer, and intact cDNAs were subcloned into pcDNA3.1 mammalian expression vectors (detailed methods are presented as Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/).

RT-PCR/Southern blotting

Total RNAs (1 μg) from primary human NBs (tissue bank of the Italian Association of Haematology and Pediatric Oncology, Genoa, Italy); pituitary, prostate, melanoma, and medullary thyroid tumors (Department of Pathology University of Rome, Italy); matched normal and breast, colon, and stomach tumor tissue (AmBion, Austin, TX); normal human fetal and adult brain and adrenal medulla and dorsal root ganglia (Clontech, Palo Alto, CA); and experimental tumors and transfected cells were subjected to RT-PCR using primers specific for *TrkA*; bFGF; VEGF; Tsp-1; Ang-1; Ang-2; MMP-9; TIMPs 1, 2, and 3; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

PCR analysis of genomic TrkA sequence

Genomic SH-SY5Y DNA was subjected to PCR using *TrkA*-specific primers spanning *TrkA* intron/exon 6 to the intron 7/exon splice junction and exon/intron 7 to exon 8/intron splice junction (Greco et al., 1996) and directly sequenced in an ABI prism automated DNA sequencer, as detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Proliferation assay

Cellular proliferation was determined using an MTS proliferation assay, as directed by the manufacturer (Promega, Madison, WI) and detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Immunoprecipitation and Western blots

Immunoprecipitation and Western blots were performed on tumor and cell extracts using appropriate immunoprecipitating antibodies or preimmune IgG and Protein G Sepharose (Fast flow, Sigma-Aldrich), as detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Stable transfections

Cells were stable transfected with pcDNA 3.1Zeo vectors bearing coding TrkAI, TrkAIII, or Trk-T3 cDNAs or with empty vector using Lipofectamine as directed by the manufacturer (Invitrogen). Stable transfectants, isolated by zeocin resistance, were clonally expanded and characterized as detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Nuclear extracts, EMSA, and luciferase reporter gene assays

Nuclear extracts were prepared, and EMSAs were performed as previously described (Farina et al., 1999). Transient transfection of NF-κB luciferase and pRSVβgal reporter genes was performed using Lipofectamine as directed by the manufacturer (Invitrogen), and β-galactosidase assays (Farina et al., 1999) and luciferase assays are detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Growth in soft agar, tumor growth in nude mice, and tumor histology

Detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Chemically induced hypoxia

Cell cultures were incubating for up to 24 hr with 150 μM $CoCl_2$ in complete medium, as previously described (Miyazaki et al., 2002).

Rabbit enolase TK, inositol IP3K, and Ras activity assays

Tk and IP3K assays were performed as previously described (Meakin and Shooter, 1991; Obermeier et al., 1993b), and Ras activation assays were performed using a Ras activity kit as directed by the manufacturer (Upstate Biotech, Lake Placid, NY), as detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Cellular ^{125}I-NGF binding assay

Steady-state binding studies with ^{125}I-NGF were performed as previously described (Mahadeo et al., 1994) and are detailed in the Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/347/DC1/.

Statistical analysis

Mann-Whitney and Student's t tests were employed to provided values with associated probabilities, considered to be statistically significantly if less than 0.05, by convention.

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